



**HAL**  
open science

## **Serotonin 5-HT<sub>2B</sub> receptors are required for bone-marrow contribution to pulmonary arterial hypertension.**

Jean-Marie Launay, Philippe Hervé, Jacques Callebert, Ziad Mallat, Corinne Collet, Stéphane Doly, Arnaud Belmer, Silvina L. Diaz, Sarah Hatia, Francine Côté, et al.

► **To cite this version:**

Jean-Marie Launay, Philippe Hervé, Jacques Callebert, Ziad Mallat, Corinne Collet, et al.. Serotonin 5-HT<sub>2B</sub> receptors are required for bone-marrow contribution to pulmonary arterial hypertension.. Blood, 2012, 119 (7), pp.1772-80. 10.1182/blood-2011-06-358374 . inserm-00996752

**HAL Id: inserm-00996752**

**<https://inserm.hal.science/inserm-00996752v1>**

Submitted on 3 Mar 2016

**HAL** is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

## **Serotonin 5-HT<sub>2B</sub> receptors are required for bone-marrow contribution to pulmonary arterial hypertension**

Jean-Marie Launay<sup>1</sup>, Philippe Hervé<sup>2</sup>, Jacques Callebert<sup>1</sup>, Ziad Mallat<sup>3</sup>, Corinne Collet<sup>1</sup>, Stéphane Doly<sup>4</sup>, Arnauld Belmer<sup>4</sup>, Silvina L. Diaz<sup>4</sup>, Sarah Hatia<sup>5</sup>, Francine Côté<sup>5</sup>, Marc Humbert<sup>6</sup> and Luc Maroteaux<sup>4</sup>.

**Short title:** Serotonin 5-HT<sub>2B</sub> receptor in bone-marrow and lung

<sup>1</sup>AP-HP, Hôpital Lariboisière, Service de Biochimie, Paris, F-75010 France; INSERM U942, Paris, F-75010 France. <sup>2</sup>Centre Chirurgical Marie Lannelongue, Le Plessis-Robinson – France. <sup>3</sup>INSERM U970, PARCC, Hopital Européen Georges Pompidou, Paris, F-75015, France. <sup>4</sup>INSERM UMR-S 839, F75005, Paris, France; Université Pierre et Marie Curie, F75005, Paris; Institut du Fer à Moulin, F75005, Paris. <sup>5</sup>CNRS UMR 8147, Paris, F-75015 France; Université Paris Descartes, F75015, Paris. <sup>6</sup>Université Paris-Sud 11, INSERM U999, Service de Pneumologie et Réanimation Respiratoire, Hôpital Antoine Bécclère, AP-HP, Clamart – France.

Correspondence and requests for materials should be addressed to L.M. (INSERM UMR-S 839, Institut du Fer à Moulin, 17 rue du Fer a moulin 75005, Paris FRANCE, Tel 331 45 87 61 23, Fax 331 45 87 61 32, E-mail: luc.maroteaux@upmc.fr).

**Classification:** Vascular biology

**word counts text 5100w, abstract 188w, figure count 6, reference count 47**

**Abstract**

Pulmonary arterial hypertension (PAH) is a progressive disease characterized by lung endothelial dysfunction and vascular remodeling. Recently, bone-marrow progenitor cells have been localized to PAH lungs, raising questions about their role in disease progression. Independently, serotonin (5-HT) and its receptors have been identified as contributors to the PAH pathogenesis. We hypothesized that one of these receptors, the 5-HT<sub>2B</sub> receptor, is involved in bone-marrow stem cell mobilization that participate to the development of PAH and pulmonary vascular remodeling. A first study revealed expression of 5-HT<sub>2B</sub> receptors by circulating *c-kit*<sup>+</sup> precursor cells, while mice lacking 5-HT<sub>2B</sub> receptors showed alterations in platelets and monocyte-macrophages number and in myeloid lineages of bone-marrow. Strikingly, mice with restricted expression of 5-HT<sub>2B</sub> receptors on bone-marrow cells developed hypoxia- or monocrotaline-induced increase in pulmonary pressure and vascular remodeling, while restricted elimination of 5-HT<sub>2B</sub> receptors on bone-marrow cells confers a complete resistance. Moreover, *ex vivo* culture of human CD34<sup>+</sup> or mice *c-kit*<sup>+</sup> progenitor cells in the presence of a 5-HT<sub>2B</sub> receptor antagonist resulted in altered myeloid differentiation potential. Thus, we demonstrate that activation of 5-HT<sub>2B</sub> receptors on bone-marrow lineage progenitors is critical for the development of PAH.

Keywords: Bone-marrow transplant; genetics; pulmonary hypertension; receptors; hypoxia.

## Introduction

Pulmonary arterial hypertension (PAH) is a rare but fatal disease, often of unknown origin, characterized by progressive increase in pulmonary vascular resistance and remodeling associated with vasoconstriction <sup>1</sup>.

PAH is histologically characterized by a neomuscularization of small pulmonary arteries with intimal thickening, medial hypertrophy, adventitial proliferation, and abnormal extracellular matrix deposition. The progression of vascular remodeling results in vascular lumen narrowing, increased pulmonary artery resistance, hypoxia, and right heart hypertrophy, although the molecular pathways initiating this remodeling are not clearly established.

On the one hand, stem cells, resident or not, may give rise to a significant proportion of differentiating/proliferating smooth muscle cells (SMC) that contribute to intimal hyperplasia in lung vascular remodeling <sup>2</sup>. Moreover, genetic ablation of the transmembrane tyrosine kinase receptor for stem cell factor/*c-kit* pathway results in a marked reduction in intimal hyperplasia in animal models of vascular injury; conversely, wild-type (WT) bone-marrow reconstitution in *c-kit* mutant mice led to intimal hyperplasia comparable to WT animals <sup>3</sup>. Pharmacological antagonism of the *c-kit* pathway with STI-571 (imatinib mesylate-Gleevec) also results in a marked reduction in hyperplasia <sup>3</sup>. Mobilization of *c-kit* expressing cells from bone-marrow to blood circulation is a physiological response to hypoxia. Increasing evidence supports the idea that these progenitor cells of bone-marrow origin may also contribute to vascular wall remodeling that is characteristic of PAH <sup>4-8</sup>. It is unclear, however, whether this entry of progenitors represents a protective or a worsening process in the development of PAH <sup>9</sup>. Other observations have also identified an association between PAH and bone-marrow-related hematological disorders <sup>10</sup>: in proliferative disorders of the hematopoietic stem cells such as myeloproliferative cancers, there is a high unexplained incidence of PAH; PAH is now a recognized complication of bone-marrow transplantation for leukemia <sup>11</sup>, chronic myeloproliferative disorders <sup>12</sup>, or in the treatment of malignant infantile osteopetrosis <sup>13</sup>.

On the other hand, serotonin (5-Hydroxytryptamine, 5-HT) is associated to the pathogenesis of PAH<sup>14</sup>. Therapeutic drugs with PAH as a side effect, like the amphetamine derivative and anorexigen dexfenfluramine, are potent 5-HT releasers acting at 5-HT transporter (SERT) and/or agonists at 5-HT receptors (5-HTRs)<sup>15</sup>. An over-expression of 5-HT<sub>2B</sub>Rs is observed in PAH<sup>16</sup>. Blockade of 5-HT<sub>2B</sub>Rs using independent approaches, either genetic (5-HT<sub>2B</sub>R knockout mice; 5-HT<sub>2B</sub><sup>-/-</sup>) or pharmacologic (5-HT<sub>2B</sub> antagonist RS-12744) inactivation, completely prevented the development of hypoxia-induced pulmonary hypertension in mice<sup>16</sup>. Using the monocrotaline (MCT)-induced pulmonary hypertension rat model, recent studies confirmed that other 5-HT<sub>2B</sub> antagonists (terguride, PRX-08066, or C-122) significantly reduced pulmonary pressure, arterial wall thickening and lumen occlusion but maintained cardiac function<sup>17-19</sup>. Independently, 5-HT was shown to stimulate human bone-marrow stromal cells and synergize with other pleiotropic growth factors that promote hematopoietic stem and progenitor cells<sup>20</sup>. The 5-HT action on hematopoiesis or bone-marrow microenvironment at patho-physiological conditions warrant further investigation. 5-HT is a potent vasoconstrictor of pulmonary arteries but also to stimulate pulmonary SMC proliferation. It may thus affect various processes associated to pulmonary vascular remodeling, but its exact contribution remains unclear.

The prognosis of PAH remains unsatisfying<sup>21</sup>, although the number of therapeutic options has increased over the past years, and several novel therapeutic targets are under active investigation<sup>1</sup>. At present, currently available vasodilator therapies for PAH, although helpful in improving exercise tolerance and quality of life, are only moderately effective in improving survival. These therapies are targeted to ameliorate the physiologic consequences of the remodeled pulmonary arterial vasculature and probably do not directly alter the underlying defects in the pulmonary vascular remodeling. Here, we investigated a possible contribution of 5-HT signaling pathways to bone-marrow-derived progenitors cells in animal models of PAH. We found that the expression of 5-HT<sub>2B</sub>Rs restricted to bone-marrow cells is necessary and sufficient for pulmonary hypertension to develop via an action at hematopoietic stem cells differentiation.

