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## **Function of the serotonin 5-hydroxytryptamine 2B receptor in pulmonary hypertension.**

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**Primary pulmonary hypertension is a progressive and often fatal human disorder that results from an increase in pulmonary blood pressure associated with abnormal vascular proliferation. The use of dexfenfluramine increases the risk of pulmonary hypertension in humans and its active metabolite is a selective serotonin 5-HT<sub>2B</sub>-receptor agonist. We have therefore investigated a possible contribution of 5-HT<sub>2B</sub>-receptor to pulmonary hypertension pathogenesis. Using the chronic hypoxic mice model of pulmonary hypertension, we found that the hypoxia-dependent increase in pulmonary blood pressure and lung remodeling are associated with a rise in vascular proliferation, elastase activity and TGF- $\beta$  level and these parameters are potentiated by dexfenfluramine treatment. By contrast, hypoxic mice with genetically or pharmacologically inactive 5-HT<sub>2B</sub> receptors manifested no change in any of these parameters. In both humans and mice, pulmonary hypertension is associated with a striking increase in 5-HT<sub>2B</sub> receptor expression in pulmonary arteries. These data show that activation of 5-HT<sub>2B</sub> receptors is a limiting step in the development of pulmonary hypertension.**

Primary pulmonary hypertension (P-PH) is a rare condition characterized by an elevation in pulmonary arterial pressure that is associated with irreversible pulmonary remodeling<sup>1,2</sup>. The hypoxia-induced changes in pulmonary artery pressure that result from vascular cell proliferation recapitulate, at least in part, the changes observed in P-PH indicating that it is a relevant model for this pathology<sup>3,4</sup>. Several studies have suggested a role for serotonin (5-hydroxytryptamine, 5-HT) in the aetiology of PH<sup>5-7</sup>. In response to hypoxia, 5-HT is released from pulmonary neuroendocrine cells and neuroepithelial bodies distributed throughout the airways. A sustained increase of 5-HT is observed in the plasma of PH patients<sup>8,9</sup>. Furthermore, isolated smooth muscle and endothelial cells from pulmonary arteries express mRNAs for 5-HT<sub>1B</sub>, 5-HT<sub>2A</sub>, 5-HT<sub>7</sub> and 5-HT<sub>2B</sub> receptors<sup>10</sup>. As a potent pulmonary vasoconstrictor<sup>5</sup>, 5-HT might have a dual effect on pulmonary circulation, contributing to both hypoxia-induced acute vasoconstriction and chronic vascular remodeling by activating its cognate receptors.

When used for more than 3 months, the appetite-suppressants (anorexigens) derived from amphetamines, fenfluramine and dexfenfluramine (DF), increase the risk of developing PH by a factor of between 3.7 to 23-fold depending on the study consulted<sup>11,12</sup>. DF binds weakly to the 5-HT<sub>2A</sub>, 5-HT<sub>2B</sub>, and 5-HT<sub>2C</sub> receptors. However, the main metabolite of DF, norDF, behaves as a high affinity ligand for 5-HT<sub>2B</sub> receptors (5-HT<sub>2BR</sub>) and more moderately for 5-HT<sub>2A</sub> and 5-HT<sub>2C</sub> receptors<sup>13</sup>. Furthermore, in cells expressing recombinant 5-HT<sub>2BR</sub>, norDF acts as a potent agonist, which stimulates phosphoinositide hydrolysis, intracellular Ca<sup>2+</sup> levels and the mitogen-activated protein (MAP) kinase cascade<sup>13</sup>. MAPkinase activation mediates the mitogenic actions of 5-HT, which is accomplished *via* the 5-HT<sub>2BR</sub> by stimulating p60<sup>Src</sup> and p21<sup>ras</sup> signaling<sup>14,15</sup>. Functionally and immunohistologically, 5-HT<sub>2BR</sub>s are detected in lung endothelial and smooth muscle cells of various species including humans<sup>16,17</sup>. We have therefore tested the hypothesis of a direct implication of 5-HT<sub>2BR</sub>s in the development of PH. This work has revealed that 5-HT<sub>2BR</sub> expression is significantly increased in PH and that the blockade of 5-HT<sub>2BR</sub>s using two independent approaches prevents completely the development of hypoxia-induced PH in mice.

### **The hypoxia-induced rise in pulmonary artery pressure is 5-HT<sub>2BR</sub>-dependent.**

We first assessed the effect of a chronic exposure of mice to hypoxia (10% O<sub>2</sub>, 5 weeks) by monitoring their hemodynamic parameters. When compared with mice maintained under normoxic conditions, control mice (129PAS wild-type, <sup>+/+</sup>) exposed to hypoxia exhibited gradual increases in right ventricular systolic pressure (RVSP) such that, after 5 weeks, values were higher than those recorded after 2 weeks (not shown). After 5 weeks under hypoxia, the mean RVSP was significantly higher (165% of the control value) than in normoxic mice (Fig. 1a).

We recently generated mutant mice by homologous recombination of the 5-HT<sub>2BR</sub> gene (5-HT<sub>2B</sub><sup>-/-</sup>). These mutant mice display cardiac defects leading to partial embryonic lethality<sup>18</sup>. Living adult 5-HT<sub>2B</sub><sup>-/-</sup> mice have normal systolic blood pressure despite their dilated cardiomyopathy<sup>19</sup>. In a first attempt to evaluate a potential role for the 5-HT<sub>2BR</sub> in PH, we treated 5-HT<sub>2B</sub><sup>-/-</sup> adult mice with the same hypoxic protocol as described above. Surprisingly, none of the 5-HT<sub>2B</sub><sup>-/-</sup> mice exposed to hypoxia exhibited an increase in RVSP (Fig. 1a). The right-ventricle weight over left-ventricle-plus-septum weight ratio (RV/LV+S) was significantly increased only in <sup>+/+</sup> mice exposed to hypoxia, reasserting the hypoxia-induced modifications of RVSP in <sup>+/+</sup> mice and the absence of hypoxia-induced responses of 5-HT<sub>2B</sub><sup>-/-</sup> mice (Table I); the basal value of 5-

HT<sub>2B</sub><sup>-/-</sup> mice, higher than in control animals, reflects directly their left ventricle dysfunctions. Our results indicate that the 5-HT<sub>2B</sub>R is necessary for the chronic hypoxic response leading to increased RVSP.

Since DF increases the risk of developing PH in humans<sup>12</sup>, we evaluated the putative effect of "therapeutic" concentrations of DF in hypoxia-induced increases in pulmonary pressure in mice. The chronic exposure of <sup>+/+</sup> mice to hypoxia and DF further increased significantly the RVSP (229% and 138% of normoxic and hypoxic values respectively) (Fig. 1a). The RV/LV+S was also significantly increased in <sup>+/+</sup> mice exposed to hypoxia in the presence of DF when compared with both normoxic and hypoxic <sup>+/+</sup> mice (Table I). Importantly, hypoxia in the presence of DF generated high lethality: out of 20 mice treated, four were found dead from obvious signs of PH. Furthermore, chronic exposure of 5-HT<sub>2B</sub><sup>-/-</sup> mice to DF in the presence of hypoxia or normoxia had no significant effect on the RVSP (Fig. 1a) or on the RV/LV+S (Table I).

