Ineffective erythropoiesis with reduced red blood cell survival in serotonin-deficient mice

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Serotonin (5-HT) has long been recognized as a neurotransmitter in the central nervous system, where it modulates a variety of behavioral functions. Availability of 5-HT depends on the expression of the enzyme tryptophan hydroxylase (TPH), and the recent discovery of a dual system for 5-HT synthesis in the brain (TPH2) and periphery (TPH1) has renewed interest in studying the potential functions played by 5-HT in nonnervous tissues. Moreover, characterization of the TPH1 knockout mouse model (TPH1\textsuperscript{-/-}) led to the identification of unsuspected roles for peripheral 5-HT, revealing the importance of this monoamine in regulating key physiological functions outside the brain. Here, we present in vivo data showing that mice deficient in peripheral 5-HT display morphological and cellular features of ineffective erythropoiesis. The central event occurs in the bone marrow where the absence of 5-HT hampers progression of erythroid precursors expressing 5-HT\textsubscript{a} and 5-HT\textsubscript{b} receptors toward terminal differentiation. In addition, red blood cells from 5-HT-deficient mice are more sensitive to macrophage phagocytosis and have a shortened in vivo half-life. The combination of these two defects causes TPH1\textsuperscript{-/-} animals to develop a phenotype of macrocytic anemia. Direct evidence for a 5-HT effect on erythroid precursors is provided by supplementation of the culture medium with 5-HT that increases the proliferative capacity of both 5-HT-deficient and normal cells. Our thorough analysis of TPH1\textsuperscript{-/-} mice provides a unique model of morphological and functional aberrations of erythropoiesis and identifies 5-HT as a key factor for red blood cell production and survival.

Erythropoiesis is the process through which the hematopoietic tissue of the bone marrow (BM) produces red blood cells (RBCs). The RBC originates from a multipotent hematopoietic stem cell through a series of differentiating divisions. The first committed erythroid progenitor is the erythroid burst-forming unit (BFU-E), which matures into an erythroid colony-forming unit (CFU-E) before reaching the proerythroblast stage. As the proerythroblast differentiates further through the basophilic, polychromatic, and orthochromatic erythroblast stages, it exhibits well-defined morphological changes, including a reduction in size, acquisition of hemoglobin, and expulsion of its nucleus, giving rise to a reticulocyte (1). Finally, the reticulocyte released into the circulation sheds its RNA and mitochondria and becomes a mature RBC (2). In humans, anemia is the most common blood disorder, defined as a reduction in either the number of RBCs or the amount of hemoglobin in the blood. If the anemia is caused by transient blood loss or hemolysis, the erythropoietic system, sensitive to reduced oxygen delivery, quickly responds by increasing erythropoietin (EPO) production. Increased EPO levels lead to an increase in erythropoietic activity that results in erythroid expansion in the BM and ultimately a restoration of normal circulating RBC counts (3). In individuals with ineffective erythropoiesis, a defect prevents erythroid cells from proliferating or differentiating normally, leading to abnormal production of mature RBCs and failure to compensate for anemia (4). Under certain circumstances, premature death or elimination of abnormal circulating RBCs also can contribute to this process. Erythropoiesis thus is a dynamic and tightly regulated process depending on the cooperation among mitogenic, differentiating, and antiapoptotic factors to ensure efficient erythropoiesis and production of a sufficient number of RBCs (5).

Serotonin (5-hydroxytryptamine or 5-HT) is a monoamine originally purified from the blood as a vasoactive agent and known for its role in the coagulation process. Its action is mediated through the activation of several receptors, encoded by at least 15 different genes, which can be found in various tissues ranging from the gut to the central nervous system (6, 7). Accordingly, and depending on its location, 5-HT may act as a neurotransmitter, a hormone, or a growth factor to regulate a wide range of cellular processes. Through activation of receptors of the 5-HT\textsubscript{a} subtype, 5-HT is known to have a mitogenic effect on a number of cell types, including vascular smooth muscle cells, trophoblast cells, osteoblasts, and cardiomyocytes (8–11). Availability of 5-HT depends on the expression of the enzyme tryptophan hydroxylase (TPH), which catalyses the first and rate-limiting step in the biosynthesis of 5-HT (12). Two genes encode TPH: the Tph2 gene, whose expression is restricted to neuronal cells, and the Tph1 gene, expressed in a broad range of nonneuronal cells (13–15). Most of the 5-HT found throughout the body is synthesized by TPH1 in enterochromaffin cells of the gastrointestinal tract (16), and targeted disruption of the Tph1 gene gives rise to a mouse with very low levels of circulating and tissue 5-HT (13). Using the TPH1 knockout (TPH1\textsuperscript{-/-}) mouse, we and others identified unsuspected roles for peripheral 5-HT in mammary gland homeostasis, cardiac function, proper embryogenesis, liver regeneration, bone formation, and pancreatic function (13, 17–21). Hence, the TPH1\textsuperscript{-/-} mouse is the best model for studying the role of 5-HT in periphery.