## Methods

### Reagents.

RS-127445, 2-amino-4-(4-fluoronaphth-1-yl)-6-isopropylpyrimidine, STI-571, 4-[(4-methylpiperazin-1-yl)methyl]-N-[4-methyl-3-[(4-pyridin-3-yl)pyrimidin-2-yl]amino]-phenyl]-benzamide and all other chemicals were reagent grade, purchased from Sigma-Aldrich and Tocris. The radioactive compounds [1,2-<sup>3</sup>H]-5-HT binoxalate (specific activity 1.11 TBq/mmol), [methyl,1',2'-<sup>3</sup>H]-thymidine (specific activity 4.44 TBq/mmol) and (6)-[<sup>125</sup>I]1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane hydrochloride ([<sup>125</sup>I]DOI, specific activity 81.4 TBq/mmol) were purchased from NEN Perkin Elmer. Monoclonal rat anti-*c-kit* antibody and the polyclonal rabbit anti-5-HT<sub>2B</sub>R were from AbCam.

### Animals.

The mice strains used in all experiments, including 5-HT<sub>2B</sub>R deficient mice (5-HT<sub>2B</sub><sup>-/-</sup>) are in a 129S2/SvPAS background<sup>22</sup>. Adult (7- 9 week-old) 5-HT<sub>2B</sub><sup>-/-</sup>, and wild-type (WT) control mice (originally obtained at Charles River Laboratories, L'Arbresle France) were all derived from heterozygote crosses bred at our animal facilities. All mice were maintained according to the EC directive 86/609/CEE, and housed in groups of 3-5 of the same genetic background after weaning.

### Bone-marrow transplantation.

As previously described<sup>23</sup>, 6 males and 6 females 8 weeks-old WT and 5-HT<sub>2B</sub><sup>-/-</sup> mice were subjected to 9.5 Gray lethal total body irradiation. The day after, mice were reconstituted by direct intravenous injection with 2.5x10<sup>6</sup> cells of freshly isolated bone-marrow from femurs and tibias of age and sex-matched WT or 5-HT<sub>2B</sub><sup>-/-</sup> mice. All lethally irradiated and transplanted mice survived revealing the efficiency of bone-marrow reconstitution. After 4 weeks of recovery, transplanted mice were then exposed to hypoxia as a validated pulmonary hypertension inducer in mice.

**Pulmonary hypertension induction.**

As previously observed <sup>24</sup>, following bone-marrow transplantation, mice were more sensitive to pulmonary hypertension inducers. Control and test mice were thus exposed to progressive hypoxia (20 to 10% O<sub>2</sub>) for 3 weeks. Control, normoxic mice were kept in the same 12/12 light-dark cycle. The vehicle or drug was delivered by miniosmotic pump (Alzet) at the beginning of the hypoxic (or normoxic) treatment.

**Cardiovascular evaluations.**

As previously described <sup>16</sup> mice were anesthetized with intraperitoneal ketamine hydrochloride (60 mg/kg) and xylazine (8 mg/kg). The arterial pulmonary pressure was estimated by assessing the cardiac right ventricular systolic pressure (RVSP), measured by insertion into the heart right ventricle of a 26-gauge needle connected to a pressure transducer. The pulmonary artery was cannulated through an incision in the right ventricle and perfused with Earle's balanced salt solution (37°C, 20 cm H<sub>2</sub>O pressure). The heart and lungs were removed and the airways were distended with 10% formaldehyde solution and fixed for 3 days. Measurements of the 5-HT<sub>2B</sub>R expression was performed using 5-HT<sub>2B</sub>R-specific iodinated radioligand (<sup>125</sup>I-DOI) sensitive to RS 127445 as previously described <sup>22</sup>.

**Cell preparations, FACS analysis and sorting.**

Murine bone-marrow cells were recovered and suspended in culture medium, as described <sup>25</sup>. Briefly, bone-marrow cells, flushed from femur and tibia, were resuspended in Hank's buffer before being passed through a 70µm strainer. Cells were then incubated with appropriately labeled antibodies (CD11b, Gr1, CD31, *c-kit* antibodies BD Biosciences, eBioscience) and acquisition was carried out on a FACS CantoII. Murine *c-kit*<sup>+</sup> cells (>95% purity) were next sorted electronically using a FACSVantage™ cell sorter (Becton

Dickinson). Peripheral blood was collected from mouse tails and cells were counted on a MS9-5 Hematology Counter (Melet Schloesing Laboratories, Osny, France).

### **Cell cultures**

Murine hematopoietic progenitors were quantified in MethoCult M3334 (StemCell Technologies) supplemented with IL-3, IL-6, SCF and in MethoCult GF H4434 (complete with growth factors), respectively. Cells were plated in a final volume of 1 ml at a concentration of  $2-5 \times 10^4$  total BM cells/culture dish (Falcon 1008) for murine and 5000 CD34<sup>+</sup> cells/ml for human progenitors. Colonies were scored on day 7 and day 14. All cultures were incubated at 37°C in a humidified chamber under 5% CO<sub>2</sub>. For methylcellulose cultures, human CD34<sup>+</sup> cells were cultured in BFU-E/CFU-E medium with EPO, IL-3, SCF (Stem Cell 4434) and murine total or *c-kit*<sup>+</sup> cells were cultured in CFU-GEMM/CFU-GM, and CFU-E/BFU-E medium with IL-3, IL-6, SCF, and EPO (Stem Cell 03434).

### **Statistics.**

The reported data represent the mean of individual values  $\pm$  SEM (n = number of individuals at the end of treatments as indicated in the text). For simple comparisons, unpaired t test was used. Significance was set at  $P < 0.05$ . For the groups, statistical comparisons were made by one way ANOVA. When statistical significance was attained ( $P < 0.05$ ), difference between groups was established using the Bonferroni multiple comparison test.



## Results

**Alterations of blood composition in 5-HT<sub>2B</sub>R mutant mice.** Starting from the previous observations that 5-HT may act on hematopoiesis<sup>20</sup>, we first investigated the blood composition of WT and 5-HT<sub>2B</sub><sup>-/-</sup> mice. Interestingly, we found a significant decrease in platelet number and increase in circulating granulocyte / macrophage population in 5-HT<sub>2B</sub><sup>-/-</sup> compared to WT mice, but not in other parameters, white blood cells, red blood cells, mean red blood cell volume, hematocrit, or hemoglobin content (**Fig 1a**). Flow cytometric cell sorting (FACS) was performed using antibodies to identify different populations, Mac-1 (CD11b) for monocytes / macrophages, Gr-1 (Gr+) for Granulocytes, PECAM (CD31) for endothelial cells / platelets / macrophages and determine the blood cell composition of WT and 5-HT<sub>2B</sub><sup>-/-</sup> mice. Completing our previous result, we found a significant reduction in CD11b-/CD31+ population that labeled immature endothelial progenitor cells in 5-HT<sub>2B</sub><sup>-/-</sup> mice (**Fig 1b**). Interestingly, by FACS of peripheral blood mononuclear cells, we also identified expression of 5-HT<sub>2B</sub>Rs in *c-kit*<sup>+</sup> (CD117 positive) cells. Importantly, *c-kit* negative cells were negative for 5-HT<sub>2B</sub>R expression (**Fig 1c**). Together, these observations support that 5-HT<sub>2B</sub>Rs may affect the differentiation of hematopoietic and bone-marrow precursor cells.

**Alterations of bone-marrow composition in 5-HT<sub>2B</sub>R mutant mice.** From these blood defects in 5-HT<sub>2B</sub><sup>-/-</sup> mice, we could expect associated alterations in bone-marrow cell composition. Using FACS analysis, we determined the bone-marrow composition of WT and 5-HT<sub>2B</sub><sup>-/-</sup> mice (**Fig 2**). In accordance with our blood results, we found in 5-HT<sub>2B</sub>R mutant bone-marrow a significant increase in Cd11b+/Gr+ that represents granulocyte precursors (**Fig 2a**). Interestingly, this was associated with a significant reduction in Cd11b-/Cd31+ population that labeled immature endothelial progenitor cells in 5-HT<sub>2B</sub><sup>-/-</sup> mice (**Fig 2d**). Together, these observations support that the lack of 5-HT<sub>2B</sub>Rs alters the differentiation of myeloid precursor cells; these precursors may be required for endothelial progenitor cells that have been proposed to participate to the development of pulmonary hypertension and pulmonary vascular remodeling<sup>26</sup>.

**Mice with restricted ablation of 5-HT<sub>2B</sub>Rs to bone-marrow are resistant to pulmonary hypertension.**

We investigated if 5-HT<sub>2B</sub>Rs interact with bone-marrow derived stem cells in the development of pulmonary hypertension. For this, we generated mice with restricted deletion or rescue of 5-HT<sub>2B</sub>Rs in bone-marrow cells using lethal total body irradiation to eliminate all stem cells combined to bone-marrow transplantation (**Fig 3a- Methods -Supplementary Methods**). Strikingly, following this procedure, we found that only mice transplanted with WT bone-marrow (i.e. expressing 5-HT<sub>2B</sub>Rs) responded to chronic hypoxia in increasing their pulmonary arterial pressure as measured in the heart by the right ventricular systolic pressure (RVSP). Irradiated mice transplanted with WT bone-marrow presented a significant increase in RVSP, including 5-HT<sub>2B</sub><sup>-/-</sup> host mice after 3 weeks of hypoxia. By contrast, all mice having received bone-marrow from 5-HT<sub>2B</sub><sup>-/-</sup> mice, were completely insensitive to hypoxia and never showed any sign of RVSP increase, including WT host mice (**Fig 3b**). Mice with restricted 5-HT<sub>2B</sub>R deletion to bone-marrow thus behaved as RS-127445-treated WT, or full 5-HT<sub>2B</sub><sup>-/-</sup> mice<sup>16</sup>. To exclude putative bias in these results due to the hypoxic model of pulmonary hypertension, we tested the effects of bone-marrow transplantation using another animal model of pulmonary hypertension induced by a single MCT injection. Strictly similar results were obtained in MCT injected mice: only mice with 5-HT<sub>2B</sub>R expressing WT bone-marrow developed an increase in RVSP (**Fig S1**). These results support a need for 5-HT<sub>2B</sub>R-dependent processes in bone-marrow for the development of pulmonary hypertension.

