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## Evolutionary considerations on 5-HT<sub>2</sub> receptors

Imane Moutkine<sup>1</sup>, Emily Lauren Collins<sup>1</sup>, Catherine Béchade<sup>1</sup>, and Luc Maroteaux<sup>1</sup>

### Affiliations:

<sup>1</sup>: INSERM UMR-S839, Paris, 75005, France; Sorbonne Université, Paris, 75005, France; Institut du Fer à Moulin, Paris, 75005, France.

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**Correspondence should be addressed to:** Luc Maroteaux INSERM UMR-S839, 17 rue du Fer a Moulin Paris, 75005, France; E-mail : luc.maroteaux@upmc.fr

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### Abstract

Serotonin is a neurotransmitter widely conserved from ancient organisms lacking nervous systems through man, and its presence precedes the appearance of nervous systems on both developmental and evolutionary time scales. Serotonin receptor subtypes diversified approximately at the time period during which vertebrates diverged from invertebrates. The biological and clinical importance of serotonin receptors, may benefit from studies on their evolution. Although potentially informative about their pathophysiological functions, reviews on this topic are sparse. Several observations support basic functions mediated by serotonin, both in periphery and central nervous system. In particular, 5-HT<sub>2B</sub> receptors have been implicated in embryonic development, including cell proliferation, survival, and/or differentiation, in either neural crest cell derivatives, myeloid cell lineage, or heart embryogenesis. In this review, we collected existing data about the genomic association between the RPN2 proteasome subunit gene *Psm1* and the 5-HT<sub>2B</sub> receptor gene *Htr2b*. We discuss about the possibility that, during genome duplications, a single copy of this pair of genes has been conserved, suggesting a strong selective pressure. Many basic physiological functions in which serotonin system is involved could be linked to the early association of these two genes in pre-vertebrates. Their evolutionary association highlights the possibility that the 5-HT<sub>2B</sub> receptor gene, *Htr2b*, is the common ancestor of 5-HT<sub>2A/2B/2C</sub>-receptor subfamily. Disentangling these possibilities could bring new understanding of the respective importance of these receptors in pathophysiology of serotonin.

**Introduction.**

Biogenic amines including serotonin can be detected in virtually all metazoans and exert many behavioral and physiological functions. In vertebrates, most receptors, which mediate biogenic amine physiological activities, belong to the broad family of G protein-coupled receptors (GPCRs), one of the largest eukaryotic receptor families. Indeed, GPCRs are mediating such a diversity of biological functions that they evolutionarily expanded in such a way that they are considered as one of the most successful gene families [1]. Within the Rhodopsin-like GPCR family, biogenic amine receptors likely emerged together with the Fungi and Metazoan lineage. In Metazoan, the diversification of biogenic amine GPCRs has largely contributed to the physiological expansion of peripheral and of central nervous system complexity [2].

Serotonin receptors have the highly-conserved structure of seven transmembrane domains common to all GPCRS, including three extracellular loops involved in ligand recognition and three intracellular loops involved in intracellular signaling mainly through Gi-, Gs-, or Gq-protein-mediated pathways. Many pharmaceuticals used to treat diseases such as cardiac diseases, hypertension, migraine, schizophrenia, or depression are directly acting at serotonin receptors. Although biologically and clinically important, studies on the evolution of sub families of serotonin receptors are limited.

Comparisons among orthologous proteins in various species is a way to estimate the gene evolutionary relationships. The percentage of amino acid homology between species allows to estimate the rate of gene evolution. For GPCRS, this rate has been estimated at approximately 1% per 10 million years [3]. From this calculation, it can be hypothesized that primitive serotonin receptors have diverged more than 800 million years ago since the 3 major classes of serotonin receptors (i.e. 5-HT<sub>1</sub>, 5-HT<sub>2</sub> and 5-HT<sub>4-6-7</sub> receptors) show less than 25% homology and orthologous receptor are present in many invertebrates. Other serotonin receptor subtypes differentiated approximatively at the time period during which vertebrates diverged from invertebrates [3]. The serotonin receptor subtypes have further differentiated over the vertebrate evolution although their respective relationships have not been characterized.