Little is known regarding the mechanism of action of 5-HT during hematopoiesis, and only scattered in vitro reports have described the influence of 5-HT on RBCs or the erythropoietic function (22, 23). Recently, an in vitro study showed that 5-HT enhances the expansion of human CD34\textsuperscript{+} cells to early-multi-lineage committed progenitors including CFU-E and BFU-E.
(24). However, no clear in vivo demonstration of the role played by 5-HT in erythropoiesis has been reported that would substantiate its capacity to regulate this critical process. Here, we show that 5-HT–deficient mice display a phenotype of ineffective erythropoiesis. Our in vivo analysis of TPH1-mutant animals supports a model whereby 5-HT is implicated in both RBC formation and survival. Our study also reveals that erythroid precursors express 5-HT_{2A} and 5-HT_{7} receptors, and low levels of 5-HT lead to an aregenerative macrocytic anemia caused by an impaired expansion of these cells. In vitro, the impaired expansion of erythroblasts can be rescued by the addition of 5-HT to the culture medium, suggesting a direct effect of the monoamine on erythroid precursors.

**Results and Discussion**

**Macrocytosis with Anemia in 5-HT–Deficient Mice.** To define the effect of 5-HT deficiency on erythropoiesis, peripheral blood cell analyses of WT and TPH1 −/− mice were performed. A significant decrease in the number of RBCs, hematocrit, and hemoglobin levels was observed in TPH1 −/− animals compared with WT mice (Fig. 1A–C). The slight decrease in hematocrit compared with the robust reduction in the total number of RBCs can be attributed to a substantial increase in the mean corpuscular volume of RBCs from 5-HT–deficient mice (Fig. 1D). Increased forward light scatter, used as a measure of cell-volume changes, provided additional evidence for increased RBC size in TPH1 −/− mice (Fig. 1E). Finally, analysis of blood parameters also revealed an increase in mean corpuscular hemoglobin (Fig. 1F), although the mean concentration of corpuscular hemoglobin was not affected (Fig. 1G), indicating that the decrease in hemoglobin levels was caused by a decrease in RBC counts. To explore further the nature of the anemia in 5-HT–deficient mice, we performed reticulocyte counts and EPO measurements. It is well established that in cases of anemia, secretion of EPO by the kidney will rapidly stimulate RBC production in the BM. In addition, elevated EPO levels often are associated with an increased number of circulating reticulocytes (25, 26), which is a quantitative measure of the BM’s production of new RBCs. We observed a 2.4-fold increase in EPO levels in TPH1 −/− animals compared with WT mice, whereas no significant rise in the number of circulating reticulocytes occurred (Fig. 1H and I).

These results indicate that 5-HT is necessary for normal erythropoiesis because TPH1 −/− mice develop a phenotype of macrocytic anemia. The data also imply that the anemic phenotype was not caused by an inadequate production of EPO, but the compensatory response did not seem sufficient to maintain a normal RBC count.

**Decreased RBC Survival and Increased Splenic Siderosis in 5-HT–Deficient Mice.** In nondisease states, there is a balance between production and destruction of RBCs, but during anemia excessive blood loss, excessive breakdown of RBCs (hemolysis), or deficient RBC production (ineffective erythropoiesis) can occur (4). To evaluate whether the anemic phenotype observed in TPH1-mutant animals may be a consequence of increased hemolysis, we measured bilirubin and lactate dehydrogenase levels, which are markers of intravascular hemolytic processes (27). There was no evidence of a significant hemolytic component to the anemia, because serum bilirubin and lactate dehydrogenase levels were not elevated in 5-HT–deficient animals compared with WT mice. Another possibility would be that TPH1 −/− RBC loss occurs through a “silent” pathway via their ingestion by macrophages. Along this line, it has been proposed that RBCs with exposed phosphatidylserine (PS) in their outer layer increase their sensitivity to phagocytosis by macrophages (28). To validate this hypothesis, we first measured by FACS the binding of fluorescein labeled annexin V (which binds specifically to PS) and observed a 2.2-fold increase in basal PS exposure in TPH1 −/− RBCs compared with WT RBCs when incubated in a normal buffer (Fig. 2A). TPH1 −/− RBCs also were more sensitive to hypertonic shock (Fig.