**Lung 5-HT<sub>2B</sub>R- and *c-kit*-expressing cells are of bone-marrow origin.** Since an increase in lung 5-HT<sub>2B</sub>R expression had been previously documented in PAH<sup>16,17</sup>, we tested lung 5-HT<sub>2B</sub>R expression after irradiation and bone-marrow replacement. Noteworthy, irradiated 5-HT<sub>2B</sub><sup>-/-</sup> mice having received WT bone-marrow and exposed to hypoxia showed a similar increase in lung 5-HT<sub>2B</sub>R expression (about 7 fold) as irradiated WT host mice having received WT bone-marrow (**Fig 3c-d**). To the opposite, WT mice having

received 5-HT<sub>2B</sub><sup>-/-</sup> bone-marrow showed no increase in 5-HT<sub>2B</sub>R expression in the lung after exposure to hypoxia as 5-HT<sub>2B</sub><sup>-/-</sup> mice having received 5-HT<sub>2B</sub><sup>-/-</sup> bone-marrow. Strictly similar results were obtained in MCT injected mice: only mice with 5-HT<sub>2B</sub>R expressing WT bone-marrow developed an increase in lung 5-HT<sub>2B</sub>R overexpression (**Fig S1**). These results support that lung cells overexpressing 5-HT<sub>2B</sub>R during pulmonary hypertension are originating from bone-marrow precursors and not from lung resident cells. Since *c-kit* has been shown to be expressed in lungs of PAH patients<sup>26,27</sup>, we completed these investigations by performing *c-kit* and 5-HT<sub>2B</sub>R immuno-histochemistry of lung tissues. Interestingly, a partially overlapping increase in both *c-kit* and 5-HT<sub>2B</sub>R expression was observed in small arteries of hypoxic WT or 5-HT<sub>2B</sub><sup>-/-</sup> mice transplanted with 5-HT<sub>2B</sub>R expressing WT bone-marrow, but not with 5-HT<sub>2B</sub><sup>-/-</sup> bone-marrow (**Fig 3c-e**). These combinations of bone-marrow transplantation into different background confirm that, *c-kit* and 5-HT<sub>2B</sub>R expressing cells in the diseased lung are requiring active 5-HT<sub>2B</sub>R in bone-marrow stem cells. In addition, these results support that the initial trigger of pulmonary hypertension is originating from bone-marrow progenitors.

**Alterations of bone-marrow differentiation after 5-HT<sub>2B</sub>R inhibition.** Previously, 5-HT has been shown to enhance the expansion of CD34<sup>+</sup> cells to early stem/progenitors (CD34<sup>+</sup> cells, colony-forming unit-mixed [CFU-GEMM]) and committed progenitors (burst-forming unit/colony-forming unit-erythroid [BFU/CFU-E]<sup>20,28</sup>. We have assessed the effect of 5-HT<sub>2B</sub>R blockade using the selective antagonist, RS-127445, during the differentiation of total bone-marrow cell cultures from mice. When mice bone-marrow was used in methylcellulose colony-forming assay, we observed a significant reduction in both CFU-GEMM/CFU-GM and CFU-E/BFU-E colonies with the 5-HT<sub>2B</sub>R antagonist (**Fig. 4a**). Similar results were obtained when analyzing the clonogenic potential of isolated *c-kit*<sup>+</sup> cells from mice bone-marrow that showed also a reduction in both CFU-GEMM/CFU-GM and CFU-E/BFU-E colonies with the 5-HT<sub>2B</sub>R antagonist (**Fig. 4b**). To validate these findings in humans, we then analyzed the clonogenic potential of

human blood cord CD34<sup>+</sup> cells in the methylcellulose colony-forming assay. The most immature CD34<sup>+</sup> cells isolated from cord blood, were expanded for 14 days in methylcellulose, with or without 5-HT<sub>2B</sub>R or 5-HT<sub>7</sub>R antagonists. The number of BFU-E/CFU-E colonies arising from CD34<sup>+</sup> cells was strongly reduced (5 fold) upon exposure to the antagonist RS-127445, while no significant difference was observed using the 5-HT<sub>7</sub>R antagonist, SB-269970 (**Fig. 4c**). By flow cytometric purification of bone-marrow cells, we also identified expression of 5-HT<sub>2B</sub>Rs in *c-kit*<sup>+</sup> (CD117 positive) cells. Importantly, *c-kit*<sup>-</sup> cells, as *c-kit*<sup>+</sup> cells after chronic STI-571 treatment (5 weeks, see below) or *c-kit*<sup>+</sup> cells from 5-HT<sub>2B</sub><sup>-/-</sup> mice were negative for 5-HT<sub>2B</sub>R expression (**Fig 4d**). Interestingly, in cultures performed in the presence of GM-CSF, we found that the lack of 5-HT<sub>2B</sub>R reduced the apparition of granulocyte precursors (CD11b<sup>+</sup>/Gr<sup>+</sup>) lineage by 50% (**Fig 4e**). Since GM-CSF controls the differentiation of monocyte/macrophages and all granulocytes<sup>29</sup>, this finding strongly supports a role of 5-HT<sub>2B</sub>Rs at precursor stage differentiation toward these lineages. These results confirm the need of the 5-HT<sub>2B</sub>R action including in human stem cells for early stem/progenitor cells and multilineage committed progenitors differentiation of myeloid and erythroid lineages that may ultimately participate in the pulmonary hypertension pathogenesis.

### **Blocking tyrosine kinase *c-kit* activity prevents pulmonary hypertension and 5-HT<sub>2B</sub>R**

**overexpression.** Among compounds that are under investigation for PAH treatment, the tyrosine kinase inhibitor, STI-571 has been shown to be effective<sup>30</sup>. We thus compared the 5-HT<sub>2B</sub> selective antagonist RS-127445 with STI-571 in their ability to prevent pulmonary hypertension in the mouse hypoxic model and found they were both equally effective for tested parameters (**Fig. 5a, Fig S2a-d**). However, we found that only STI-571 was able to prevent the increase in lung 5-HT<sub>2B</sub>R expression (**Fig. 5b**). We therefore investigated the kinetic in establishment of hypoxic pulmonary hypertension with that of 5-HT<sub>2B</sub>R overexpression. Interestingly, we found that STI-571 was able to prevent both the increase in RVSP and the

5-HT<sub>2B</sub>R overexpression with similar kinetic (**Fig 5c,d**). These results support an action of both inhibitors at a common pathway that ultimately participate in the pulmonary hypertension pathogenesis.

## Discussion

Both 5-HT and bone-marrow-derived stem cells have been shown to participate to some extent in PAH. Our work establishes, for the first time, a causal link between these two issues by showing (i) the initial functions in pulmonary hypertension of 5-HT<sub>2B</sub>R restricted to bone-marrow cells, (ii) the contribution to stem cells differentiation/proliferation of 5-HT<sub>2B</sub>Rs, and (iii) the critical functions of bone-marrow-derived cells for pulmonary hypertension development.

Several independent investigations have described a mobilization of bone-marrow-derived cells during pulmonary hypertension <sup>2</sup>. Not only cells expressing *c-kit* are mobilized from bone-marrow in the circulation in response to hypoxia, but they are also found in the remodeled lung vessel wall in PAH <sup>(7,8,12,24,31 and Fig 3)</sup>. Activation of *c-kit* was reported as necessary for mobilization of reparative bone-marrow progenitor cells <sup>32</sup> and for the remodeling of blood vessels from these progenitor cells <sup>3,27</sup>. Recently, a role of *c-kit*<sup>+</sup> progenitors in hypoxia-induced vascular remodeling <sup>26,33</sup> was evidenced: stromal derived factor-1 (SDF-1/CXCL12) and its receptors CXCR4 and CXCR7 have been shown to be critical for homing of hematopoietic *c-kit*<sup>+</sup> progenitor cells in the perivascular niche, including in chronic hypoxia-exposed mice. These mice showed increased lung expression of CXCR4, CXCR7 and CXCL12, associated with significantly increased RVSP, vascular remodeling and perivascular *c-kit*<sup>+</sup>/*sca-1*<sup>+</sup> progenitor cell accumulation <sup>26</sup>. In humans, pulmonary arterial lesions are also associated with expression of CXCL12 that may recruit *c-kit*<sup>+</sup> cells <sup>27</sup>. As we previously reported <sup>16</sup>, 5-HT<sub>2B</sub>Rs were found overexpressed (as in mice) on human vascular SMC layer in lungs from PAH patients and at least partially colocalized with SMC alpha-actin <sup>17</sup>. Independently, *c-kit* was shown to colocalize partially with SMC alpha-actin <sup>26</sup>. We show, here, that *c-kit* and 5-HT<sub>2B</sub>R staining are also partially overlapping in hypoxic lung arteries, supporting a common link to SMC lineage. Further work will be need in order to clarified the exact contribution of these different partners into vascular remodeling during PAH.