In this review, we started from the observation that the *Htr2b* gene, coding for the 5-HT<sub>2B</sub> receptor is encoded within an intron of a large subunit of the proteasome *Psm1*, in humans, mice and rats. Existing data from genome sequencing support the hypothesis that association between these two genes appeared in pre-vertebrates. Previous published work indicated that serotonin and 5-HT<sub>2B</sub> receptors regulate neural crest cells, heart, and bone marrow stem cells differentiation and proliferation. In addition, these data revealed that during genome duplications, a strong selective pressure seems to keep a single copy of this genetic association to the proteasome *Psm1* gene. Together with its early appearance in pre-vertebrates and its persistence throughout evolution, this association may have functional consequences. These data raised the possibility that the 5-HT<sub>2B</sub> receptor is the common ancestor of the 5-HT<sub>2A/2B/2C</sub>-receptor subfamily.

**Genomic organization of the 5-HT<sub>2B</sub> receptor gene.**

In humans, the *HTR2B* gene is located on the reverse strand at the tip of the second chromosome at 2q37.1. The 5-HT<sub>2B</sub> receptor's cDNA, which is 2246 base pairs (bp) long, is encoded by four exons, three of which are coding, and produce a pre-mRNA transcript of approximately 51 Kbp before splicing [4-7]. Interestingly, the *PSMD1* gene, which encodes the non-ATP regulatory subunit 1 (RPN2) of the 26S proteasome, is located at the same locus on the forward strand and overlaps *HTR2B*, which is entirely encoded within intron 16 as revealed in the Ensembl database [8] (**Fig. 1**). Similar organization with the 5-HT<sub>2B</sub> receptor's cDNA encoded in the reverse strand of a large intron of the proteasome RPN2 can be found not only in humans, rats, and mice but also

in all mammals. In all other vertebrates from Teleost fishes, such as zebrafish *Danio rerio*, to jawless fishes, such as lamprey *Petromyzon marinus*, similar association can be observed (**Fig. 1**). Interestingly, in ascidian pre-vertebrates such as *Ciona savignyi* (ascidian, tunicates, chordates), a simple survey of the largest intron of the *Psm1*-like gene (ENSCAVG00000000318) revealed that it also includes a coding region that encodes for a protein very close to 5-HT<sub>2</sub> receptors (**Fig. 1**). None of the other serotonin receptor genes is embedded in other genes.

### **Evolutionary relationship within *Htr2* genes.**

Focusing on 5-HT<sub>2</sub> receptor genes in all tetrapods including mammals, three *Htr2* genes can be identified, when, in teleost fishes such as zebrafish, five *Htr2* genes are present. Closer relationships can be found between *Htr2a*, and to *Htr2c* compared to *Htr2b* genes as shown by the Ensembl gene tree comparison [8] (**Fig. 2A-B**). In more primitive species, two *Htr2* genes have been identified in the jawless fish agnatha, Lamprey. Only one *Htr2*-like gene can be found encoded in the reverse strand of *Psm1*-like gene (ENSCAVG00000000318) in *Ciona savignyi*. Concerning *Psm1* evolutionary relationships, an almost identical evolutionary tree to that of *Htr2b* gene is found in the Ensembl gene tree comparison [8] (**Fig. 2C**). One of the two lamprey *Htr2* genes is associated to *Psm1* as well as the uniquely identified *Ciona Htr2*-like gene, which is embedded in *Psm1* gene. Furthermore, only one of the five *Htr2* genes found in teleost such as zebrafish is associated to *Psm1* gene and identified as *Htr2b*. Importantly, none of the invertebrate *Htr2* genes is associated with *Psm1*/RPN2 coding gene in fruit fly or nematode.