![Fig. 1.](https://www.pnas.org/cgi/doi/10.1073/pnas.1103964108)  

TPH1 −/− mice display a phenotype of aregenerative macrocytic anemia. (A–C) A significant decrease in the number of RBCs (7.5 ± 0.2 vs. 8.8 ± 0.1 x 10^{6} cells/μL), hematocrit (41.5 ± 0.8 vs. 45.2 ± 0.7%), and hemoglobin levels (15.1 ± 0.3 vs. 16.6 ± 0.2 g/dL) is seen in TPH1 −/− vs. WT mice and indicates an anemic phenotype. (D and E) A significant increase in mean corpuscular volume (MCV) (55.0 ± 0.3 vs. 51.2 ± 0.2 fl), associated with a general increase in RBC cell size, is observed in TPH1 −/− vs. WT mice and is consistent with macrocytosis. (F) Mean corpuscular hemoglobin (MCH) is increased significantly (20.2 ± 0.2 vs. 18.8 ± 0.1 pg), but (G) no difference is seen in mean corpuscular hemoglobin concentration (MCHC) (36.7 ± 0.2 vs. 36.8 ± 0.3 g/dL) in TPH1 −/− vs. WT mice. (H) A significant increase in EPO level is observed in plasma of TPH1 −/− mice (541 ± 81 pg/mL, n = 30) compared with WT mice (229 ± 49 pg/mL, n = 17) and indicates a response to the anemia. (I) No reticulocytosis is observed in TPH1 −/− mice (184,000 ± 20,000 reticulocytes/mL, n = 23) compared with WT mice (164,000 ± 18,000 reticulocytes/mL, n = 17) and points to aregenerative anemia. P was calculated using an unpaired t test in G and I, an unpaired t test with Welch’s correction in B and H, and a Mann–Whitney test in A, C, D, and F. P < 0.05 was considered significant. In A–D, F, and G each dot represents a single blood count (WT, n = 42; TPH1 −/−, n = 56; **P < 0.01; ***P < 0.001).
RBCs, an issue we addressed in more staining. (12) Mice, suggesting Jolly bodies Decreased RBC survival and increased splenic iron deposits in TPH1 cells (Fig. 3 compared with WT mice (Fig. 2 mice show a significant reduction in the hematopoietic cellularity of the BM (Fig. 3D). To assess whether the decrease in BM cellularity of TPH1−/− mice was caused by a blockade of erythroid differentiation, we performed methylcellulose colony-forming assays and found an increase in the frequency of the erythroid-committed progenitors BFU-E and CFU-E in TPH1−/− mice (Fig. 3B Left). However, the reduced cellularity in the BM of TPH1−/− mice, the absolute number of BFU-E/CFU-E was the same in animals of both genotypes (Fig. 3B Right). It is well established that CFU-E requires the presence of EPO as a growth factor (32). Thus, the increase in the frequency of erythroid progenitors in the colony assay is in agreement with the observed increase in EPO level and, again, reflects a compensatory response to the anemia present in TPH1−/− mice. However, inability of the 5-HT–deficient animals to respond to the anemia despite the increase in EPO level and in the number of erythroid progenitors led us to predict that the 5-HT–sensitive step would occur after the BFU-E/CFU-E stages of RBC development. Hence, we examined the erythroid maturation from the proerythroblast to late orthochromatic erythroblast stage, just before enucleation in the BM, using the expression of cell-surface markers CD71 and Ter119, which are associated with distinct stages of erythroid differentiation (1). No accumulation at any specific maturation stage was observed, and the same percentage of different subsets of the erythroblast population (Ter119+/CD71−) cells was found in TPH1−/− and WT mice (Fig. 3C Left and Center). Nonetheless, the significant reduction in the total cellularity of the BM in 5-HT–deficient mice resulted in a substantial decrease in the total number of Ter119+/CD71+ cells (Fig. 3C Right). Finally, no difference was observed in annexin V binding to Ter119+/CD71+ cells from TPH1−/− and WT mice, ruling out the likelihood of increased cell apoptosis as a cause of the anemia.

