

Overexpression of the 5-Hydroxytryptamine Transporter Gene

Effect on Pulmonary Hemodynamics and Hypoxia-Induced Pulmonary Hypertension

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Background—Increased serotonin (5-hydroxytryptamine, 5-HT) transporter activity has been observed in human familial pulmonary hypertension.

Methods and Results—We investigated pulmonary hemodynamics and the development of hypoxia-induced pulmonary hypertension and pulmonary vascular remodeling in mice overexpressing the gene for the 5-HT transporter (5-HTT+ mice). Right ventricular pressure was elevated 3-fold in normoxic 5-HTT+ mice compared with their wild-type controls. Hypoxia-induced increases in right ventricular hypertrophy and pulmonary vascular remodeling were also potentiated in the 5-HTT+ mice. 5-HTT-like immunoreactivity, protein, and binding sites were markedly increased in the lungs from the 5-HTT+ mice. Hypoxia, however, decreased 5-HT transporter immunoreactivity, mRNA transcription, protein, and binding sites in both wild-type and 5-HTT+ mice.

Conclusions—Increased 5-HT transporter expression causes elevated right ventricular pressures, and this occurs before the onset of right ventricular hypertrophy or pulmonary arterial remodeling. Hypoxia-induced remodeling is, however, increased in 5-HTT+ mice, whereas hypoxia inhibits 5-HTT expression. This provides a unique model that demonstrates differential mechanisms for familial pulmonary arterial hypertension and pulmonary arterial hypertension with hypoxemia. (*Circulation*. 2004;109:2150-2155.)

Key Words: hypertension, pulmonary ■ hypoxia ■ remodeling ■ risk factors ■ genes

Chronic hypoxia induces a sustained increase in pulmonary arterial pressure and pulmonary vascular smooth muscle cell proliferation.¹ Serotonin (5-hydroxytryptamine, 5-HT) is a pulmonary vasoconstrictor and co-mitogen, and studies suggest a role for 5-HT both in remodeling of the pulmonary circulation and in increased pulmonary vascular tone, associated with exposure to hypoxia.² 5-HT-induced proliferation of pulmonary artery smooth muscle cells (PASMCs) is inhibited by selective inhibitors of the 5-HT transporter (5-HTT) such as paroxetine and fluoxetine but not by the 5-HT_{2A} receptor antagonist ketanserin.^{3,4} The relationship between hypoxia-induced pulmonary arterial hypertension (PAH) and 5-HTT expression remains controversial. Previous studies in rats show that hypoxia either decreases 5-HTT activity and gene transcription^{5,6} or increases 5-HTT activity.⁴ In mice, hypoxia decreases 5-HT uptake.⁷ Hypoxia-induced PAH is, however, attenuated in mice lacking the 5-HTT gene.⁸ There is 5-HTT overexpression in pulmonary arteries and platelets from patients with PAH.⁹ 5-HTT is

encoded by a single gene on chromosome 17q11.2, and a variant in the upstream promoter region of the 5-HTT gene has been described.¹⁰ This polymorphism with long (L) and short (S) forms affects 5-HTT expression and function, with the L allele inducing an increased rate of 5-HTT gene transcription over the S allele. This L-allelic variant was found to be present in homozygous form in 65% of PAH patients but in only 27% of control subjects.⁹ Patients who exhibit PAH linked to a genetic polymorphism or mutation have recently been classified as having familial PAH (fPAH), as opposed to idiopathic PAH (iPAH), in which no underlying genetic defect is known (Venice classification). However, it is accepted that more than one modifying factor probably contributes to the development of the disease in fPAH patients. Indeed, changes in 5-HTT may be a modifying factor that triggers fPAH in patients with mutations in the bone morphogenetic protein type 2 (BMP2) receptor.^{11,12} Other factors such as hypoxia may modify the effects of overexpression of the 5-HTT. Because patients present at the

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clinic with well-established PAH, it has not been possible to identify the early changes associated with increased expression of the 5-HTT.

Hence, we investigated the effect of overexpression of 5-HTT on the pulmonary arterial system per se and the influence of a potential modifying factor such as hypoxia.

Methods

The 5-HTT+ Mouse

The C57BL/6×CBA wild-type strain was used to generate the 5-HTT+ mouse and used as controls in this study. The transgene was a 500-kb yeast artificial chromosome (YAC35D8) containing the h5-HTT gene flanked by 150 kb of 5' and 300 kb of 3' sequence, with the "short" allele of the 5-HTTLPR in the promoter region and the 10-repeat allele of the VNTR in intron 2.¹³ The YAC was modified to include a hemagglutinin epitope tag at the C-terminus of the 5-HTT protein and a *lacZ* reporter gene downstream of an internal ribosomal entry site as described previously.¹⁴ Analysis by in situ hybridization showed that h5-HTT mRNA is expressed in a pattern that closely resembles that of the endogenous mouse 5-HTT gene.