### **Evolutionary considerations in the context of *Htr2* genes.**

A whole genome duplication event has been proposed to arise at the non-vertebrate chordate divergence that gave rise to the jawless vertebrates (Agnatha- today lampreys and hagfish) [9]. A gene present in the ancestral prochordate lineage would give rise to two copies of that gene in agnathans. Function of the ancestral gene can be retained by duplicated genes (sub functionalization), could acquire new function (neo functionalization), or could lose its original function and become a pseudogene. At the divergence of the agnathans toward the jawed vertebrates (Gnathostomata), a second whole genome duplication has been proposed to occur [9]. From these two genome duplication events, a potential for four copies of the original ancestral prochordate gene could be present in gnathostomes. Whole genome duplication events were followed by loss of some redundant genes. Interestingly, in the non-vertebrate chordate *Ciona*, *Psm1* gene is found associated with a *Htr2*-like gene. Later, in jawless fishes, the *Psm1* gene is found associated with only one of the two *Htr2* gene. Then, the *Htr2b* gene of cartilaginous fishes, of all tetrapods, and of zebrafish is associated with the *Psm1* gene (**Fig. 3**). Tetrapods have one *Htr2a* and one *Htr2c* gene, whereas teleosts such as zebrafish display two *Htr2a*- and two *Htr2c*-like genes. One likely explanation would be that an association between *Htr2* and *Psm1* gene appeared in pre-vertebrates like *Ciona*. Following the scheme proposed in reference [9], this ancestral gene could have been duplicated in jawless fishes and only one of the two resulting *Htr2* gene remained associated with *Psm1*, giving rise to one *Htr2a*- and one *Htr2b*-like gene in lamprey. Following the subsequent whole genome duplication that gave rise to tetrapods, the *Htr2a*-like gene could have been duplicated, giving rise to the *Htr2a* and *Htr2c* of vertebrates; the *Htr2b* associated to *Psm1* having lost its duplicated copy (**Fig. 3**). Finally, the most recent whole genome duplication that appeared at the origin of teleost fishes [9] may have led to two *Htr2a* and two *Htr2c* genes, the *Htr2b* associated to *Psm1* having likely again lost its duplicated copy. Thus, this interpretation would support that a strong selective pressure exists in order to keep only one copy of *Psm1*/*Htr2*. This would make *Htr2b* as the common ancestor of the other *Htr2* genes in

vertebrates; existing data could support that *Htr2b* is associated with basic functions of serotonin in embryonic differentiation and development.

### **Evolutionary consideration about early vertebrates and serotonin implication**

At the transition from invertebrates to vertebrates, the shift from a passive to an active mode of predation led to the appearance of many features specific to vertebrates:

**1-Neural crest-** The acquisition of the neural crest cells and its derivatives in vertebrate lineage was of high evolutionary importance [10, 11]. The neural crest cells are a population of multipotent stem-cells originating from the neuroectodermal cells located at the junction between neural plate and future epidermis. Concomitant to the neural tube closure, neural crest cells delamination generates migratory cells that will populate various regions of the embryo. An epithelio-mesenchymal transition makes cells ready to migrate within embryonic tissues. Neural crest cells differentiate into melanocytes, craniofacial bones, or enteric neurons, glia, and smooth muscle [12]. Neural crest cells generate many features specific to vertebrates like jaw-based predation [10]. It was recently shown that, in the tunicate *Ciona intestinalis*, a cephalic melanocyte lineage appears to be similar to neural crest since expression of the transcription factor Twist was sufficient to reprogram this lineage into migrating 'ectomesenchyme'. This work supported that the neural crest melanocyte lineage appeared before the divergence of tunicates and vertebrates [13]. Thus, features considered as specific to vertebrates can be identified in the common ancestor of agnathans and gnathostomes [14].

The question is to know if serotonin could be implicated in such evolutionary processes. Interestingly, Jean Lauder, in late 1970<sup>th</sup>, described the participation of serotonin to embryogenesis of many species [15]. In vertebrates, she described that serotonin participates in neural crest cell migration [16]. Our group showed about 20 years ago that *Htr2b* was expressed by migratory neural crest cells in mice (**Fig. 4A-B**) and that its blockade induced defects in embryonic development affecting neural crest cells survival [17]. Subsequently, the group of Michaela Ori, working with *Xenopus* embryos, showed that the 5-HT<sub>2B</sub>-receptor signaling had a crucial role in neural crest cell derivatives and craniofacial morphogenesis, being involved in shaping the first branchial arch and the jaw joint [18]. More recently the group of Michel Bader confirmed in Zebrafish an organizing role of serotonin in pharyngeal arch morphogenesis since the 5-HT<sub>2B</sub> receptor was found to be expressed in the pharyngeal arches and its pharmacological inhibition also induced defects in pharyngeal arch morphogenesis [19]. These results have been completed by the group of Michael Gershon who showed that the 5-HT<sub>2B</sub> receptor is expressed by enteric neurons, another neural crest derivative, and suggested that by stimulating 5-HT<sub>2B</sub> receptors, serotonin affects the fate of the large subset of enteric neurons [20]. Furthermore, serotonin can stimulate proliferation of melanocytes, also neural crest derivatives, via 5-HT<sub>2B</sub> receptors [21]. These studies indicated that serotonin via 5-HT<sub>2B</sub> receptors regulates proliferation, differentiation, survival, and/or apoptosis of neural crest cells, which were already acquired by pre-vertebrates.