Erythroid BM Cells from 5-HT-Deficient Mice Display a Cell-Cycle Defect in Vivo and Express 5-HT2A and 5-HT2B Receptors. Together, our data indicate that neither a blockade in the differentiation scheme nor increased apoptosis can account for the decrease in the total number of Ter119+/CD71+ cells in mutant animals but imply a low production efficiency of RBC precursors. Moreover, during erythroid differentiation, cell division is tightly linked with reduction in cell size (33), and reduced cell division could result in the macrocytosis observed in TPH1−/− mice. By analogy, in humans, macrocytic anemia may give rise to defective DNA synthesis in the BM, leading to alteration of the normal differentiation–proliferation pathway of the erythroid lineage and release of
macrophages into the blood stream (34). To determine whether the lack of 5-HT causes a defective proliferation of RBC precursors, WT and mutant mice were injected with BrdU for 2 h as a measure of entry into the S-phase of the cell cycle and proliferation of Ter119+/CD71+ cells. Our analysis did not reveal a difference in the DNA content between WT and TPH1−/− animals, but a marked decrease in the percentage of cells incorporating BrdU was observed in 5-HT−/− deficient animals (19.3 ± 1.4%) vs. WT animals (24.7 ± 1.4%) (Fig. 4A and B). In parallel, careful microscopic examination of BM smears from TPH1−/− and control mice substantiated the notion that lack of 5-HT is associated with a defect in cell proliferation and not with a blockade of differentiation or with cell death during erythroblast proliferation. Indeed, significant abnormalities, including binucleated erythroblasts with increased parachromatin separating dense heterochromatin and erythroblasts with fragmented nuclei, were seen more frequently in mutant mice (arrow, Fig. 4C).

As mentioned previously, 5-HT is known to regulate proliferation of various cell types by activating receptors of the 5-HT2 subtype (8–11). To evaluate whether 5-HT has a direct effect on erythroid precursors, we checked for the presence of these 5-HT receptors on Ter119+/CD71+ cells. Binding studies identified the 5-HT2A and 5-HT2B receptors, but not the 5-HT2C receptor, on Ter119+/CD71+ cells (Table 1). The presence of these receptors on erythroid precursors favors the possibility that the low production efficiency of RBC precursors observed in Tph1−/− mice could result from a direct action of 5-HT on these cells.

Several observations in experimental mice models of macrocytic anemia have suggested that a defect in S-phase entry and progression results in ineffective erythropoiesis similar to that observed in TPH1 mutant mice (35–37). Particularly in the E2f4 mouse mutant, the delayed cell-cycle progression of erythroblast cells results from the activation of a DNA damage response (37). In TPH1−/− mice, the presence of Howell–Jolly bodies in the peripheral blood and the decreased number of cells present in S-phase also could be indicative of increased DNA damage or of an inability to repair DNA properly. The macrocytic anemia of 5-HT−/− mice also shares a number of striking similarities with human megaloblastic anemia: erythroid macrocytosis, an increased presence of Howell–Jolly bodies within mature erythrocytes, and the presence in the BM of morphologically abnormal erythroblasts, mainly binuclear erythroblasts. In humans, the cellular defects characteristic of megaloblastic anemia often are linked to slow replication caused by decreased dietary or metabolic availability of folate or vitamin B12 (38). We do not suspect a defect in folate metabolism to be the underlying cause of the erythroid phenotype in TPH1−/− mice, because no difference was seen in the level of folate in 5-HT−/− deficient vs. WT mice. Also, we did not observe the large increase in apoptosis in erythroid precursors that has been reported previously for mouse models of megaloblastic anemia arising from dietary folate deficiency (39).

**Table 1.** Erythroid precursors express 5-HT2A and 5-HT2B receptors

<table>
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<tr>
<th>Receptor type</th>
<th>Ligand binding to Ter119+/CD71+ cells (fmol/mg protein)</th>
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<tr>
<td>5-HT2A receptor</td>
<td>32.0 ± 1.7</td>
</tr>
<tr>
<td>5-HT2B receptor</td>
<td>44.4 ± 1.6</td>
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<tr>
<td>5-HT2C receptor</td>
<td>&lt; 0.5</td>
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Binding was performed on membranes of sorted Ter119+/CD71+ cells isolated from the BM. Data are mean ± SEM (n = 5).