STI-571 is a protein-tyrosine kinase inhibitor, which selectively blocks *Abl*, *c-kit* and PDGFR<sup>34</sup>, but has no 5-HT<sub>2B</sub>R agonist or antagonist properties (see supplementary data of reference<sup>35</sup>). Expression of PDGFR was found to be significantly increased in lung tissue from pulmonary arterial hypertension patients and beneficial action of STI-571 has been proposed to act directly at lung PDGFR<sup>36</sup>, which is well known to mediate SMC proliferation. However, in experimental vascular injury, expression of PDGFR $\beta$  by medial SMCs is upregulated only several days after vascular injury<sup>3</sup>, while *c-kit*<sup>+</sup> cells appear within the vessel medial wall by the first day and early intervention with STI-571 can significantly attenuate intima hyperplasia formation. Finally, the STI-571-mediated inhibition of hyperplasia appears to act through the *c-kit* pathway, since the blocking anti-*c-kit* receptor monoclonal antibody ACK2 generated similar results<sup>3</sup>. In the present work, we show that the 5-HT<sub>2B</sub>R-overexpression in lungs is fully prevented by STI-571 treatment or by lack of 5-HT<sub>2B</sub>R in bone-marrow, both of which can hardly be explained only by a direct inhibition of lung resident cells expressing PDGFR. Our result strongly supports that cells, which give rise to 5-HT<sub>2B</sub>R expressing cells in lungs, are *c-kit*<sup>+</sup> progenitor cells originating from bone-marrow, although contribution of other receptors-tyrosine kinase sensitive to STI-571 cannot be excluded.

Plasma 5-HT is increased in PAH patients, even after lung transplantation<sup>37</sup>, which suggests that 5-HT either is an extra pulmonary causative factor in PAH or is associated with such a factor. Deletion of tryptophan hydroxylase 1 (the limiting enzyme in peripheral 5-HT synthesis), SERT or 5-HT<sub>1B</sub>Rs reduces pulmonary vascular remodeling and hypoxic pulmonary hypertension<sup>38</sup>. Locally, 5-HT has been shown to be a potent vasoconstrictor of isolated pulmonary arteries but also to stimulate pulmonary SMC proliferation. It has thus been implicated in various processes associated to pulmonary vascular remodeling. We found here 5-HT<sub>2B</sub>R expression in both *c-kit*<sup>+</sup> bone-marrow-derived cells and *c-kit*<sup>+</sup> circulating blood cells. In addition, the results of bone-marrow transplantation establish for the first time that 5-HT<sub>2B</sub>R-expressing cells in hypertensive lungs are exclusively of extra-pulmonary origin probably deriving from *c-*

*kit*<sup>+</sup> bone-marrow precursors. These results clearly reveal that the initial requirement for 5-HT<sub>2B</sub>Rs in PAH is extrinsic to the lung and bone-marrow-derived, independently of resident lung cells.

Although likely not instructive in stem cell lineages, 5-HT may rather modulate the balance between different lineages by participating at the proliferation and/or survival of specific lineage via its receptors (**Fig 6**). Similar to what we found here using 5-HT<sub>2B</sub>R antagonist RS-127445, a decrease in colony-forming capacity was observed with STI-571 with inhibition of both CFU-GEMM and BFU-E formation attributed to a reduction of cell proliferation and/or an apoptotic effect<sup>39</sup>. In addition, we found 5-HT<sub>2B</sub>R expression in *c-kit*<sup>+</sup> bone-marrow cells, but not in these cells after STI-571 treatment. To the opposite, 5-HT significantly enhanced the expansion of CD34<sup>+</sup> cells to early stem/progenitors (CFU-GEMM) and committed progenitors (BFU/CFU-E)<sup>20</sup>. Recently, the absence of 5-HT was shown to reduce erythroid precursors in bone-marrow via 5-HT<sub>2A</sub> and 5-HT<sub>2B</sub>Rs since a 5-HT<sub>2</sub> agonist, PNU 22394, produced the same proliferative effect on erythroid precursors as observed with 5-HT<sup>28</sup>. At early stages of megakaryocytopoiesis, 5-HT regulates proliferation and survival by antiapoptotic effects on megakaryoblastic cells<sup>40</sup>. On megakaryocytic cell line HEL, pretreatment with 5-HT protected subsequent nitric oxide-induced apoptosis<sup>41</sup>. We previously showed that 5-HT<sub>2B</sub>Rs were required for proliferation of embryonic and survival of newborn cardiomyocytes via regulation of mitochondrial membrane permeability, caspase activation, and Akt/ERK1/2 pathways<sup>42</sup>. Combined with these observations, the present data clearly highlight the importance of 5-HT via 5-HT<sub>2B</sub>Rs at various levels of the myeloid lineages as summarized on the scheme (**Fig 6**). Our data support their requirement for proliferation/survival after the *c-kit*-dependent mobilization of precursors from bone-marrow.

Bone-marrow progenitor cell mobilization is a physiological response to hypoxic conditions<sup>4,6</sup>, and altered circulating bone-marrow-derived precursors have been reported in PAH as contributors to vascular remodeling<sup>7,8,24,31</sup>. Patients with myeloproliferative diseases often develop PAH, but this secondary form of PAH has been reported to resolve with treatment of the underlying myeloproliferative process<sup>43</sup>. Other data



highlight a potential interdependence of dysregulated hematopoiesis and abnormal pulmonary vascular endothelial behavior that may play a key role in the development of PAH<sup>10</sup>. It was previously demonstrated that cells from the mononuclear lineage could differentiate into endothelial- and SMC-like cells at injured vessels<sup>44-47</sup> and that circulating precursors of this lineage can contribute to hypoxia-induced pulmonary vascular remodeling<sup>6</sup>. In this context, a stimulus-dependent generation of pro-angiogenic and bone-marrow-derived early progenitors could be required for physiological repair response to ongoing pulmonary vascular shear stress and endothelial injury. That this process ultimately contribute to the abnormal proliferation of SMC and endothelial cells leading to progression of pulmonary hypertension was still hypothetical<sup>9</sup>. We validate here the interdependence of dysregulated hematopoiesis and abnormal pulmonary vascular remodeling that play key roles in the development of PAH.

Studies in patients and experimental models have led to a dichotomy of views regarding the role of bone-marrow progenitor cells in PAH. On the one hand, a relative deficiency of circulating endothelial precursor cells might contribute to pulmonary vascular pathology; and the transfusion of native autologous or genetically modified endothelial precursor cells could offer a novel cell-based therapy; long-term pharmacologic augmentation of endogenous progenitors represents an additional strategy. On the other hand, dysfunctional, apoptotic-resistant, and proliferative pulmonary vascular cells are implicated in the pathogenesis of PAH<sup>12</sup>; and the inclusion of progenitor cells in vascular lesions may have adverse long-term consequences<sup>9</sup>. Our work reveals that the absence of 5-HT<sub>2B</sub>Rs generates permanent alterations of blood and bone-marrow composition in myeloid lineages and in particular, in endothelial/SMC progenitors. The present finding clearly supports a role of 5-HT<sub>2B</sub>Rs in the maturation of different myeloid progenitors including immature endothelial/SMC precursors required for PAH pathogenesis.

Our work supports the concept that bone-marrow-derived cells contributing to pulmonary vascular remodeling may be the link between PAH and bone-marrow-related hematological disorders<sup>4</sup>. By demonstrating a causal role for 5-HT<sub>2B</sub>Rs in bone-marrow progenitors, which controls precursor cells

contributing to pulmonary hypertension vascular remodeling, this work switches the attention about PAH initiating events from an intrinsic lung issue to a bone-marrow originating problem.

**Acknowledgements**

We thank O. Hermine and Anne Roumier for critical reading of the manuscript, K. Boutourlinsky and Imane Moutkine for excellent technical assistance.

**Footnotes****Source of funding**

This work has been supported by funds from the Centre National de la Recherche Scientifique, the Institut National de la Santé et de la Recherche Médicale, the Université Pierre et Marie Curie, and by grants from the Fondation de France, the Fondation pour la Recherche Médicale, and the European Union (DEVANX). S. Diaz is supported by a Region Ile de France DIM STEM and S. Doly by a Lefoulon-DeLalande fellowship.

**Authors contributions**

Conceived and designed the experiments: JML, FC, LM. Performed the experiments: PH, JC, ZM, CC, AB, SD, SLD, SH. Analyzed the data: ZM, FC, JML, MH, LM. Contributed reagents/materials/analysis tools: PH, JC, ZM, CC, SD, SLD, SH. Wrote the paper: ZM, FC, JML, MH, LM.

**Disclosures**

None.