**2-Heart structure-** The transition from invertebrates to vertebrates was associated with an increased sophistication of the heart. From a simple single-monolayered heart, the ascidian heart started being multilayered before the apparition of multichamber heart in vertebrates. The embryonic development of vertebrate heart requires a complex coordination between genetic programs and cellular and morphogenetic events. The genetic coordination of the heart embryonic development and anatomy in pre-vertebrates may be informative of important basic biological mechanisms. In the tunicate *Ciona*, the heart displays the basic cardiac genetic program and developmental cell specification events of the chordate heart [22]. Pre-vertebrate tunicate heart is

composed of a single-chamber peristaltic pump formed by a single layer of myocardium surrounded by a pericardium. In vertebrates, the heart is composed of at least a two-chambered myocardium with cardiac myocytes, an endocardium, valves that separate inflow and outflow chambers and a surrounding pericardium; the embryonic heart derives from different embryonic territories, including primary and secondary heart fields. Embryonic heart development starts with the formation of a heart tube; looping of the tube initiates the formation of heart chambers through a conserved process among vertebrates [23]; then, the secondary heart field gives rise to the right ventricle and outflow tract. The organization of *Ciona* heart represents a simplified version of the cardio-pharyngeal program in which progenitor cells produce the first and second heart field including pharyngeal muscles precursors and associated heart stem cells [22]. Heart progenitor cells of simple chordates also generate precursors of the atrial siphon muscles. It appears thus that the last common ancestor between tunicates and vertebrates already possessed multipotent cardio-pharyngeal precursors that contributed to the emergence of the secondary heart field [24].

We showed that the 5-HT<sub>2B</sub> receptor, which is expressed by embryonic cardiomyocytes, was involved in heart development (**Fig. 4C-D**) [25]. The *Htr2b* inactivation leads to the appearance of dilated cardiomyopathy in surviving mice. Serotonin protects newborn cardiomyocytes against apoptosis via cross-talks between phosphatidylinositol-3 kinase (PI3K)/Akt and extracellular signal-regulated kinase 1/2 (ERK1/2) signaling pathways that regulate mitochondrial functions [26]. These findings support a role for serotonin via 5-HT<sub>2B</sub> receptor regulatory events during heart development, which also started appearing at the pre-vertebrate stage.

**3-Blood system-** The transition from invertebrates to vertebrates was associated with improved blood system formation. In reaction to the environment, immune system evolved to protect individuals from microbial infection and to recycle their damaged tissues. The two components of the immune system consist in innate and adaptive immunity. Innate immunity is common to all species while adaptive immune system appeared with vertebrates. Adaptive immune system relies on near limitless genetic diversity with long-term memory of lymphocytes. In *Ciona*, chitin-binding proteins containing variable regions, display high allelic complexity that may reflect functional specificity [27]. Agnathans have completed innate responses with adaptive elements; in lampreys, leucine rich repeat-containing proteins appear as variable lymphocyte receptors. In higher vertebrates, the major histocompatibility complex, T-cell and B-cell receptors (immunoglobulins) also assists innate responses as adaptive elements [28].

Hematopoietic stem cells initially derive from blood islands on the yolk sac at E7.5 in mice from endothelial progenitor cells (hemangioblasts). Hematopoiesis then occurs later in the aorta-gonad-mesonephros (AGM) region at E10.5 in mice and moves to fetal liver at E12.5 before migrating to bone marrow around birth. Hematopoietic stem cells give rise to blood cells, including platelets, erythrocytes, myeloid and lymphoid cells. In mice, when looking at the expression of 5-HT<sub>2B</sub> receptors in embryos, there were mRNA and protein located at the distal end of the embryo and along the neural tube similar to AGM (**Fig. 4AB**). In *Htr2b*<sup>-/-</sup> mice, the E12.5 embryos are smaller and paler than controls embryos with light pink liver (**Fig. 4E**). AGM and liver are sites of embryonic hematopoiesis. Furthermore, the absence of 5-HT<sub>2B</sub> receptors generates permanent changes in the composition of the blood and bone-marrow lineage, particularly in myeloid lineage and in endothelial cell progenitors in adult mice [29] (**Fig. 4F**). Serotonin participates in pulmonary hypertension [30] and valvulopathies [31]; we and other have shown that the initial functions of 5-HT<sub>2B</sub> receptors in these diseases are restricted to bone-marrow precursor cells [29, 32].

Together these data pointed *Htr2b* as a regulator of neural crest cell, heart development and bone marrow stem cells differentiation and proliferation. These features appeared in pre-vertebrates and remained throughout evolution, supporting a putative early appearance of the functions mediated by this receptor subtype in evolution. It has to be noticed that no developmental defect has been described in either *Htr2a*<sup>-/-</sup> or *Htr2c*<sup>-/-</sup> mice.