To validate these observations, we used an independent pharmacological approach. We challenged <sup>+/+</sup> mice with the recently developed compound, RS-127445 (2-amino-4-(4-fluoronaphth-1-yl)-6-isopropylpyrimidine, RS). This compound has a sub-nanomolar affinity for the 5-HT<sub>2B</sub>R (pK<sub>i</sub> = 9.5 ± 0.1) and potently antagonizes the 5-HT<sub>2B</sub>R with a 1,000 fold selectivity over numerous other molecules including all known 5-HT receptors and transporter<sup>20</sup>. In complete agreement with our first observations on 5-HT<sub>2B</sub><sup>-/-</sup> mice, simultaneous exposure of <sup>+/+</sup> mice to hypoxia in the presence of RS prevented an increase in RVSP (Fig. 1a). Furthermore, chronic exposure to RS in the presence of normoxia in <sup>+/+</sup> mice and of hypoxia or normoxia in 5-HT<sub>2B</sub><sup>-/-</sup> mice had no significant effects on the RVSP (Fig. 1a). Combined, these data support the initial findings that the 5-HT<sub>2B</sub>R is required for the chronic hypoxia-induced increase in RVSP.

### **The histological remodeling triggered by hypoxia requires 5-HT<sub>2B</sub>R.**

Exposure to hypoxia dramatically increases structural remodeling of the pulmonary arteries resulting from vascular cell proliferation that generates most of the RVSP increase<sup>21</sup>. Chronic hypoxic exposure of <sup>+/+</sup> mice induced obvious modifications of lung morphology revealed by the degree of pulmonary artery muscularization (Fig. 1b-2ghi); the statistical analysis of lung morphology revealed that increased RVSP is associated with a significant increase in pulmonary artery muscularization; DF further and significantly enhanced this remodeling (Fig. 1b-2jklmp). Alternatively, no significant modification of the pulmonary artery muscularization could be revealed in hypoxic 5-HT<sub>2B</sub><sup>-/-</sup> mice with, or without, RS or DF, or following RS treatment of hypoxic <sup>+/+</sup> mice (Fig. 1b-2def). In addition, staining of lung sections revealed that the hyperplastic cell population consisted mainly of smooth muscle cells as they expressed smooth muscle-specific actin (Fig. 2n) and also demonstrated the presence of 5-HT<sub>2B</sub>R expression in hypoxic <sup>+/+</sup> mice (Fig. 2p).

To get further insights into the molecular pathways involved in hypoxic responses, we developed isolated lung vasculature explant culture of hypoxia- (or normoxia-) treated mice. Compared with normoxic animals, basal thymidine incorporation was significantly increased (1.95 times basal) in the lung vessels from hypoxic <sup>+/+</sup> mice (Fig. 3a). This increase was completely prevented in the hypoxic lungs of <sup>+/+</sup> mice treated by RS, while DF treatment significantly potentiated DNA synthesis (2.5 times *vs.* basal and 1.3 times *vs.* hypoxia). By contrast, lung vessels of 5-HT<sub>2B</sub><sup>-/-</sup>

mice did not exhibit any hypoxia-induced nor DF-potentiated increase in DNA synthesis. These data support the notion that 5-HT<sub>2B</sub>R is a limiting factor in chronic hypoxia-induced pulmonary vascular proliferation.

Progression of PH is associated with increased serine elastase activity<sup>22</sup>, which can activate storage sites for growth factors such as basic fibroblast growth factor and transforming growth factor-beta (TGF- $\beta$ )<sup>23</sup>. Supernatants of lung vessels cultures from hypoxic<sup>+/+</sup> mice revealed a significant increase in elastase activity (11.6 times basal) and in TGF- $\beta$  levels (3.9 times basal), both of which were further potentiated after DF treatment (16.9 and 4.9 times basal, respectively). Conversely, there was no detectable change in hypoxia-induced elastase activity and TGF- $\beta$  levels in supernatants from vessels of hypoxic<sup>+/+</sup> mice in the presence of RS or of 5-HT<sub>2B</sub><sup>-/-</sup> mice after either treatment (Fig. 3b-c). These results indicate that both hypoxia-increased remodeling factors, pulmonary elastase activity and TGF- $\beta$  levels, are dependent on 5-HT<sub>2B</sub>R activity.

### **The 5-HT<sub>2B</sub>R is not involved in acute hypoxia-dependent vasoconstriction**

Acute hypoxic responses are important triggers in the establishment of chronic PH<sup>24</sup> and thus, the limiting functions of 5-HT<sub>2B</sub>R in chronic response to hypoxia could rely on direct acute effects of hypoxia on 5-HT<sub>2B</sub>R. We therefore investigated whether 5-HT<sub>2B</sub>R participates in acute responses to hypoxia. Following repeated acute hypoxic stimuli<sup>25</sup>, we observed a moderate but significant increase in RSVP in both<sup>+/+</sup> and 5-HT<sub>2B</sub><sup>-/-</sup> mice, indicating that 5-HT<sub>2B</sub>Rs are not required for short-term increases in RVSP (Fig. 3d). The result of the same experimental setup in the presence of DF was also independent of 5-HT<sub>2B</sub>Rs. Plasma DF and norDF measurements (Supplementary Data) clearly demonstrated that short term exposure is not sufficient enough to produce significant amounts of norDF and confirmed that the effect of DF is mediated by its metabolite norDF at 5-HT<sub>2B</sub>R. When testing for elastase activity following the same acute hypoxic stimuli, we observed a significant increase in elastase activity in<sup>+/+</sup> but not in 5-HT<sub>2B</sub><sup>-/-</sup> mice lungs (Fig. 3e), indicating that effectors of the 5-HT<sub>2B</sub>R (after chronic hypoxia) are regulated by acute hypoxic stimulus. These results clearly suggest that 5-HT<sub>2B</sub>R influence is not at the level of acute vasoconstrictive responses but rather that of the signaling mechanisms that lead to the expression of factors important in vascular remodeling.

### **Pulmonary artery 5-HT<sub>2B</sub>R expression is increased during PH.**

The absence of 5-HT transporter (5-HTT) in pulmonary vessels reduces chronic hypoxia-induced PH<sup>26</sup>. We tested therefore the 5-HT uptake activity in cultures of lung vasculature. In<sup>+/+</sup> hypoxic vessels, we observed a trend toward a decrease in 5-HT uptake while, in 5-HT<sub>2B</sub><sup>-/-</sup> mice, an increase in basal 5-HTT activity was detected. The 5-HT uptake activity was almost entirely blocked by DF in both<sup>+/+</sup> and 5-HT<sub>2B</sub><sup>-/-</sup> hypoxic vessels (Fig. 4a). From this latter observation, it can be concluded that defective 5-HT uptake is not sufficient to induce pulmonary hypertensive response in 5-HT<sub>2B</sub><sup>-/-</sup> mice.