Exposure to Hypoxia

Mice (wild-type and 5-HTT+, 5 to 6 months, female) were exposed to 14 or 28 days of hypobaric hypoxia as described previously.¹⁵ Appropriate aged-matched control groups were also used.

Assessment of PHT

Measurement of Right Ventricular Hypertrophy

Right ventricular hypertrophy (RVH) was assessed by measuring the RV free wall (RV) and left ventricle together with the septum (LV+S). The ratio RV/LV+S was calculated.

Lung Histology

Sections were stained with elastica–van Gieson (EVG) stain and microscopically assessed for muscularization of small pulmonary arteries associated with airways distal to respiratory bronchioles (20- to 60- μ m OD) as described previously.¹⁵ In total, 133±29 wild-type and 110±16 5-HTT+ vessels were counted per mouse lung section (n=5 to 6 lungs studied per group). Fully muscularized vessels with an external diameter <80 μ m were also analyzed by computer-assisted imaging. The percentage wall thickness was calculated as described previously.¹⁵ Two to 4 vessels were analyzed per lung section, and the results were expressed as mean±SEM (n=6 lungs studied per group).

In Vivo Hemodynamic Measurements

Anesthesia was induced with 2% to 4% halothane. Anesthesia was maintained with halothane (1% to 1.5%) and a mixture of nitrous oxide and oxygen (2:1). Pressure and heart rate measurements were measured and analyzed as described previously.¹⁵

Systemic arterial pressure was obtained via a cannula (Portex, 0.75-mm OD) inserted into a carotid artery. A 25-gauge needle was advanced into the RV by use of a transdiaphragmatic approach for measurement of RV pressure (RVP).

Radioligand Binding

Membrane Preparation

Lungs were washed and pulverized under liquid nitrogen and resuspended in assay buffer: in mmol/L, 150 NaCl, 50 Tris-HCl, 1 EDTA, 10 MgCl₂, containing 500 μ g/mL soybean trypsin inhibitor, 10 mmol/L benzamidine, 1 μ g/mL leupeptin, bacitracin, pepstatin A, antipain, and 10% glycerol, pH 7.4. After homogenization, homogenate was filtered and centrifuged at 1200g for 5 minutes at 4°C. The supernatant fraction was centrifuged twice at 56 000g for 30 minutes at 4°C, and the resulting membrane pellet was resuspended in

Tris-HCl buffer and homogenized. Protein estimation was by Pierce protein assay kit (Pierce Chemical Co).

Saturation Binding Studies

Saturation studies were performed with membranes (wild-type, 50 μ g/mL; 5-HTT+, 25 μ g/mL) incubated in duplicate with [³H]citalopram (83 Ci/mmol) (Amersham) (0.025 to 20 nmol/L) in Tris-HCl assay buffer. For each assay, membranes were prepared from n=8 to 10 lungs. The reaction mixture was incubated in a final volume of 0.5 mL for 60 minutes at 22°C for the measurement of total binding. Nonspecific binding was defined in the presence of 10 μ mol/L fluoxetine-hydrochloride (Tocris Cookson Ltd). The reaction was terminated with a Brandel cell harvester, and bound [³H]citalopram was separated from free by vacuum filtration over Whatman GF/C filters. Binding isotherms were analyzed by a nonlinear least-squares parametric curve-fitting program, GRAPHPAD Prism, to derive a dissociation constant (K_D) and receptor number (B_{max}).

Immunohistochemistry

Paraffin sections 5 μ m thick were mounted on poly-L-lysine slides. Slides were dewaxed and sections rehydrated by immersion in ethanol (100%, 95%, and 70%) and then in distilled water. After antigen retrieval, endogenous peroxidase activity was blocked with 3% H₂O₂ in methanol for 30 minutes. After washing, sections were preincubated in PBS supplemented with 0.5% BSA, 10% normal horse serum for 1 hour. Endogenous biotin was blocked by use of an avidin/biotin blocking kit (Vector Laboratories) then incubated overnight with goat polyclonal anti-5-HTT antibody (Ab) (Santa Cruz Biotechnology Inc) diluted 1:50 in PBS containing 0.5% BSA, 15% normal horse serum. The sections were exposed (1 hour) to biotin-labeled anti-goat secondary Abs (Vector Laboratories) diluted 1:100, then to streptavidin-biotin–horseradish peroxidase solution. Peroxidase staining used 3',3'-diaminobenzidine tetrahydrochloride dihydrate and hydrogen peroxide. Nickel enhancement was used for optimizing contrast. Finally, the sections were stained with hematoxylin.