#### **Putative link between *Htr2b* and *Psm1***

The 26S proteasome is a highly conserved multicatalytic protease from yeast to mammals, which functions to degrade proteins following ubiquitination via the ubiquitin-proteasome system. The proteasome is required for critical cellular functions including cell cycle control and protein homeostasis. The 26S proteasome contains many distinct subunits and is predominantly composed of two large structures; the 19S complex (regulatory particle), within which RPN2 is found, functions to recognize ubiquitinated proteins; it then processes and transports them to the 20S complex (core particle), where proteolysis occurs. RPN2 functions as a molecular scaffold and mediates the binding of a variety of factors including additional proteasome subunits. RPN1 and RPN2 emerge as the primary coordinators of the ubiquitin processing machinery at the base of the 26S proteasome. RPN1 and RPN2 are characterized by eleven tandem copies of a 35-40 amino acid repeat motif termed the proteasome/cyclosome repeat, and RPN13, the ubiquitin receptor, binds to the C-terminal 20 residues of RPN2 [33]. As a result, the RPN2 protein plays a crucial role as it is a major site of proteasomal regulation.

The genomic arrangement between *Htr2b* and *Psm1*, which has been evolutionarily conserved, could indicate a functional interaction between these two genes. As overlapping genes are relatively common in DNA and RNA viruses, they are quite rare in non-viral organisms, with only few examples described in mammalian genomes. The most common form of overlap seen in eukaryotes between protein coding genes is a nested structure whereby, the internal gene, like *Htr2b*, lies entirely within an intron of the external gene, here *Psm1*. It is thought that this genetic arrangement may result in a co-regulatory relationship [34], leading to a negative correlation due to transcriptional collusion or having a neutral effect; previous studies have shown significant negative correlation between nested genes with little positive correlation [35]. Several potential mechanisms could be suggested including direct competition for the transcriptional apparatus or complementary binding between the pre-mRNA of *Htr2b* and *Psm1* intron. Therefore, it remains to be determined if the two genes are expressed coordinately.

#### **Conclusion.**

Several observations indicate that some embryonic functions attributed to serotonin are performed by 5-HT<sub>2B</sub> receptors. Existing data support that the association of *Htr2b* with *Psm1* appeared early in evolution. Many developmental physiological functions, in which serotonin system is involved, could be linked to the early association of these two genes in pre-vertebrates. More work is needed to clearly establish the importance of this association and the possibility that *Htr2b* is the common ancestor of 5-HT<sub>2</sub>-receptor genes. Determining the molecular basis if this gene association may provide new insight into the embryonic, differentiation, and/or physiological functions of serotonin both in the brain and periphery and bring new understanding of their respective importance in pathophysiology of serotonin.