To elucidate how 5-HT receptors control hypoxic responses, we investigated their expression in PH lung vasculature. While 5-HT<sub>2A</sub>R expression was not modified by exposure of<sup>+/+</sup> mice to hypoxia and DF, 5-HT<sub>1B</sub>R expression increased  $1.75 \pm 0.1$  times ( $P < 0.01$ ,  $n = 6$ ), though this could be prevented by RS. More strikingly, hypoxia itself and in the presence of DF or of RS increased significantly the mean 5-HT<sub>2B</sub>R-

specific binding ( $2.6 \pm 0.2$  times,  $2.9 \pm 0.2$  times, and  $2.2 \pm 0.1$  times, respectively) in  $^{+/+}$  mice (Fig. 4b). Furthermore, we observed significant ( $P < 0.0001$ ) correlations between the entire individual 5-HT<sub>2B</sub>R binding and RVSP values (Fig. 4c) and between the basal thymidine incorporation indexes in lungs and both 5-HT<sub>2B</sub>R binding and RVSP values but only in mice submitted to hypoxia and in the presence of active 5-HT<sub>2B</sub>R (Fig. 4de). These correlations further substantiate the evidence that 5-HT<sub>2B</sub>R is required for a hypoxic increase in RVSP and thus for the PH pathogenesis. Accordingly, a preliminary screening of 5-HT receptors expression in biopsies of human pulmonary artery indicates that the numbers of binding sites are markedly higher in PH patients than in non-PH patients for 5-HT<sub>1B</sub>R and 5-HT<sub>2B</sub>R ( $1.98 \pm 0.05$  times and  $4.32 \pm 0.76$  times, respectively,  $P < 0.01$ ,  $n = 5$ ), but not for 5-HT<sub>2A</sub>R ( $0.99 \pm 0.10$  times,  $n = 5$ , NS).

## Discussion

The identification of specific pathways involved in the development of PH is dependent upon pharmacological, transgenic or targeted gene disruption approaches on animal models. The only commonly utilized mouse model of PH is the chronic hypoxic case. Mice with genetic ablation of known vasodilators, endothelial NO synthase<sup>27</sup> or prostacyclin receptor<sup>28</sup>, appear more sensitive to hypoxia. By contrast, targeted disruption of genes involved in pulmonary vasoconstriction appears to reduce hypoxia-induced PH in mice. Mice deficient for 5-HT<sub>1B</sub>R (5-HT<sub>1B</sub><sup>-/-</sup>) developed less severe PH and vascular remodeling than  $^{+/+}$  mice<sup>29</sup>. The 5-HT<sub>1B</sub>Rs play a role in the development of PH *via* enhanced 5-HT<sub>1B</sub>R-mediated contraction of the pulmonary arterial circulation<sup>24</sup>, but this activity is not limiting in the development of PH since 5-HT<sub>1B</sub><sup>-/-</sup> mice are still responding to hypoxia<sup>29</sup>. However, using the chronic hypoxic mice model, we are able to demonstrate the complete absence of PH and vascular remodeling in hypoxic 5-HT<sub>2B</sub>R-inactive mice indicating that 5-HT<sub>2B</sub>Rs are limiting for chronic hypoxic responses, though independently of acute hypoxic vasoconstrictive responses.

Although congenital cardiac defects increase the risk of developing PH<sup>30</sup>, LV dysfunction in 5-HT<sub>2B</sub><sup>-/-</sup> mice<sup>19</sup> could impact on the cardiovascular remodeling responses, but this can be ruled out in 5-HT<sub>2B</sub><sup>-/-</sup> mice since: (i) The cardiac phenotype of 5-HT<sub>2B</sub><sup>-/-</sup> mice is characterized by LV dysfunctions but no apparent RV alterations<sup>19</sup>. (ii) Basal pulmonary pressure in 5-HT<sub>2B</sub><sup>-/-</sup> mice is indistinguishable from  $^{+/+}$  mice. (iii) No PH developed in  $^{+/+}$  mice exposed to chronic hypoxia in the presence of 5-HT<sub>2B</sub>R antagonist (RS), clearly demonstrating that the activation of 5-HT<sub>2B</sub>Rs is required for hypoxia-induced PH independently of any prior cardiac phenotype. Furthermore, some mutant mice strains developing cardiomyopathy such as mice lacking natriuretic peptide receptor A have an increased sensitivity to hypoxia of their lung vasculature<sup>31</sup>. Combined, these observations strongly support the conclusion that the absence of hypoxic response of 5-HT<sub>2B</sub><sup>-/-</sup> mice cannot be attributed to their cardiomyopathy.

Some 5-HTT blockers and substrates such as DF are implicated in PH pathogenesis, while others, *e.g.* m-CPP are not<sup>32,33</sup>. The DF effect on PH was initially suggested to result from 5-HTT inhibition since a change in platelet 5-HT uptake could explain the raised 5-HT plasma level observed in PH patients<sup>7-9,34</sup>. Other 5-HTT blockers, however, such as 5-HT-specific-reuptake-inhibitors or tricyclic antidepressants, have not been associated with pulmonary pathology. Upon 5-HT<sub>2B</sub>Rs, the EC<sub>50</sub> of DF is over 1  $\mu$ M, but the major metabolite of DF, norDF, which acts as a potent agonist with nanomolar affinity ( $pEC_{50} = 8.06 \pm 0.16$ )<sup>13</sup>, requires chronic

treatments to be detected (Supplementary Data). Other high affinity agonists at 5-HT<sub>2B</sub>Rs do not appear to be associated with PH in humans<sup>33</sup> and thus a combinatorial effect at different receptors with transporters may be required for PH development. However, the significant DF potentiation of a hypoxia-dependent increase in RVSP and remodeling is completely eliminated in 5-HT<sub>2B</sub><sup>-/-</sup> mice. Since the 5-HT uptake activity is largely reduced in lungs of 5-HT<sub>2B</sub><sup>-/-</sup> mice exposed to hypoxia and DF and which do not develop PH, blocking 5-HT uptake activity is not sufficient to trigger hypoxia-dependent PH. Consistent with this observation is the fact that mice deficient for 5-HTT (5-HTT<sup>-/-</sup>) are still responsive to hypoxia though they develop less severe PH and vascular remodeling when compared with the <sup>+/+</sup> mice<sup>26</sup>.

In animal models, DF does not induce PH *per se*, but it can potentiate hypoxia-induced PH: dogs chronically treated with DF revealed increased pulmonary vascular resistance upon exposure to hypoxia<sup>35</sup>. Rats exposed to a 4-week DF treatment in normoxia followed by 2-week hypoxia developed stronger PH than the controls, suggesting also that DF pre-treatment promotes the development of PH in rats<sup>36</sup>. The recently proposed mechanism by which DF-like drugs may promote pulmonary vascular remodeling by stimulating 5-HTT expression<sup>37</sup> cannot fully explain why patients developing PH after DF treatment reveal the same proportion of 5-HTT promoter polymorphism as do the PH patients in general. The lack of a higher incidence of the 5-HTT polymorphism in the group that ingested DF and developed PH suggests also that other factors are required for DF-induced PH to develop<sup>38</sup>. In our hypoxic mouse model, the need of active 5-HT<sub>2B</sub>Rs to reveal the DF potentiating action demonstrates that, although a contribution of 5-HTT to DF effects is likely, the DF potentiation of hypoxia-induced PH requires 5-HT<sub>2B</sub>R activation.