Lung 5-HTT Immunoblotting

Lung homogenates were subjected to SDS-PAGE. Proteins were transferred onto MXB Nylon (Bio-Rad Laboratories Inc) by electroblotting in a Hoeffer electroblotter for 1.5 hours at 680 mA in transfer buffer (25 mmol/L Tris, 0.21 mol/L glycine, 20% methanol, pH 8.0). After transfer, the membrane was incubated in blocking solution (5% nonfat dry milk in TBST [Tris, NaCl, Tween-20], pH 7.6) for 1 hour at room temperature. Primary antibodies (goat anti-5-HTT, 1:500, Santa Cruz Biotechnology) were applied overnight at 4°C. The membrane was washed 6 times in TBST at room temperature for 15 minutes before addition of secondary Ab (donkey anti-goat, 1:5000) for 1 hour at room temperature. Membranes were washed, incubated with enhanced chemiluminescence reagent (Amersham), and exposed to film.

TaqMan Reverse Transcription–Polymerase Chain Reaction

After extraction of total RNA from lung with Trizol reagent (Life Technologies), real-time fluorogenic reverse transcription–polymerase chain reaction (PCR) was performed using Assays on Demand gene expression probes for human and mouse SERT (Hs00169010 and Mm00439391, respectively; Applied Biosystems) according to the manufacturer's instructions. Relative mRNA abundance was determined by use of the comparative delta-CT method using 18S ribosomal RNA as internal control.

Statistical Analysis

Statistical comparisons were made by 1-way ANOVA, and differences ($P<0.05$) were established by use of Tukey's multiple-comparison test.

TABLE 1. Mean Systemic Arterial Pressure, Systolic RVP, and Right Ventricular/Left Ventricular Plus Septum Ratios in Wild-Type and Hypoxic Wild-Type and 5-HTT+ Mice

Group	Mean SAP, mm Hg	Systolic RVP, mm Hg	RV/LV+S	Heart Rate, bpm	n
Normoxic					
Wild-type	76±3.4	13±1.2	0.259±0.003	508±10	9
5-HTT+	82.9±3.8	36.4±7.1*	0.262±0.003	497±22	7
Hypoxic					
Wild-type	79.5±3.9	31.1±4.1*	0.327±0.005†	484±13	11
5-HTT+	77.1±5.5	46.8±5.2‡	0.426±0.006§	503±12	7

SAP indicates systemic arterial pressure; RV/LV+S, right ventricular/total ventricular plus septum; and n, number of animals. Data are shown as mean±SEM.

* $P<0.01$, † $P<0.001$ vs wild-type normoxic; ‡ $P<0.05$, § $P<0.001$ vs wild-type hypoxic; || $P<0.001$ vs transgenic normoxic.

Results

Hemodynamics and RV/LV+S Ratio

Mean aortic pressure and heart rate were unaffected in 5-HTT+ mice or by exposure to hypoxia (Table 1). RVP was elevated in the normoxic 5-HTT+ mice compared with the wild-type controls, but there was no evidence for RVH or any pulmonary vascular remodeling in either the wild-type or 5-HTT+ normoxic mice. Hypoxia induced an increase in both RVP and RVH in both groups of mice, and the latter effect was exaggerated in the 5-HTT+ mice (Table 1). RVH was elevated by ≈26% in the wild-type but by ≈63% in the 5-HTT+ mice. The final RVP was higher in the 5-HTT+ than in the wild-type mice, although the percent change was less because the basal normoxic pressures were already markedly elevated. Body weights were not markedly different at death. The effects of 28 days of hypoxia were also examined, but there was no further increase in RVP in either the wild-type or 5-HTT+ mice (data not shown).

Pulmonary Vascular Remodeling

There was no remodeling in control wild-type or 5-HTT+ mice. However, hypoxia caused the appearance of remodeled vessels in the wild-type mice, and the percentage of these was doubled in the 5-HTT+ mice (Figure 1). The wall thickness of these vessels was also increased in the 5-HTT+ mice (32±2% versus 24±3%, n=6, $P<0.05$).

Immunohistochemistry

Moderate endothelial and smooth muscle 5-HTT-like immunostaining was observed in the wild-type mouse pulmonary arteries, whereas there was dense staining in pulmonary arteries of 5-HTT+ mice, particularly in the PASMCs bordering the adventitia (Figure 2, a and b). This staining was also evident in small remodeled pulmonary arteries (Figure 2c).