**References**

1. Archer SL, Weir EK, Wilkins MR. Basic Science of Pulmonary Arterial Hypertension for Clinicians: New Concepts and Experimental Therapies. *Circulation*. 2010;121:2045-2066.
2. Stenmark KR, Fagan KA, Frid MG. Hypoxia-induced pulmonary vascular remodeling: cellular and molecular mechanisms. *Circ Res*. 2006;99:675-691.
3. Wang CH, Anderson N, Li SH, et al. Stem cell factor deficiency is vasculoprotective: unraveling a new therapeutic potential of imatinib mesylate. *Circ Res*. 2006;99:617-625.
4. Asosingh K, Aldred MA, Vasanji A, et al. Circulating angiogenic precursors in idiopathic pulmonary arterial hypertension. *Am J Pathol*. 2008;172:615-627.
5. Marsboom G, Pokreisz P, Gheysens O, et al. Sustained endothelial progenitor cell dysfunction after chronic hypoxia-induced pulmonary hypertension. *Stem Cells*. 2008;26:1017-1026.
6. Frid MG, Brunetti JA, Burke DL, et al. Hypoxia-induced pulmonary vascular remodeling requires recruitment of circulating mesenchymal precursors of a monocyte/macrophage lineage. *Am J Pathol*. 2006;168:659-669.
7. Hayashida K, Fujita J, Miyake Y, et al. Bone marrow-derived cells contribute to pulmonary vascular remodeling in hypoxia-induced pulmonary hypertension. *Chest*. 2005;127:1793-1798.
8. Davie NJ, Crossno JT, Frid MG, et al. Hypoxia-induced pulmonary artery adventitial remodeling and neovascularization: contribution of progenitor cells. *Am J Physiol Lung Cell Mol Physiol*. 2004;286:L668-678.
9. Diller G-P, Thum T, Wilkins MR, Wharton J. Endothelial progenitor cells in pulmonary arterial hypertension. *Trends Cardiovasc Med*. 2010;20:22-29.
10. Yoder M, Rounds S. Bad blood, bad endothelium: ill fate? *Blood*. 2011;117:3479-3480.
11. Limsuwan A, Pakakasama S, Rochanawutanon M, Hong-Eng S. Pulmonary arterial hypertension after childhood cancer therapy and bone marrow transplantation. *Cardiology*. 2006;105:188-194.

12. Farha S, Asosingh K, Xu W, et al. Hypoxia-inducible factors in human pulmonary arterial hypertension: a link to the intrinsic myeloid abnormalities. *Blood*. 2011;117:3485-3493.
13. Steward CG, Pellier I, Mahajan A, et al. Severe pulmonary hypertension: a frequent complication of stem cell transplantation for malignant infantile osteopetrosis. *Br J Haematol*. 2004;124:63-71.
14. Chan SY, Loscalzo J. Pathogenic mechanisms of pulmonary arterial hypertension. *J Mol Cell Cardiol*. 2008;44:14-30.
15. Weir EK, Obreztkhikova M, Hong Z. Fenfluramine: riddle or Rosetta stone? *Eur Respir J*. 2008;31:232-235.
16. Launay JM, Hervé P, Peoc'h K, et al. Function of the serotonin 5-hydroxytryptamine 2B receptor in pulmonary hypertension. *Nat Med*. 2002;8:1129-1135.
17. Dumitrascu R, Kulcke C, Königshoff M, et al. Terguride ameliorates monocrotaline-induced pulmonary hypertension in rats. *Eur Respir J*. 2011;37:1104-1118.
18. Zopf DA, Neves LAAd, Nikula KJ, Huang J, Senese PB, Gralinski MR. C-122, a novel antagonist of serotonin receptor 5-HT(2B), prevents monocrotaline-induced pulmonary arterial hypertension in rats. *Eur J Pharmacol*. 2011;670:195-203.
19. Porvasnik SL, Germain S, Embury J, et al. PRX-08066, a novel 5-hydroxytryptamine receptor 2B antagonist, reduces monocrotaline-induced pulmonary arterial hypertension and right ventricular hypertrophy in rats. *J Pharmacol Exp Ther*. 2010;334:364-372.
20. Yang M, Li K, Ng PC, et al. Promoting effects of serotonin on hematopoiesis: ex vivo expansion of cord blood CD34+ stem/progenitor cells, proliferation of bone marrow stromal cells, and antiapoptosis. *Stem Cells*. 2007;25:1800-1806.
21. Farber HW, Loscalzo J. Pulmonary arterial hypertension. *N Engl J Med*. 2004;351:1655-1665.
22. Nebigil CG, Choi D-S, Dierich A, et al. Serotonin 2B receptor is required for heart development. *Proc Natl Acad Sci U S A*. 2000;97:9508-9513.

23. Potteaux S, Combadiere C, Esposito B, et al. Role of bone marrow-derived CC-chemokine receptor 5 in the development of atherosclerosis of low-density lipoprotein receptor knockout mice. *Arterioscler Thromb Vasc Biol.* 2006;26:1858-1863.
24. Spees JL, Whitney MJ, Sullivan DE, et al. Bone marrow progenitor cells contribute to repair and remodeling of the lung and heart in a rat model of progressive pulmonary hypertension. *FASEB J.* 2008;22:1226-1236
25. Schneider E, Machavoine F, Pleau JM, et al. Organic cation transporter 3 modulates murine basophil functions by controlling intracellular histamine levels. *J Exp Med.* 2005;202:387-393.
26. Gambaryan N, Perros F, Montani D, et al. Targeting of c-kit+ haematopoietic progenitor cells prevents hypoxic pulmonary hypertension. *Eur Respir J.* 2011;37:1392-1399.
27. Montani D, Perros F, Gambaryan N, et al. C-kit-positive cells accumulate in remodeled vessels of idiopathic pulmonary arterial hypertension. *Am J Respir Crit Care Med.* 2011;184:116-123.
28. Amireault P, Hatia S, Bayard E, et al. Ineffective erythropoiesis with reduced red blood cell survival in serotonin-deficient mice. *Proc Natl Acad Sci USA.* 2011;108:13141-13146.
29. Hercus TR, Thomas D, Guthridge MA, et al. The granulocyte-macrophage colony-stimulating factor receptor: linking its structure to cell signaling and its role in disease. *Blood.* 2009;114:1289-1298.
30. Ghofrani HA, Morrell NW, Hoeper MM, et al. Imatinib in pulmonary arterial hypertension patients with inadequate response to established therapy. *Am J Respir Crit Care Med.* 2010;182:1171-1177.
31. Sata M. Role of circulating vascular progenitors in angiogenesis, vascular healing, and pulmonary hypertension: lessons from animal models. *Arterioscler Thromb Vasc Biol.* 2006;26:1008-1014.
32. Fazel SS, Chen L, Angoulvant D, et al. Activation of c-kit is necessary for mobilization of reparative bone marrow progenitor cells in response to cardiac injury. *FASEB J.* 2008;22:930-940.

33. Gambaryan N, Perros F, Montani D, Cohen-Kaminsky S, Mazmanian GM, Humbert M. Imatinib inhibits bone marrow-derived c-kit<sup>+</sup> cell mobilisation in hypoxic pulmonary hypertension. *Eur Respir J*. 2010;36:1209-1211.
34. Buchdunger E, Cioffi CL, Law N, et al. Abl protein-tyrosine kinase inhibitor STI571 inhibits in vitro signal transduction mediated by c-kit and platelet-derived growth factor receptors. *J Pharmacol Exp Ther*. 2000;295:139-145.
35. Huang XP, Setola V, Yadav PN, et al. Parallel functional activity profiling reveals valvulopathogens are potent 5-HT<sub>2B</sub> receptor agonists: implications for drug safety assessment. *Mol Pharmacol*. 2009;76:710-722.
36. Schermuly RT, Dony E, Ghofrani HA, et al. Reversal of experimental pulmonary hypertension by PDGF inhibition. *J Clin Invest*. 2005;115:2811-2821.
37. Hervé P, Launay JM, Scrobohaci ML, et al. Increased plasma serotonin in primary pulmonary hypertension. *Am J Med*. 1995;99:249-254.
38. Morecroft I, Dempsey Y, Bader M, et al. Effect of tryptophan hydroxylase 1 deficiency on the development of hypoxia-induced pulmonary hypertension. *Hypertension*. 2007;49:232-236.
39. Pirson L, Baron F, Meuris N, et al. Despite inhibition of hematopoietic progenitor cell growth in vitro, the tyrosine kinase inhibitor imatinib does not impair engraftment of human CD133<sup>+</sup> cells into NOD/SCIDbeta2mNull mice. *Stem Cells*. 2006;24:1814-1821.
40. Liu YS, Yang M. The effect of 5-hydroxytryptamine on the regulation of megakaryocytopoiesis. *Hematology*. 2006;11:53-56.
41. Battinelli E, Loscalzo J. Nitric oxide induces apoptosis in megakaryocytic cell lines. *Blood*. 2000;95:3451-3459.