A lung vascular proliferative response occurs in response to 5-HT, but it remains debatable whether the 5-HT mitogenic effect is initiated through a 5-HT<sub>2</sub>R or the 5-HTT, or both<sup>39,40</sup>. The observation that lung tissue from PH patients without the long 5-HTT promoter polymorphism revealed proliferative response to 5-HT higher than normal<sup>37</sup>, indicates also that other components of the 5-HT pathway, such as receptors or downstream effectors, are needed to promote PH<sup>38</sup>. In lung vascular cells, 5-HT induced DNA synthesis seemed dependent on the 5-HTT, since it was prevented by some 5-HTT inhibitors, but unaltered by ketanserin, a 5-HT<sub>2A</sub>R antagonist<sup>39</sup>. However, in lung smooth muscle cells, the calcium transients due to 5-HT persisted in Na<sup>+</sup>-free conditions (no 5-HT uptake) and 5-HTT inhibitors were still inhibiting 5-HT-induced mitogenesis<sup>41</sup>. Thus, the 5-HT<sub>2</sub>R rather than the 5-HTT initiated the 5-HT effect, indicating that 5-HTT inhibitors have 5-HT<sub>2</sub>R antagonist properties as recently confirmed<sup>42</sup>. In contrast to what is observed in hypoxic <sup>+/+</sup> mice lung vasculature, the total lack of increased basal thymidine incorporation in hypoxic 5-HT<sub>2B</sub><sup>-/-</sup> mice tissue indicates an absolute requirement of 5-HT<sub>2B</sub>R for chronic hypoxia-induced mitogenesis. This finding is validated by the complete prevention of proliferation and vascular remodeling upon treatment of hypoxic <sup>+/+</sup> mice with the 5-HT<sub>2B</sub>R antagonist RS. Although a direct contribution of 5-HT<sub>2B</sub>R to the chronic hypoxia-induced mitogenesis remains to be firmly established, it is likely since (i) we have previously reported that activation of 5-HT<sub>2B</sub>R can initiate cell cycle progression<sup>15</sup> and (ii) the basal thymidine incorporation indexes in lungs are significantly correlated with both 5-HT<sub>2B</sub>R binding and RVSP values, only when mice are submitted to hypoxia and in the presence of active 5-HT<sub>2B</sub>R. Other serotonergic molecules<sup>5,26</sup> may indeed participate in the hypoxic mitogenic process of PH, but our animal model provides the first direct evidence that 5-

HT<sub>2B</sub>R activation is a limiting, non-redundant step, required for the hypoxia-induced vascular proliferation in mice.

We report that the 5-HT<sub>2B</sub>R effect is not at the level of acute vasoconstrictive responses but rather the signaling mechanisms that control factors important in vascular remodeling such as elastase. PH vascular remodeling is associated with increased serine elastase activity working either directly or *via* activation of matrix metalloproteinases, and elastase inhibitors can reverse advanced pulmonary vascular disease<sup>43</sup>. The matrix metalloproteinases are a family of proteases implicated in extracellular matrix turnover and hence contribute to vascular smooth muscle and endothelial cell migration and proliferation<sup>22</sup>. Furthermore, elastase can increase TGF- $\beta$  availability, optimizing the mitogenic response in cultured vascular cells<sup>23</sup>. Our data indicate that 5-HT<sub>2B</sub>Rs are crucial for hypoxia-induced remodeling factors by their regulation of elastase activity and TGF expression in mice lung vasculature. In humans, heterozygous mutations within the bone morphogenetic protein type II receptor (BMPRII) gene of the TGF- $\beta$  cell-signaling superfamily have recently been identified in 25% of sporadic and 55% of familial cases of PH<sup>3</sup>. The low penetrancy of these mutations (10-20%) suggests a need for other factors (genetic, environmental, pathologic) in PH pathogenesis, see for review<sup>3</sup>. Recent evidence has shown that both TGF- $\beta$  and BMPs proliferative functions are altered in smooth muscles from PH patients<sup>44</sup>. In familial forms of PH, the primary event appears to be vascular-cell proliferation<sup>3</sup>. However, hypoxia is a preponderant environmental factor that generates PH in humans<sup>4</sup>. Vascular proliferative changes, which may represent adaptive (maladaptive) responses to this exogenous stimulus are associated with increased 5-HT<sub>2B</sub>R expression and require activation of 5-HT<sub>2B</sub>Rs. In complete agreement with the mice model is the increase in 5-HT<sub>2B</sub>R expression found in lungs of PH patients. Furthermore, this finding could explain the proliferative response to 5-HT, higher than normal, observed in PH patients<sup>37</sup> and independent of their 5-HTT genotype<sup>38</sup>.

In conclusion, our work supports the scheme presented (Fig. 5) in which 5-HT<sub>2B</sub>Rs hypoxia-dependent increased expression has a central role in pulmonary hypertensive responses in mice. Subsequently, active 5-HT<sub>2B</sub>Rs are an absolute necessity for lung vascular proliferation, elastase- and TGF- $\beta$ -dependent remodeling. In addition, DF action at 5-HTT acts as an amplifier of norDF agonist action at 5-HT<sub>2B</sub>Rs. All these activities contribute toward increases in RVSP and PH pathogenesis in mice. Similar increased 5-HT receptor expression in lungs of PH patients suggests that comparable mechanisms exist in humans and indicate that 5-HT<sub>2B</sub>R expression may be used as a diagnostic criterion for susceptibility to PH. The 5-HT<sub>2B</sub>Rs should now be considered as a potential target for developing new treatments of PH in humans.

## **‘Methods’.**

### **Hypoxia.**

The 5-HT<sub>2B</sub>R mutant mice (5-HT<sub>2B</sub><sup>-/-</sup>) were generated in the 129PAS pure genetic background that was used as controls (<sup>+/+</sup>). Mice 6 weeks of age (20-25 g) were randomly divided into two groups of 5 males and 5 females, one maintained in room air (21% O<sub>2</sub>) and the other exposed to hypoxia, for 2 or 5 weeks. Normobaric hypoxic chambers (21–23°C) maintained by 5.5 l/min flow of hypoxic air (10% O<sub>2</sub>, 90% N<sub>2</sub>) were opened twice a day for 15 min for cage cleaning, food and water supplies. The oxygen concentrations were monitored continuously using blood gas analyzers and soda lime was used to reduce the concentration of carbon dioxide. Normoxic mice were kept in the same room with the same 12/12 light-dark cycle. The vehicle, RS at 1 mg/kg/day or DF at the "therapeutic" dose of 2.5 mg/kg/day was delivered by miniosmotic pump (Alzet) at the beginning of the hypoxic (or normoxic) treatment. Acute hypoxic experiments consisted of the exposure of conscious mice to five successive hypoxic (10% O<sub>2</sub>) challenges<sup>25</sup>, hemodynamic variables were recorded first while the animal was breathing air, then after 10 min of exposure to the hypoxic gas mixture and finally 20 min after returning to normoxia. Five similar successive hypoxic challenges were repeated for each mouse. All animal care and procedures were in accordance with institutional guidelines and European regulations.

### **Cardiovascular evaluations.**

Mice were anesthetized with intraperitoneal ketamine hydrochloride (60 mg/kg) and xylazine (8 mg/kg). Right ventricular systolic pressure (RVSP) was measured by insertion into the right ventricle of a 26-gauge needle connected to a pressure transducer. After a blood sample was collected by cardiac puncture for DF and norDF measurements (Supplementary Data), 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 *en bloc*, and the airways were distended with 10% formaldehyde solution and fixed for 3 days. For the assessment of right ventricular hypertrophy, weights of the right ventricular free wall (RV) and the left ventricle together with the septum (LV + S) were measured separately. For morphometric analysis, one sagittal section (5 µm in thickness) was obtained from each left lung and after paraffin embedding, stained with hematoxylin-phloxin-saffron and orcein-picric-indigo-carmin. In each section, a total of 55 to 65 vessels was examined. Pulmonary arteries (25-100 µm) associated with an airway distal to the respiratory bronchiole were counted. The arteries were considered as muscularized if they possessed a distinct double-elastic lamina visible for the complete diameter in the vessel cross-section<sup>29</sup>. If they were seen in smaller part of the arterial circumference, they were recorded as partially muscular arteries and if absent as non-muscular arteries. These observations were done in a blinded setup *vs.* genotypes and treatments.