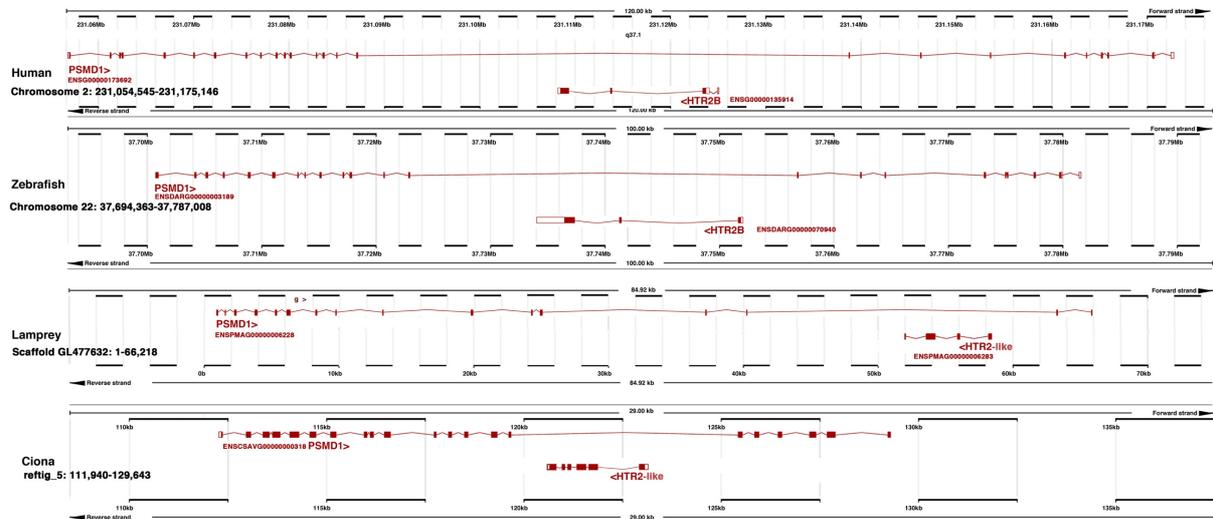
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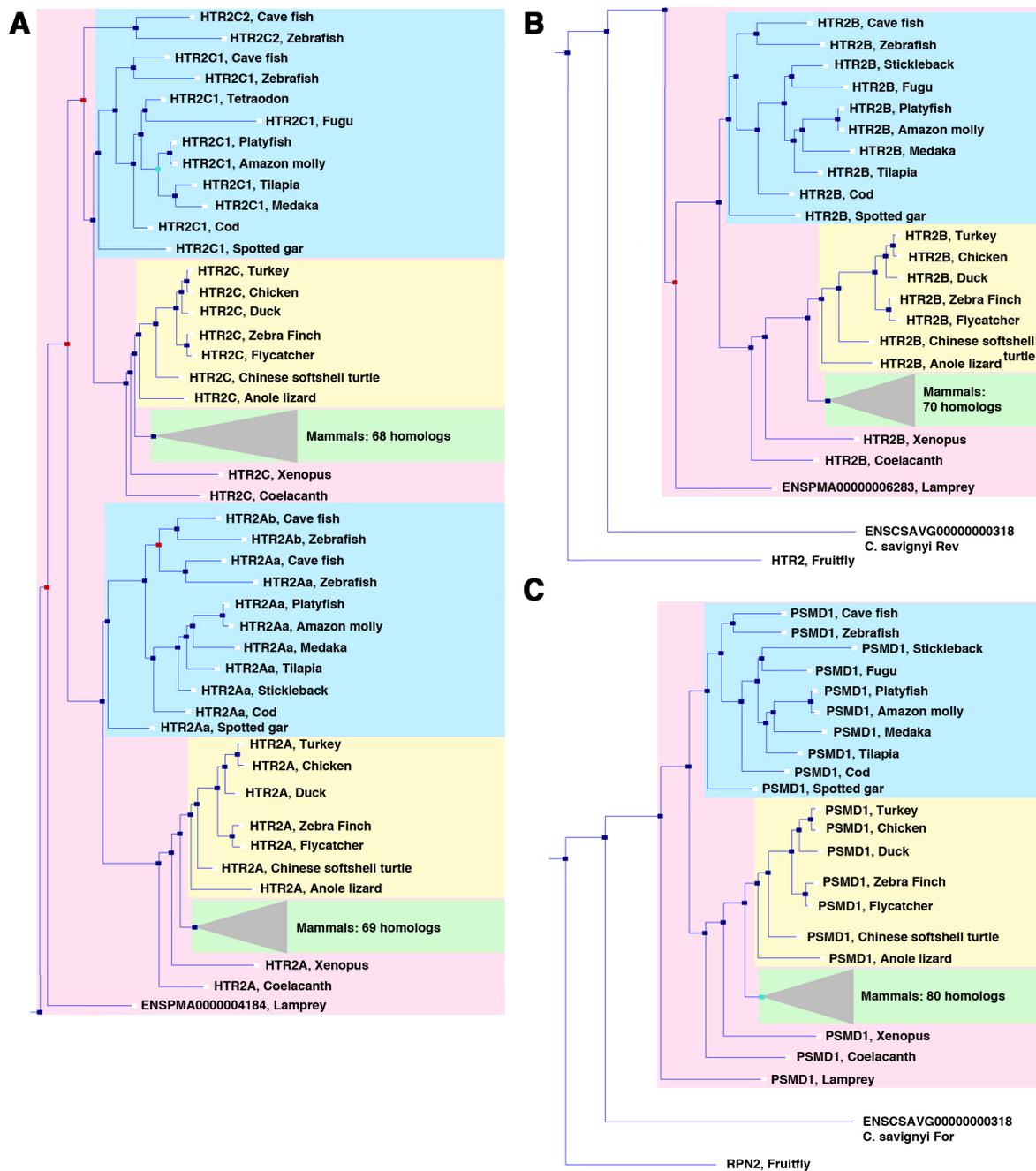
**Conflict of interest**

The authors declare no competing financial interests.