42. Nebigil CG, Etienne N, Messaddeq N, Maroteaux L. Serotonin is a novel survival factor of cardiomyocytes: mitochondria as a target of 5-HT<sub>2B</sub>-receptor signaling. *FASEB J.* 2003;17:1373-1375.
43. Popat U, Frost A, Liu E, et al. High levels of circulating CD34 cells, dacrocytes, clonal hematopoiesis, and JAK2 mutation differentiate myelofibrosis with myeloid metaplasia from secondary myelofibrosis associated with pulmonary hypertension. *Blood.* 2006;107:3486-3488.
44. Asahara T, Murohara T, Sullivan A, et al. Isolation of putative progenitor endothelial cells for angiogenesis. *Science.* 1997;275:964-967.
45. Shimizu K, Sugiyama S, Aikawa M, et al. Host bone-marrow cells are a source of donor intimal smooth- muscle-like cells in murine aortic transplant arteriopathy. *Nat Med.* 2001;7:738-741.
46. Sata M, Saiura A, Kunisato A, et al. Hematopoietic stem cells differentiate into vascular cells that participate in the pathogenesis of atherosclerosis. *Nat Med.* 2002;8:403-409.
47. Bailey AS, Willenbring H, Jiang S, et al. Myeloid lineage progenitors give rise to vascular endothelium. *Proc Natl Acad Sci USA.* 2006;103:13156-13161.



**Figure legends****Figure 1:**

(a) In total blood, the number of white blood cells (WBC), red blood cells (RBC), the mean red blood cell volume (MRBCV), the hematocrit, and the hemoglobin content were not different among genotypes, while the number of platelets was significantly reduced and the percent of neutrophil/granulocyte (%N/Gr) significantly increased in the blood of 5-HT<sub>2B</sub><sup>-/-</sup> mice. Values are means±sem (n=20, P < 0.05). Any statistical difference by unpaired t test vs. control is indicated by \*\*\*P < 0.001; \*\*P < 0.01. (b) Flow cytometric analysis of blood cells using cell-surface markers allows identification of different populations. Mac-1 (CD11b) for monocytes/macrophages, Gr-1 (Gr+) for Granulocytes, PECAM (CD31) for endothelial cells / platelets / macrophages antibodies were used to identify lin<sup>+</sup> cells or late multipotent progenitors. In blood, while the number of *c-Kit*<sup>+</sup>*Lin*<sup>-</sup> cells was unaffected, the 5-HT<sub>2B</sub><sup>-/-</sup> mice had reduced number of CD11b-/CD31+, immature endothelial progenitors. Values are means±sem (n=10, P < 0.05). Any statistical difference by unpaired t test vs. control is indicated by \*\*\*P < 0.001. (c) By flow cytometric purification of peripheral blood mononuclear cells, we first identified expression of 5-HT<sub>2B</sub>Rs in *c-kit*<sup>+</sup> cells but not in *c-kit*<sup>-</sup> cells (28.4±7.9 fmoles/mg protein vs. 4.6±3.2, p<0.05, n=14). Any statistical difference by unpaired t test vs. control is indicated by \*\*\*P < 0.001.

**Figure 2:**

(a) In bone-marrow of 5-HT<sub>2B</sub><sup>-/-</sup> mice, the number of CD11b+/Gr+ cells, corresponding to committed precursor cells of the monocyte/granulocyte lineage, was significantly increased as shown by the distribution of cells by FACS (a-left panel, representative experiment) and quantification in percent of total cells (a-right panel, n=3). No modifications of the *c-kit*<sup>+</sup>*Lin*<sup>-</sup> cells, multipotent stem cells (b) or Cd11b+CD31+ cells (c) was observed in bone-marrow of 5-HT<sub>2B</sub><sup>-/-</sup> mice. However, bone-marrow of 5-HT<sub>2B</sub><sup>-/-</sup> mice present a significant reduction of CD11b-/CD31+, immature committed endothelial/SMC

precursor cells (**d**). Values are means $\pm$ sem, n=3 independent determinations. Any statistical difference by unpaired t test vs. control is indicated by \*\*\* $P < 0.001$ .

**Figure 3:**

(a) WT mice transplanted with bone-marrow cells from WT or 5-HT<sub>2B</sub><sup>-/-</sup> (WT\_WT, WT\_KO, respectively) and 5-HT<sub>2B</sub><sup>-/-</sup> (KO) mice transplanted with bone-marrow cells from 5-HT<sub>2B</sub><sup>-/-</sup> or WT (KO\_KO, KO\_WT, respectively) were exposed to progressive hypoxia (20% to 10% for 3 weeks). (b) Pulmonary arterial pressure as assessed by RVSP increased in mice bearing WT bone-marrow either in WT (WT\_WT) or 5-HT<sub>2B</sub><sup>-/-</sup> (KO\_WT) background. Conversely, mice with 5-HT<sub>2B</sub><sup>-/-</sup> bone-marrow into WT (WT\_KO) or 5-HT<sub>2B</sub><sup>-/-</sup> (KO\_KO) background did not show any RVSP increase. (c) Immunohistochemistry of *c-Kit* and 5-HT<sub>2B</sub>R. Staining was performed with antibodies against 5-HT<sub>2B</sub>R (pink; rabbit polyclonal AbCam ) or against *c-Kit* (blue; rat monoclonal AbCam). Scale bars represent 50  $\mu$ m. (d) Lung 5-HT<sub>2B</sub>R expression (Bmax) increased in mice bearing WT bone-marrow not only in WT (WT\_WT) but also in 5-HT<sub>2B</sub><sup>-/-</sup> (KO\_WT) background. Conversely, not only mice with 5-HT<sub>2B</sub><sup>-/-</sup> bone-marrow into 5-HT<sub>2B</sub><sup>-/-</sup> (KO\_KO), but also in WT (WT\_KO) background did not show any change in lung 5-HT<sub>2B</sub>R expression compared to controls WT (18 fmoles/mg prot) or full 5-HT<sub>2B</sub><sup>-/-</sup> (less than 5 fmoles/mg prot). (e) Similarly, the pulmonary perivascular *c-kit*<sup>+</sup> bone-marrow derived progenitor cell recruitment induced by hypoxia was higher in mice with WT bone-marrow to WT (WT\_WT) or 5-HT<sub>2B</sub><sup>-/-</sup> (KO\_WT) background as revealed by quantification of the immuno-labeling. Values are means $\pm$ sem (n=12,  $P < 0.05$ ). Lines are normoxic values. Any statistical difference by one way ANOVA followed by Bonferroni post hoc test is indicated by \*\*\* $P < 0.001$ .

**Figure 4:**

(a) In mouse bone-marrow cells, methyl cellulose cultures, RS-127445 reduced CFU-GEMM/CFU-GEM/BFU-E/CFU-E (n = 3 independent cultures). Statistical difference by unpaired t test vs. control is indicated by \*\*\* $P < 0.001$ , \* $P < 0.05$  vs. Vehicle. (b) Similarly, in *c-kit*<sup>+</sup> bone-marrow cells, methyl cellulose cultures, RS-127445 reduced CFU-GEMM/CFU-GEM/BFU-E/CFU-E (n = 3 independent cultures). Statistical difference by unpaired t test vs. control is indicated by \*\*\* $P < 0.001$ , \* $P < 0.05$  vs. Vehicle. (c) On methyl cellulose cultures of human blood cord CD34<sup>+</sup> cells, the 5-HT<sub>2B</sub>R antagonist RS-127445 significantly reduced expansion of BFU-E/CFU-E, burst-forming unit/colony-forming unit-erythroid, but not the 5-HT<sub>1B</sub>R antagonist SB-269970 (n = 4 independent cultures). Any statistical difference by unpaired t test vs. control is indicated by \*\*\* $P < 0.001$  vs. Vehicle, # $P < 0.05$  vs. SB-269970. (d) After flow cytometric purification of bone-marrow cells, expression of 5-HT<sub>2B</sub>Rs was detected in *c-kit*<sup>+</sup> (CD117 positive) cells (n=8), but remained undetectable in *c-kit*<sup>-</sup> cells (n=5), *c-kit*<sup>+</sup> cells after STI-571 (STI) treatment (n=6) or *c-kit*<sup>+</sup> cells from 5-HT<sub>2B</sub><sup>-/-</sup> mice (n=5). (e) On *ex vivo* expansion of total bone-marrow cells in the presence of GM-CSF driving the monocyte/macrophages and all granulocytes differentiation, the lack of 5-HT<sub>2B</sub>Rs significantly reduced the number of CD11b<sup>+</sup>/Gr<sup>+</sup> cells (n = 3-5 mice of each genotype). Any statistical difference by unpaired t test vs. control is indicated by \*\*\* $P < 0.001$  vs. 5-HT<sub>2B</sub><sup>+/+</sup> culture.