### **Explants.**

Lung vascular bed around pulmonary arteries was collected and prepared for organ culture<sup>45</sup>. Each explant (10-15 mg) was maintained in DMEM supplemented with 5-HT-depleted serum (10%) for a maximal period of 3 days. Fragments (1-2 mg each) seeded onto tissue culture vessels for 24-h were washed with HBSS and grown for 24-h in serum-free medium {DMEM/F-12 (1:1) with 5 µg/ml insulin, 5 µg/ml transferrin, 30 nM selenium, 20 nM progesterone, and 100 µM putrescine} before thymidine

incorporation was performed within next 12-h<sup>14</sup>. Conditioned media from all cultures were collected and stored. For elastolytic activities, conditioned media were incubated at 37°C with [<sup>3</sup>H]-elastin<sup>43</sup>. TGF-β was also measured in conditioned media with an ELISA using a pan-specific anti-TGF-β polyclonal antibody (R&D Systems catalog # AB-100-NA). To measure 5-HT uptake, after incubation for 1 min with 10<sup>-7</sup> M [<sup>3</sup>H]-5-HT binoxalate, each lung fragment was lysed by addition of 0.1 N NaOH and radioactivity was counted. Specific 5-HT uptake assessed as the difference between total uptake and uptake in the presence of 1 μM paroxetine, was expressed in picomoles of 5-HT taken up per milligram of protein per minute. Measurements of the 5-HT receptor expression was performed using a 5-HT<sub>2B</sub>R-specific tritiated radioligand (LY266070), a 5-HT<sub>1B</sub>R-specific iodinated radioligand (<sup>125</sup>I-GTI), or for 5-HT<sub>2A</sub>R the fraction of 5-HT<sub>2</sub>R-specific iodinated radioligand (<sup>125</sup>I-DOI) insensitive to LY266070 as previously described<sup>18</sup>.

### Statistics.

The reported data represent the mean of individual values ± SEM (n = number of individuals at the end of treatments as indicated in the text). Comparisons were performed using the non-parametric Kruskal-Wallis test. All statistical difference vs. normoxic untreated control values is indicated by an asterisk and vs. chronic hypoxia values by a cross and significance was set at *P* < 0.05. This level of significance also applies to correlation between individual values, assessed by Kendall rank coefficients. For the groups of pulmonary artery muscularization, statistical comparisons were made by one way ANOVA. When statistical significance was attained (*P* < 0.05), difference between groups was established using the Newman-Keuls multiple comparison test.

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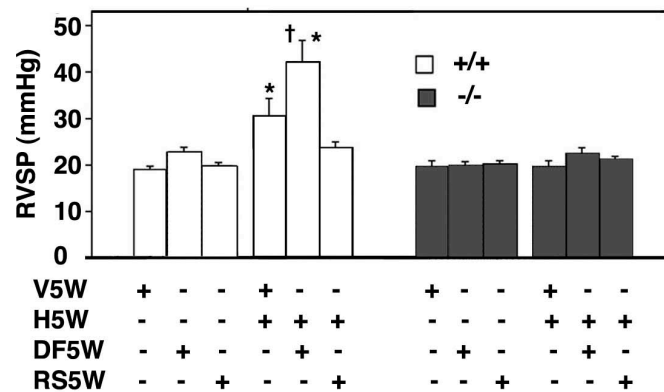
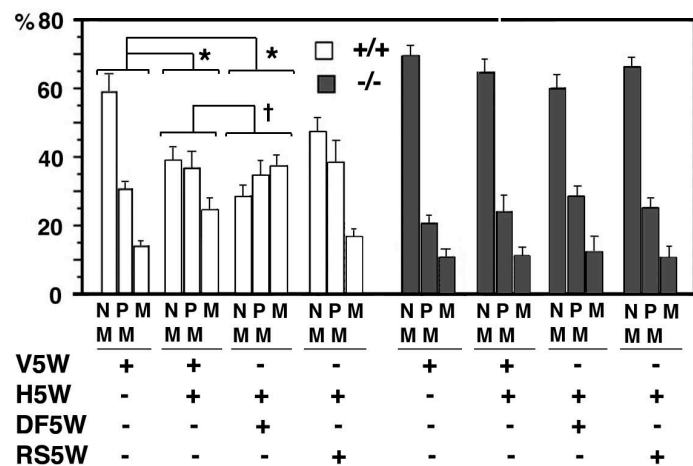
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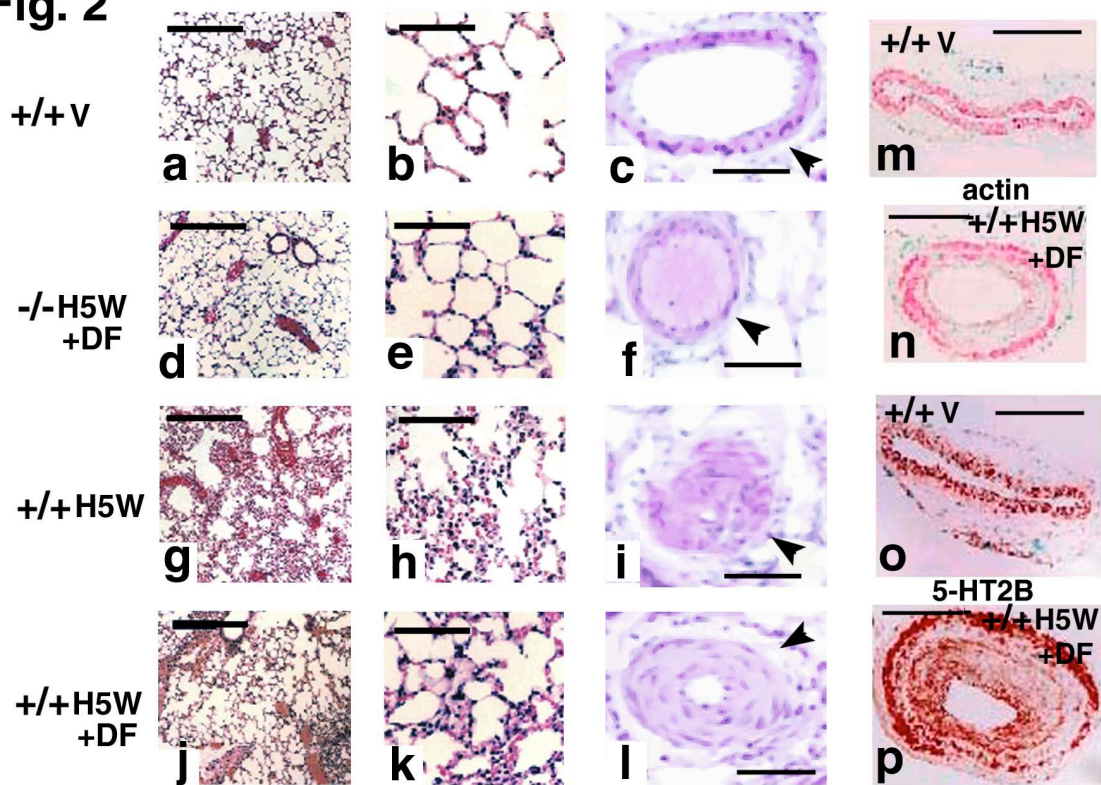
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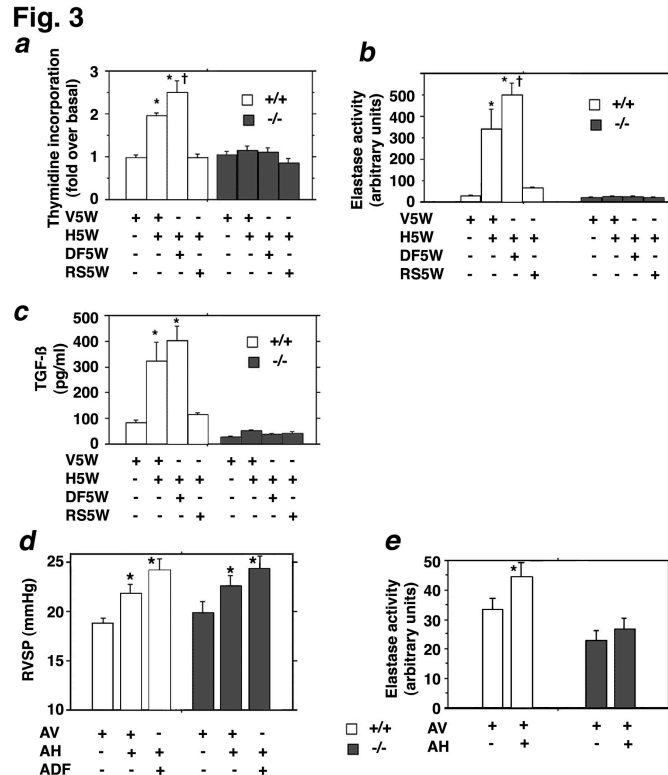
**Correspondence should be addressed to L.M. (e-mail: [lucm@igbmc.u-strasbg.fr](mailto:lucm@igbmc.u-strasbg.fr)).**