5-HT or a 5-HT2 agonist increases the proliferative capacity of erythroid precursors. To confirm the role played by 5-HT in erythroid proliferation, we used a well-defined culture system that closely mimics the proliferation and differentiation of erythroid precursors in vivo (40, 41). After 1 d in culture, the proliferation parameters of 5-HT−/− deficient erythroid precursors were significantly reduced compared with WT cultures (Fig. 5A). This decrease in the number of cells was maintained and even amplified.
during the following days. Most interestingly, the poor proliferative capacity of cells from 5-HT− deficient mice was rescued by the addition of 5-HT to the culture medium. The 5-HT supplementation likewise increased the proliferative capacity of WT cells. Involvement of 5-HT2A and 5-HT2B receptors in this proliferative effect was confirmed by supplementing the medium with a specific 5-HT2 agonist, PNU 22394, which produced the same proliferative effect on erythroid precursors as observed using 5-HT (Fig. 5B). Cell viability was similar in all cases, indicating that the decrease in cell counts in absence of 5-HT was not a consequence of decreased cell survival. These data are in agreement with the decrease in BM cellularity and the BrdU-incorporation capacity of Ter119+CD71+ cells observed in TPH1−/− mice. They confirm that the underlying cause for the macrocytosis of 5-HT− deficient mice is decreased proliferation of erythroid precursors leading to the production of macrocytic RBCs, because these cell divisions are tightly linked with a reduction in cell size (33).

Such an impaired cellular proliferation is a phenotype shared by other knockout mouse models of the serotonergic system as well as by models of altered 5-HT signaling. Indeed, pioneer work by Jean Lauder and coworkers (42, 43) revealed that 5-HT regulates basic developmental processes, including cell proliferation, in mammalian and nonmammalian species. Also, identification and characterization of a 5-HT2A signaling pathway by Sonier et al. (9) demonstrated that 5-HT regulates proliferation of a human breast cancer cell line, and Collet et al. (10) showed that decreased bone formation in aging female 5-HT2B knockout mice was associated with reduced osteoblast proliferation.

Conclusion

Our data establish a function for 5-HT in regulating in vivo erythropoiesis. 5-HT− deficient animals present a phenotype of macrocytic anemia with morphological and cellular features of ineffective erythropoiesis. In the BM, absence of 5-HT down-regulates the rate of RBC production, given that progression of erythroid precursors toward terminal differentiation is significantly hampered in 5-HT− deficient mice. Direct evidence that 5-HT acts directly on erythroid precursors in the BM is provided by the presence of 5-HT2A and 5-HT2B receptors on these cells and by in vitro experiments in which supplementing the culture medium of TPH1−/− erythroid precursors with 5-HT restored their proliferative capacity. Also TPH1−/− RBCs released into the circulation somehow are malformed, because they have a shortened in vivo half-life. It remains to be determined whether the reduced half-life of TPH1−/− RBCs results from the observed ineffective erythropoiesis, from a direct effect of 5-HT on the RBC, or both. Nevertheless, the combination of these two defects undoubtedly contributes to the emergence of the observed aregenerative macrocytic anemia. Because treatment of human anemia has limitations, a better understanding of 5-HT− sensitive mechanisms involved in erythropoiesis may help identify targets to treat this disease.

Materials and Methods

Animal Model. Targeted mutagenesis of the tph1 gene was described previously (13). Age-matched transgenic and WT animals were derived from pure C57BL/6J genetic backgrounds in our animal care facility (Pitié Salpêtrière Hospital). Animal experiments were performed according to the recommendations of the French Institutional Committee.

Blood Counts. Blood was obtained after the animal was killed and was stored in EDTA tubes until analysis using an electronic hematology particle counter (type MS9-5; Melet Schloesing Laboratories).

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PS Exposure. Tail-vein blood was harvested with an EDTA-coated capillary. RBCs were incubated for 4 h at 37 °C in Ringer’s solution, washed, and stained with allophyocyanin (APC)-conjugated annexin V. APC-annexin V binding was evaluated by FACS analysis on a FACS Calibur (BD Biosciences) (SI Materials and Methods).

RBC Survival. RBC survival was evaluated using biotinylation of the RBC cohort and monitoring for its replacement (SI Materials and Methods).

In Vivo BrdU Incorporation Assay. Mice were injected with an i.p. dose of BrdU (1 mg) dissolved in PBS. BM cells were harvested 2 h after the injection, and Ter119+CD71+ cells were isolated using a FACS-Aria cell sorter (BD Biosciences). BrdU incorporation level in Ter119+CD71+ cells was assessed using a BrdU flow kit according to the manufacturer’s instructions (BD Pharmingen).

Radioligand Binding Assays. Crude membranes for radioligand binding assays were prepared as described previously (10) on frozen pellets of BM Ter119+CD71+ cells isolated using a FACS-Aria cell sorter. Radioligands used were [3H]-MDL100907 (5-HT2A), [3H]-LY266079 (5-HT2B), and [3H]-mesulergine (5-HT4).

Erythroid in Vitro Cell Culture. Erythroid cultures were derived from BM cells flushed from femur and tibia and grown essentially as previously described (40, 41). Further details are given in SI Materials and Methods.

For a detailed description of all of procedures, see SI Materials and Methods.

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