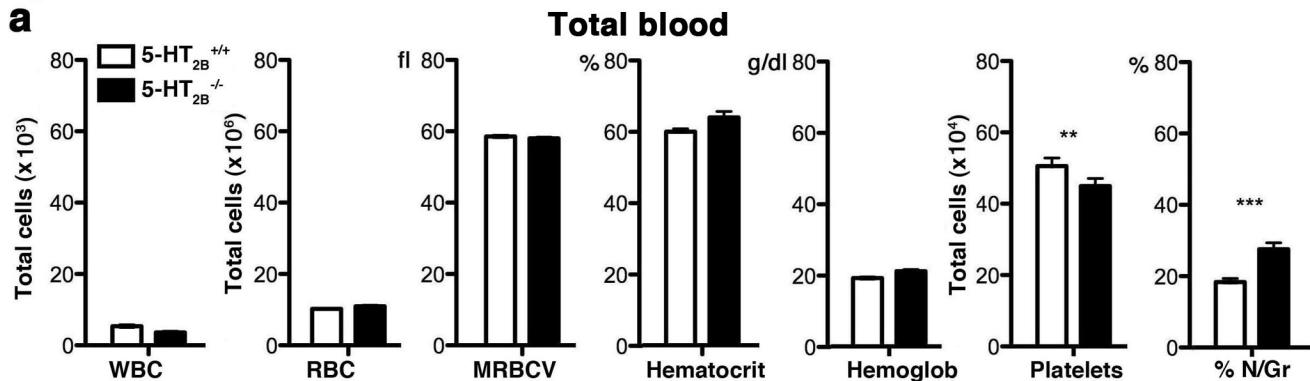
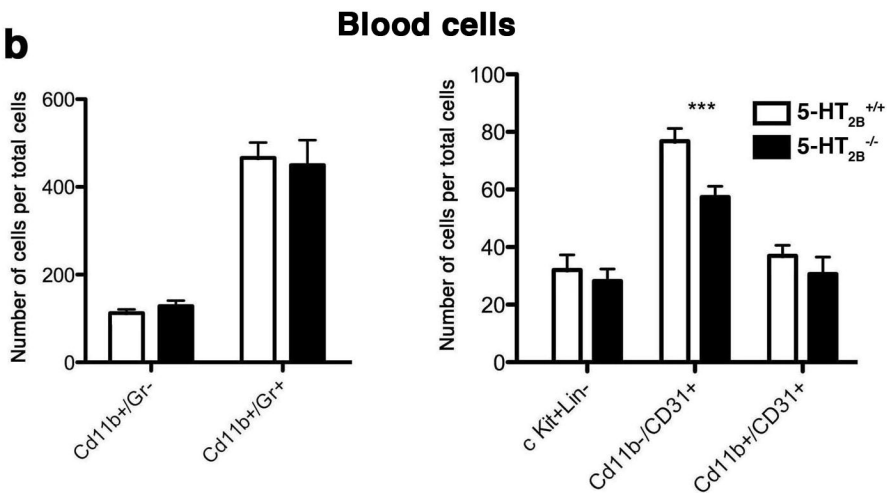
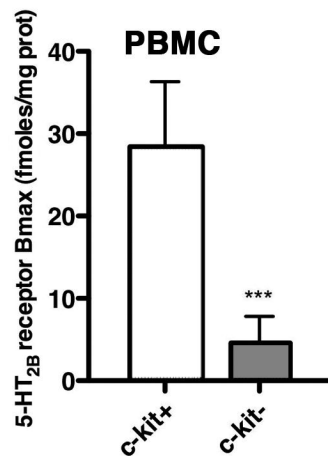
**Figure 5:**

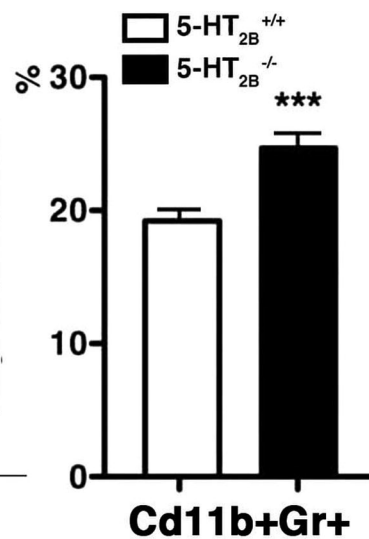
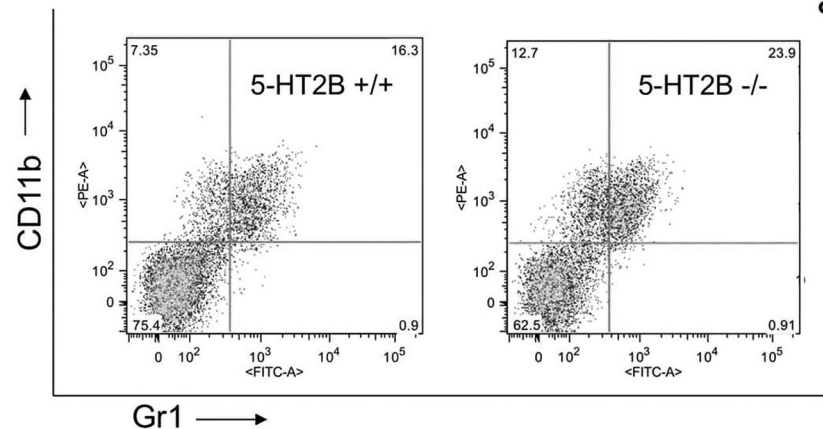
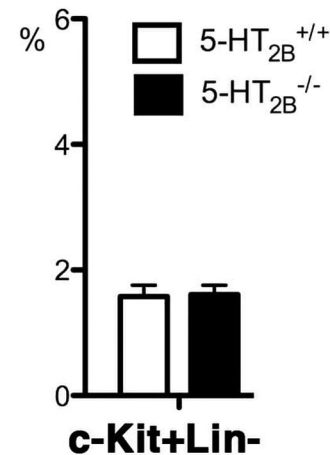
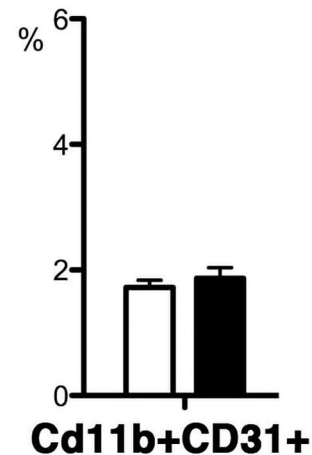
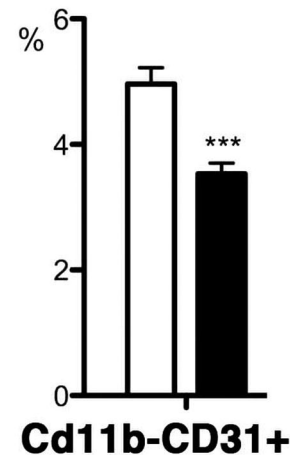
Groups of 10 WT mice were exposed to hypoxia (10% O<sub>2</sub>) for 5 weeks (H), and in the presence of either RS-127445 (0.5mg/kg-HRS), or STI-571 (1mg/kg-HST) and compared to normoxic mice (0). (a) The significant increase in hypoxia-induced RVSP was totally prevented by RS-127445 or STI-571. (b) Chronic hypoxia increased significantly the maximal number of lung 5-HT<sub>2B</sub>R specific binding sites (B<sub>max</sub>), which was not significantly prevented by RS-127445, but STI-571 did prevent this increase. (c) Groups of 10 WT

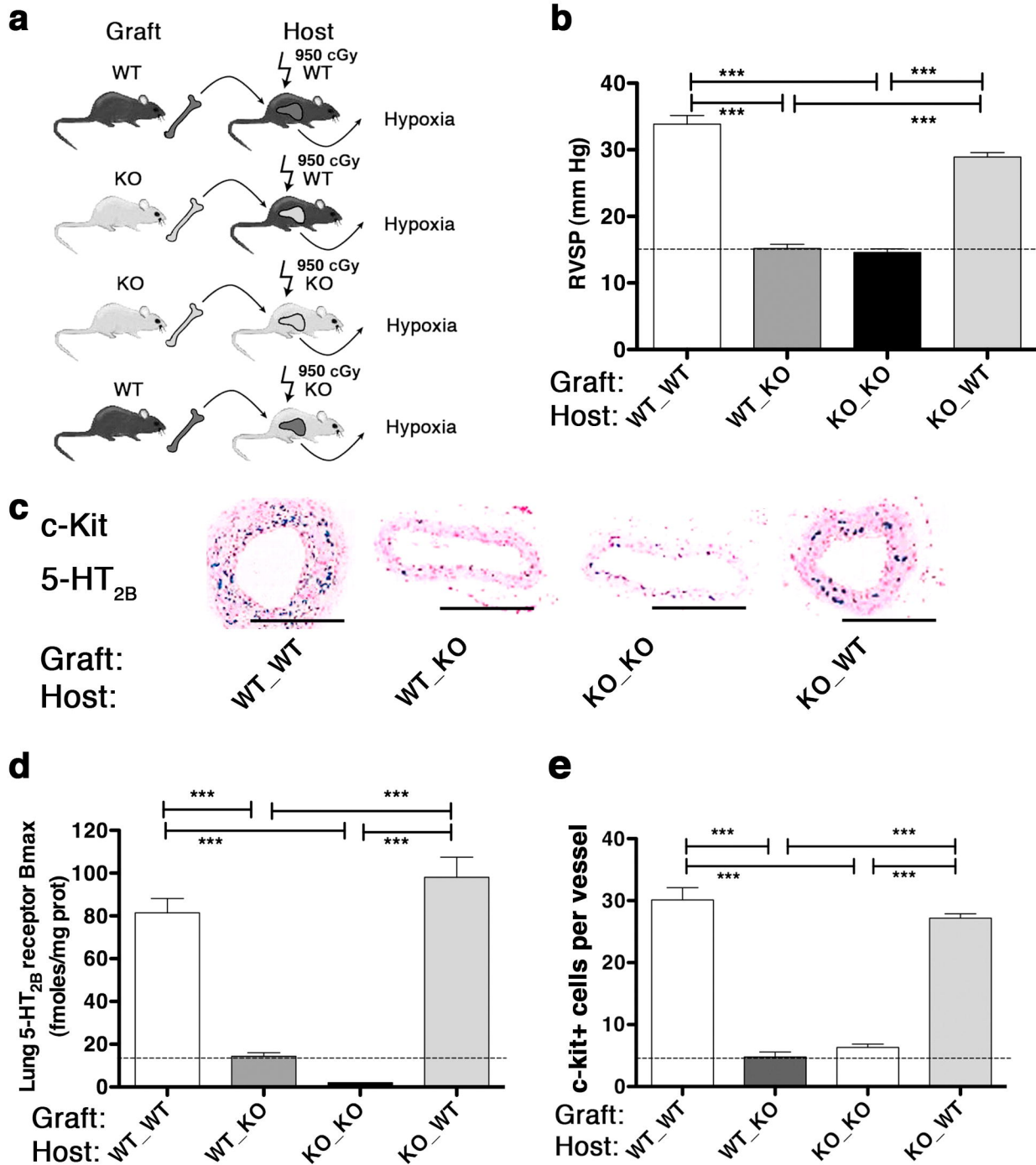
mice were exposed to hypoxia (10% O<sub>2</sub>) for 0 to 35 days in the presence of vehicle (Veh) or STI-571 (STI) (1mg/kg). The significant increase in hypoxia-induced RVSP observed at 21 and 35 days was totally prevented by STI-571 with similar kinetic. **(d)** Chronic hypoxia increased significantly the maximal number of lung 5-HT<sub>2B</sub>R specific binding sites (Bmax) at 21 and 35 days, which was significantly prevented by STI-571 with similar kinetic. Values are means±sem (n=10, P < 0.05) and are representative of at least two independent experiments. Straight lines are normoxic values. Any statistical difference by one way ANOVA followed by Bonferroni post hoc test vs. normoxic untreated control values is indicated by \* and vs. chronic hypoxia values by a #; \*\*\*P <0.001; \*\*P <0.01; \*P <0.05; ##P <0.01; #P <0.05.