**Fig. 1**  
**a****b**

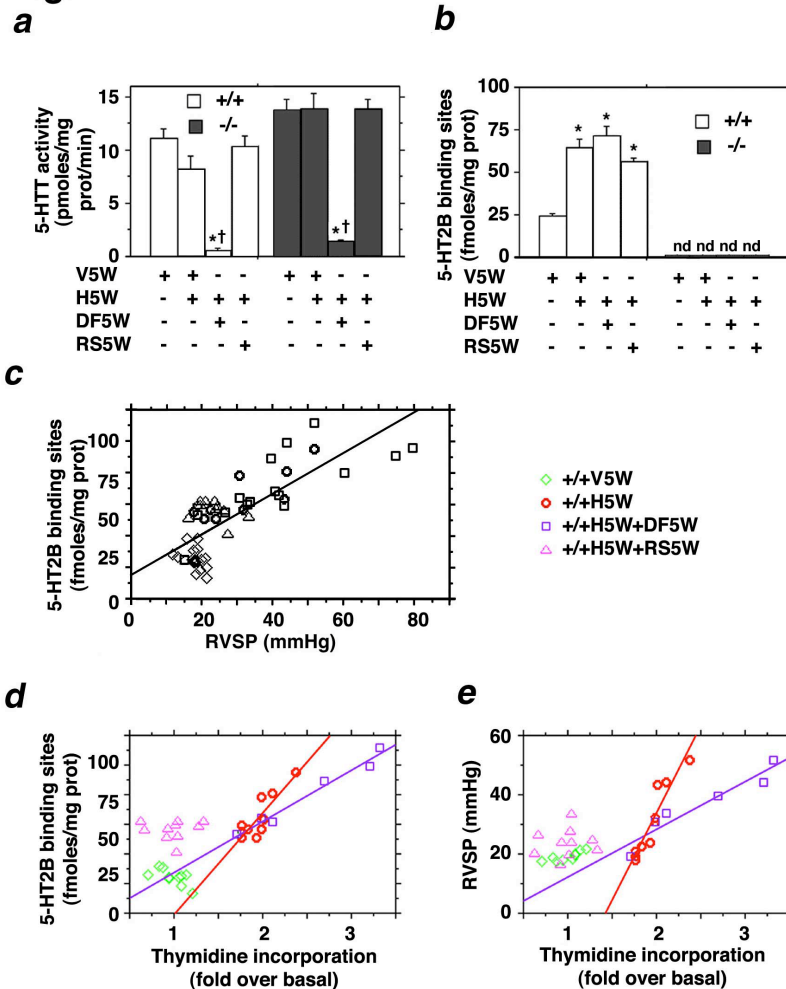
**Figure 1** Requirement of 5-HT<sub>2B</sub>R for hypoxia-induced RVSP and vascular muscularization. **a** In <sup>+/+</sup> mice exposed to hypoxia for 5 weeks (H5W), RVSP is significantly higher (30.6 ± 3.8 mm Hg, n = 10) than in vehicle-treated normoxic <sup>+/+</sup> mice (V5W, 18.5 ± 0.4 mm Hg n = 28). Simultaneous exposure of <sup>+/+</sup> mice to hypoxia in the presence of the specific 5-HT<sub>2B</sub>R antagonist RS (RS5W) prevents the increase in RVSP (23.6 ± 1.5 mm Hg, n = 10) whereas the presence of DF (DF5W) further increases significantly the RVSP (42.3 ± 4.7 mm Hg, n = 15). By contrast, none of the 5-HT<sub>2B</sub><sup>-/-</sup> mice exposed to hypoxia for 5 weeks (n = 7) exhibits any change in RVSP vs. vehicle-treated normoxic mutant mice (n = 9), neither in the presence of RS (n = 9) nor DF (n = 9). Chronic exposure of normoxia to these compounds alone is ineffective for both genotypes. Any statistical difference from normoxic untreated control values is indicated by an asterisk, and from chronic hypoxia values by a cross (P < 0.05). **b** A significantly different degree of muscularization (muscular, M, partially muscular, PM, or non-muscular, NM) is observed in hypoxic pulmonary arteries compared with normoxic <sup>+/+</sup> mice (P < 0.05). The degree of muscularization in the DF plus hypoxia mice is also significantly more severe than the plain hypoxia or the vehicle-treated mice (P < 0.05). Alternately, the degree of pulmonary artery muscularization does not differ significantly from controls after RS treatment of hypoxic <sup>+/+</sup> mice or in hypoxia-treated 5-HT<sub>2B</sub><sup>-/-</sup> mice with, or without, RS or DF.