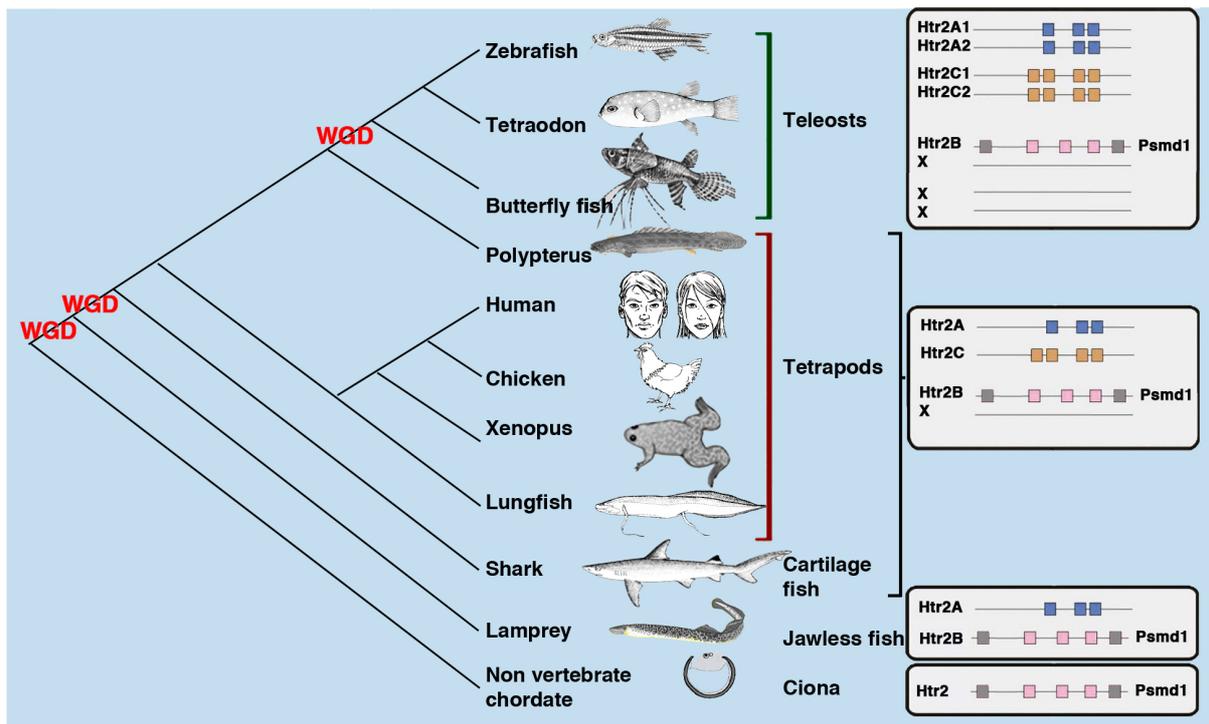
## Figure legends



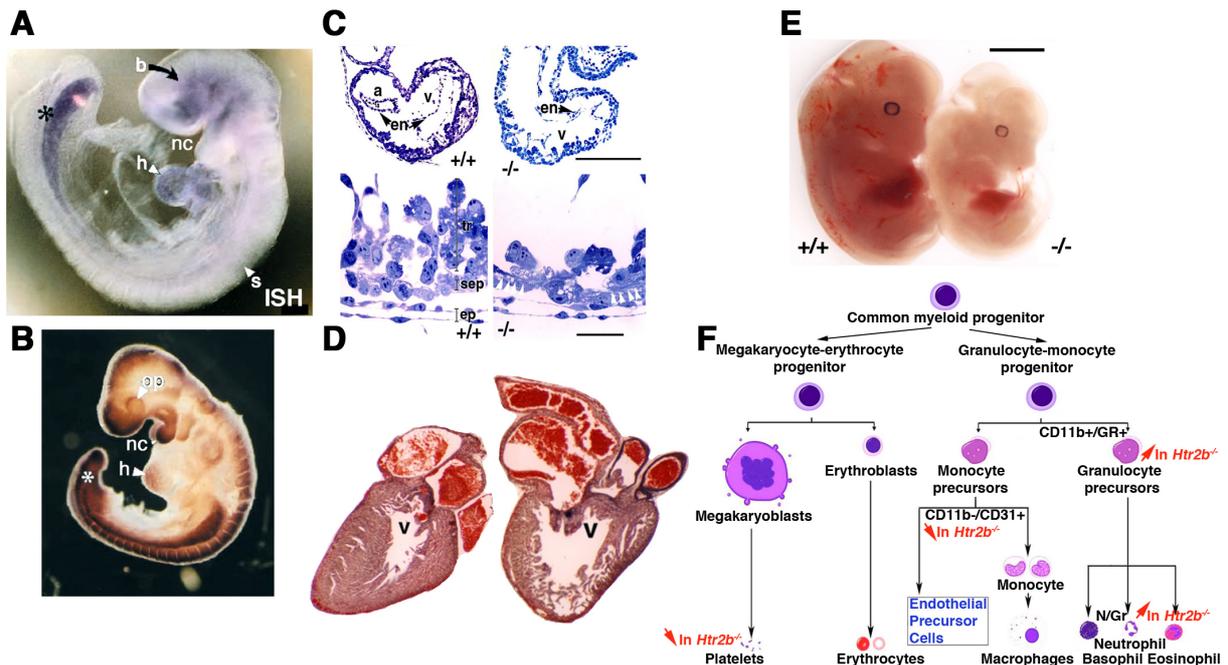
**Figure 1: Conserved gene structure of the *HTR2B* gene.** The *HTR2B* gene is encoded on the reverse strand of the *PSMD1* gene located at the same locus, which encodes the non-ATP regulatory subunit RPN2 of the proteasome 19S in human. In other vertebrates from the jawless fish lamprey to Teleost fishes such as zebrafish, similar organization can also be observed. Analysis of ascidian pre-vertebrates such as *Ciona savignyi* locus revealed identical organization with a large intron of *Psmd1*-like gene encoding a *Htr2*-like gene. All data are extracted from the Ensembl database [8].



**Figure 2: Evolutionary relationships between *Htr2* genes; comparison with *Psm1* genes.** Close but clearly distinct relationships can be found between *Htr2a*, *Htr2b*, *Htr2c* genes. (A-B) *Htr2* gene tree. (C) The *Psm1* gene tree is identical to *Htr2b* subtree (B). All data are extracted from the Ensembl database [8].



**Figure 3: Evolutionary consideration about *Htr2* genes.** Species evolution has been favored by whole genome duplications (WGD) [9]. The whole genome duplication that arrived at the divergence between chordates and jawless fishes may have generated the two *Htr2* genes in Jawless fishes. The second whole genome duplication that aroused between Jawless fish and cartilaginous fish and all tetrapods may have generate the three *Htr2* genes in tetrapods. And the later whole genome duplication that took place at the divergence of teleost fish may have generated the five *Htr2* genes in teleost fishes. Gene exons from *Htr2a* are in blue, from *Htr2c* in orange, and fom *Htr2b* in pink with exons surrounding the largest intron of *Psm1* in grey; lost copy X.