**Figure 6:**

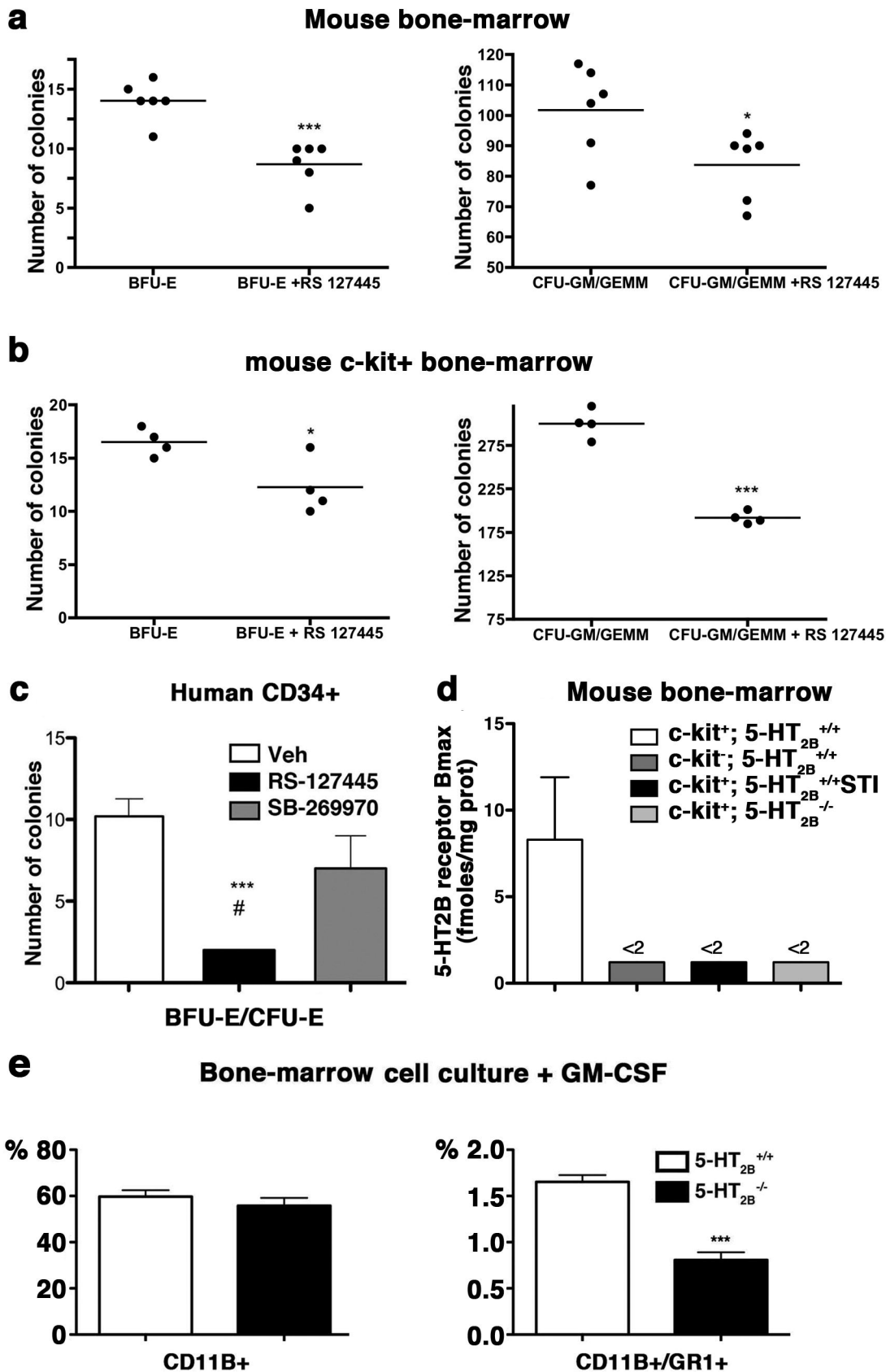
Working model illustrating the present findings. This work implicates 5-HT<sub>2B</sub> receptors at different levels of the myeloid lineage. The absence of 5-HT<sub>2B</sub>Rs in mutant mice (2B<sup>-/-</sup>) leads to a reduction in (↘) platelets and immature endothelial progenitors and lineages but to an increase (↗) in granulocyte precursors and lineages.

**Figure 1****a****b****c**

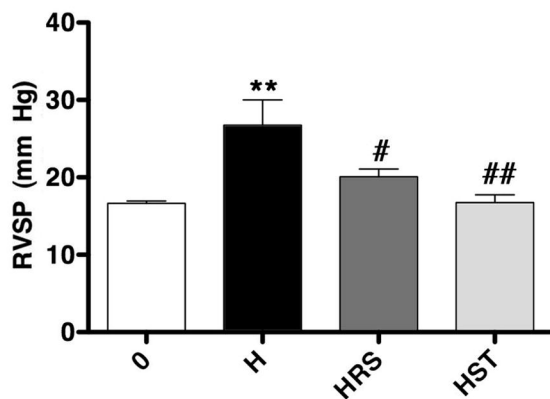
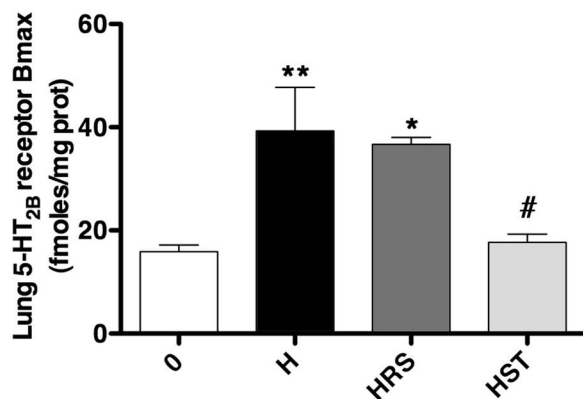
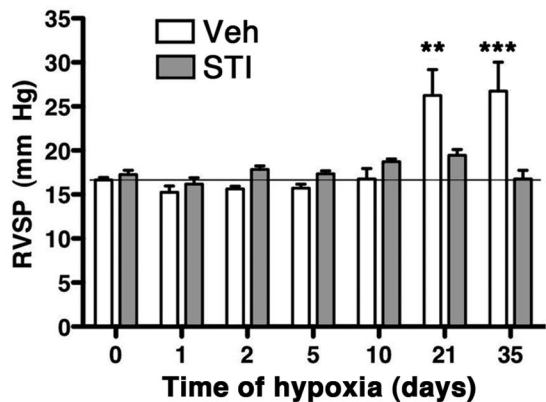
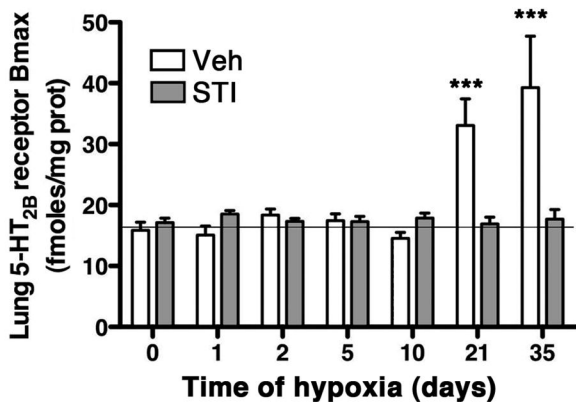
**Figure 2****Total bone-marrow cells****a****b****c****d**

**Figure 3**

# Figure 4





**Figure 5****a****b****c****d**

**Figure 6**

**model**