**Fig. 2**

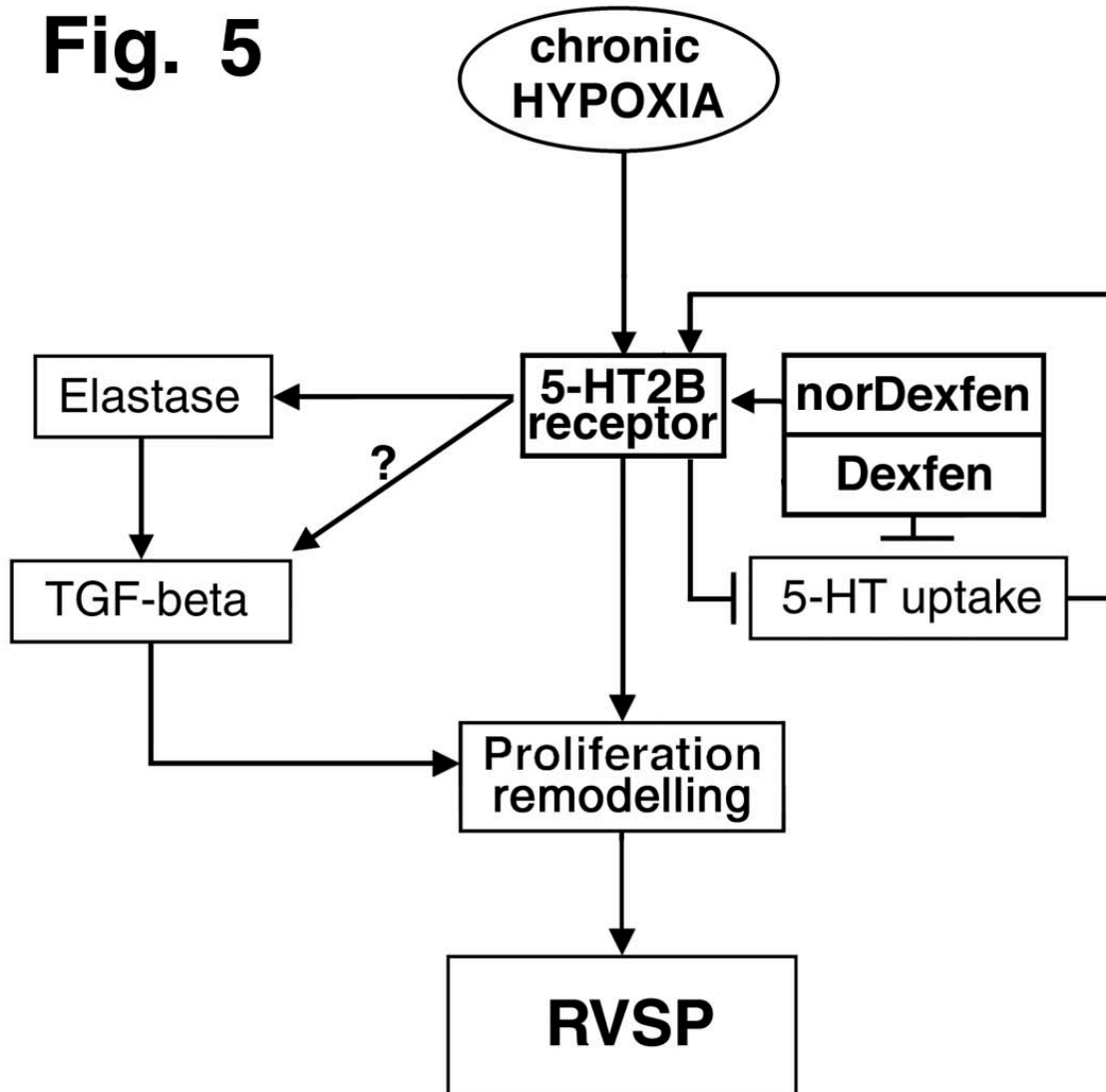
**Figure 2** Requirement of 5-HT<sub>2B</sub>R for hypoxia-induced lung remodeling. Sections of lung tissue of different genotype following different treatments, normoxic <sup>+/+</sup> mice (a, b, c, m, o, <sup>+/+</sup>V), hypoxic <sup>+/+</sup> mice (g, h, i, <sup>+/+</sup>H5W), mice treated by hypoxia in the presence of DF, <sup>+/+</sup> mice (j, k, l, n, p, <sup>+/+</sup>H5W+DF) or 5-HT<sub>2B</sub><sup>-/-</sup> mice (d, e, f, <sup>-/-</sup>H5W+DF) are presented. Sections of lung tissue stained with haematoxylin at low magnification (a, d, g, j), and intermediate magnification (b, e, h, k), illustrate the altered lung morphology and increased cell proliferation. At higher magnification, when compared with normoxic <sup>+/+</sup> mice (c, m, o, <sup>+/+</sup>V), the thickness of pulmonary vessels (arrowheads) media is clearly increased in hypoxic <sup>+/+</sup> mice (i, <sup>+/+</sup>H5W), and is further increased in the presence of DF (l, n, p, <sup>+/+</sup>H5W+DF) whereas no modification of lung morphology was detected in normoxic 5-HT<sub>2B</sub><sup>-/-</sup> mice, in 5-HT<sub>2B</sub><sup>-/-</sup> mice after hypoxia with DF (f, <sup>-/-</sup>H5W+DF) or without (not illustrated). The pulmonary vessels media is illustrated by van Giesen staining of elastin (c, f, i, l) or by staining with antibodies against smooth muscle alpha-actin (m, n, actin) or against 5-HT<sub>2B</sub>R (o, p, 5-HT<sub>2B</sub>). This staining reveals that the proliferating cell population are 5-HT<sub>2B</sub>R positive whereas only few 5-HT<sub>2B</sub>R positive cells could be observed in normoxic <sup>+/+</sup> mice. Bars = 500 μm, (a, d, g, j). Bars = 125 μm, (b, e, h, k). Bars = 25 μm, (c, f, i, l, m, n, o, p).



**Figure 3** Requirement of 5-HT<sub>2B</sub>Rs for lung remodeling factors but not for acute vasoconstriction. **a** After exposure to hypoxia, <sup>+/+</sup> mice lung vasculature exhibits a significantly increased (H5W, 1.95 ± 0.06 times basal control, n = 10) tritiated thymidine incorporation (per mg of tissue proteins), when compared normoxic with lung vessels (V5W, 0.99 ± 0.05). The basal normoxic incorporation is 2112 ± 355 cpm/mg of tissue proteins. This increase is significantly potentiated by DF (DF5W, 2.51 ± 0.27 times basal, n = 10) and completely prevented by either RS treatment (RS5W) or by the elimination of 5-HT<sub>2B</sub>R in 5-HT<sub>2B</sub><sup>-/-</sup> mice. **b** In <sup>+/+</sup> lung vessel explant supernatants, total elastolytic activity is significantly increased after chronic hypoxic exposure (344 ± 90 cpm/mg of tissue proteins, n = 10 vs. 33 ± 4 for normoxia, n = 19). A further significant increase is observed after chronic hypoxia in the presence of DF (496 ± 55 cpm/mg prot, n = 15) while near normoxic values are obtained in the presence of RS (67 ± 4 cpm/mg prot, n = 10). By contrast, hypoxia does not modify the elastolytic activity in supernatants of 5-HT<sub>2B</sub><sup>-/-</sup> mice following either treatment. **c** In <sup>+/+</sup> lung vessel explant supernatants, TGF-β levels are significantly increased upon hypoxia (322 ± 72 pg/ml vs. 83 ± 10 for normoxia, n = 10). This increase is potentiated by DF (401 ± 56 pg/ml) but blocked by RS (114 ± 8 pg/ml). No significant TGF-β level modification can be detected in supernatants of hypoxic lungs of 5-HT<sub>2B</sub><sup>-/-</sup> mice after either treatment. **d** Exposure to acute hypoxic challenges (AH) triggers a significant increase in RVSP in both 5-HT<sub>2B</sub>R<sup>-/-</sup> (22.5 ± 1.0 mm Hg, n = 10) and <sup>+/+</sup> (21.6 ± 1.0 mm Hg, n = 10) mice, when compared with vehicle treated (AV) control mice (18.9 ± 1.0 mm Hg, n = 10 and 18.4 ± 0.5 mm Hg, n = 29, respectively). DF administered 5 min before the hypoxic period (ADF) further increases these values independently of the mice genotype. **e** In <sup>+/+</sup> lungs, total elastolytic activity is significantly increased following acute hypoxic challenges (43.4 ± 6.1 cpm/mg of tissue proteins vs. 33.0 ± 3.6 for normoxia) though it is not significantly modified in 5-HT<sub>2B</sub>R<sup>-/-</sup> mice (26.7 ± 4.2 cpm/mg of tissue proteins vs. 21.9 ± 4.5 for normoxia).