**Figure 4: The 5-HT<sub>2B</sub> receptor is associated with development of neural crest cells derivatives, heart, and hematopoietic system.** (A-B) On E10.5 (embryonic day 10.5) embryos, the 5-HT<sub>2B</sub> receptor expression as seen by mRNA (A) and protein (B) is located in the cephalic region (b), neural crest cells (nc) derivatives, heart (h), and at the distal end of the embryo (\*), adapted from [17] with permission. (C) Inactivation of 5-HT<sub>2B</sub> receptors (*Htr2b*<sup>-/-</sup>) (-/-) causes cardiac development defects leading to partial embryonic and perinatal lethality, adapted from [25]. Semi-thin sagittal sections of E9.5 embryos demonstrated a severe reduction in the thickness of the ventricle (v) including both the compact zone and the trabeculae in *Htr2b*<sup>-/-</sup> (-/-) embryos. a, Atrium; en, endocardium (e). Higher magnification shows a reduction of trabecular cells (tr) in the mutant heart, whereas elongated cells (white arrowhead) are visible in the compact subepicardial layer (sep). Epicardial cells (ep) are normally developed, adapted from [25]. (D) Histological analysis of newborn mutant hearts shows an obvious loss of ventricular (v) mass (hypoplasia), adapted from [25]. (E) The *Htr2b*<sup>-/-</sup> (-/-) embryos at E12.5 are smaller and paler with light pink liver, a place for embryonic hematopoiesis, adapted from [25], "Copyright (2000) National Academy of Sciences.". (F) The absence of 5-HT<sub>2B</sub> receptors generates permanent changes in the composition of the blood and bone-marrow myeloid lineage, particularly in endothelial cell progenitors, adapted from [29] with permission.

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