**Fig. 4**

**Figure 4** Hypoxia increases 5-HT<sub>2B</sub>R expression in lungs. **a** The uptake activity of lung vasculature from <sup>+/+</sup> hypoxic mice (H5W) is non-significantly reduced by 23.4 % vs. normoxic controls (V5W), corresponding to a 28.6 % decrease of the maximal initial rate of uptake and no change in affinity for 5-HT. For the 5-HT<sub>2B</sub><sup>-/-</sup> mice, basal 5-HTT activity is slightly higher (by 24%) than normoxic <sup>+/+</sup> values but is unaffected by hypoxia or RS (RS5W). DF treatment (DF5W) almost completely blocked the 5-HT uptake in the hypoxic <sup>+/+</sup> and 5-HT<sub>2B</sub><sup>-/-</sup> mice. **b** Hypoxia increases significantly the number of 5-HT<sub>2B</sub>R specific binding sites ( $64.7 \pm 4.7$  fmoles/mg prot,  $n = 10$  vs.  $25.3 \pm 1.5$  for normoxic lungs,  $n = 19$ ). This increase in 5-HT<sub>2B</sub>R binding sites is not significantly affected by either DF ( $71.9 \pm 5.8$  fmoles/mg prot,  $n = 15$ ) or RS ( $56.0 \pm 2.0$  fmoles/mg prot,  $n = 10$ ). Indeed, 5-HT<sub>2B</sub>R-specific sites were not detected in 5-HT<sub>2B</sub><sup>-/-</sup> mice (nd). **c,d,e** Correlation between parameters involved in PH development. A highly significant correlation ( $P < 0.0001$ ) is observed between all individual values of the number of 5-HT<sub>2B</sub>R specific binding sites and the RVSP increase (**c**). Moreover, only when <sup>+/+</sup> mice are exposed to normoxia (+/+V5W, diamonds), hypoxia alone (+/+H5W, circles) or with DF (+/+H5W+DF5W, squares) but not RS (+/+H5W+RS5W, triangles) (i.e. active 5-HT<sub>2B</sub>R), the thymidine incorporation index correlates significantly with the number of 5-HT<sub>2B</sub>R binding sites ( $r = 0.987$  for +/+H5W+DF5W and  $0.868$  for +/+H5W, both  $P < 0.01$ ) (**d**) or the RVSP ( $r = 0.952$  for +/+H5W+DF5W,  $0.939$  for +/+H5W and  $0.827$  for +/+V5W, in each case  $P < 0.01$ ) (**e**).

**Fig. 5**

**Figure 5** Model of 5-HT<sub>2B</sub>R-dependent hypoxia-induced RVSP in mice. Chronic hypoxia triggers a striking increase in 5-HT<sub>2B</sub>R expression. Chronic DF (Dexfen) potentiates hypoxia by affecting RVSP through activation of 5-HT<sub>2B</sub>R by norDF (norDexfen), with an amplification from 5-HTT blockage. Lung proliferation and remodeling also appear to be dependent on the 5-HT<sub>2B</sub>R signaling activity and involve extracellular elastase activation and increase of TGF-β levels.

**Table I - Evaluation of RV/LV+S**

	+/+V	+/+DF5W	+/+H5W	+/+H5WDF	-/-V	-/-DF5W	-/-H5W	-/-H5WDF
RV/LV+S	0.21	0.19	0.29	0.39	0.46	0.45	0.47	0.46
(%±sem)	±0.01	±0.03	±0.03*	±0.04*†	±0.01	±0.04	±0.02	±0.02
n	10	10	14	15	9	10	7	8

Abbreviations: <sup>+/+</sup> mice exposed to hypoxia for 5 weeks (<sup>+/+</sup>H5W), vehicle-treated normoxic <sup>+/+</sup> mice (<sup>+/+</sup>V). <sup>+/+</sup> mice exposed to hypoxia in the presence of DF (<sup>+/+</sup>H5WDF), 5-HT<sub>2B</sub><sup>-/-</sup> mice exposed to hypoxia for 5 weeks (<sup>-/-</sup>H5W), vehicle-treated normoxic 5-HT<sub>2B</sub><sup>-/-</sup> mice (<sup>-/-</sup>V), in the presence of DF (<sup>-/-</sup>H5WDF), and mice exposed chronically to these compounds alone (<sup>+/+</sup>DF5W, <sup>-/-</sup>DF5W). Any statistical difference from normoxic untreated control values is indicated by an asterisk and from chronic hypoxia values by a cross (P < 0.05, Kruskal-Wallis test).

**Table Supplementary Data - DF and norDF plasma levels (in nM ± SEM).**

	+/+V	+/+DF5W	+/+H5WDF	+/+AHDF	-/-V	-/-5WDF	-/-H5WDF	-/-AHDF
DF	0	6,4±0,8	7,2±0,5	425±65	0	13,5±16	8,9±1,1	391±43
norDF	0	526±74	570±56	2,7±0,2	0	481±26	565±76	4,2±0,7
n	10	10	6	9	9	10	8	10

DF and norDF plasma levels were measured by HPLC (Midha, K.K. *et al.*, 1983). Abbreviations: <sup>+/+</sup> mice exposed to hypoxia for 5 weeks (<sup>+/+</sup>H5W), vehicle-treated normoxic <sup>+/+</sup> mice (<sup>+/+</sup>V). <sup>+/+</sup> mice exposed to hypoxia in the presence of DF (<sup>+/+</sup>H5WDF), 5-HT<sub>2B</sub><sup>-/-</sup> mice exposed to hypoxia for 5 weeks (<sup>-/-</sup>H5W), vehicle-treated normoxic 5-HT<sub>2B</sub><sup>-/-</sup> mice (<sup>-/-</sup>V), in the presence of DF (<sup>-/-</sup>H5WDF), and mice exposed chronically to these compounds alone (<sup>+/+</sup>DF5W, <sup>-/-</sup>DF5W), mice exposed to five successive acute hypoxic challenges in 5-HT<sub>2B</sub>R<sup>-/-</sup> (<sup>-/-</sup>AH) or <sup>+/+</sup> (<sup>+/+</sup>AH) mice and mice exposed to five successive acute hypoxic challenges in the presence of DF administered 5 min before the hypoxic period (<sup>+/+</sup>AHDF, <sup>-/-</sup>AHDF).

Reference:

Midha, K.K. *et al.* The identification of two new urinary metabolites of fenfluramine in man. *Xenobiotica* **13**, 31-38. (1983).