[³H]Citalopram Binding

Binding was increased ≈6-fold in the 5-HTT+ mice, and the binding affinity of [³H]citalopram for the native mouse transporter was higher than for the human transporter (Table 2). After exposure to hypoxia, binding decreased by ≈86% in the 5-HTT+ lungs, although the affinity increased.

Western Blotting

5-HTT protein levels were consistently raised in the lungs of the 5-HTT+ mouse compared with its wild-type control. Hypoxia consistently decreased these levels (Figure 3).

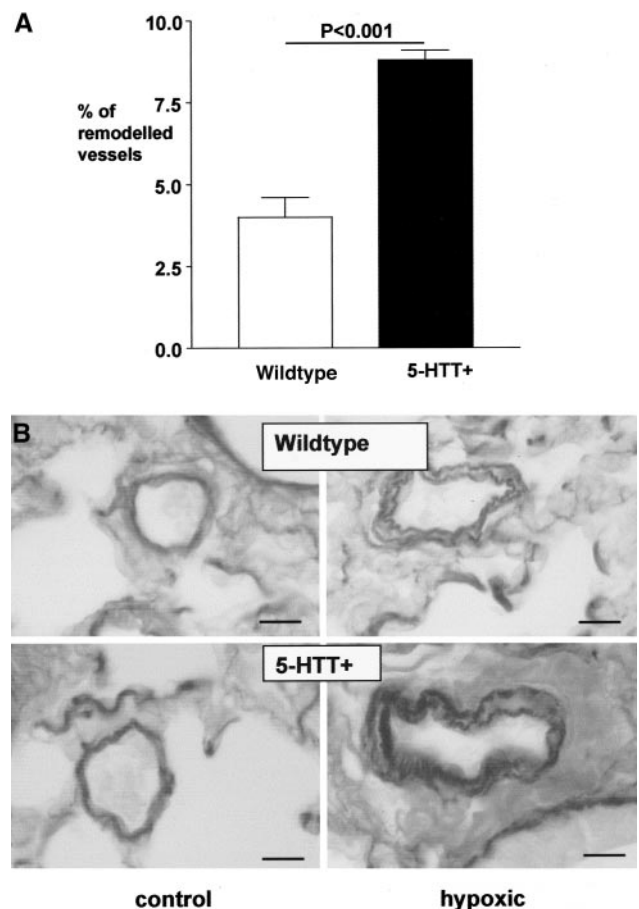


Figure 1. Remodeling in 5-HTT+ mice and their wild-type controls. A, Percentage of remodeled small pulmonary arteries in wild-type and 5-HTT+ mice after hypoxia. B, Left, Small pulmonary arteries in normoxic mice. Right, Small pulmonary arteries in hypoxic mice. Bar=10 μm.

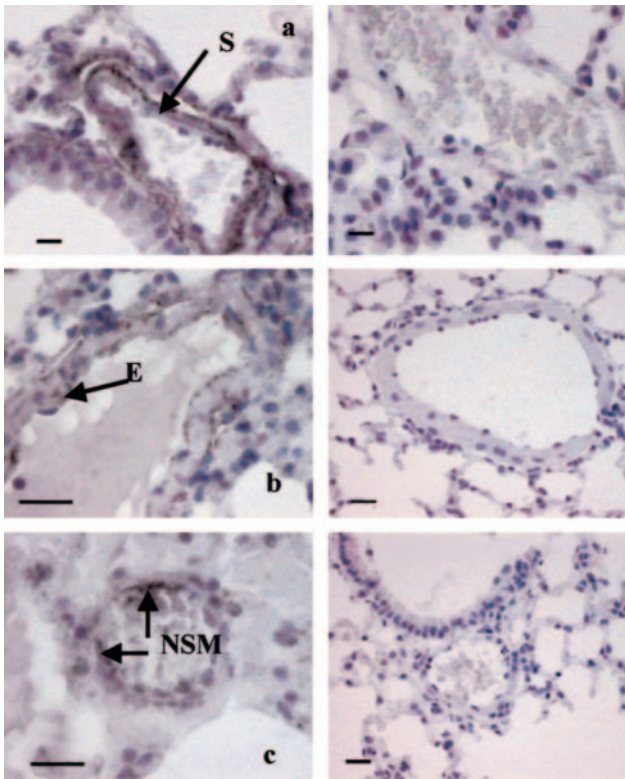


Figure 2. Immunohistochemical localization of 5-HTT in pulmonary arteries from (a) control 5-HTT+ mouse, (b) control wild-type mouse, and (c) distal pulmonary artery from 5-HTT+ mouse after 2 weeks of hypoxia. DAB staining was nickel enhanced and therefore black in nature. Left, Sections incubated with primary and secondary antibody; right, sections incubated with secondary but not primary antibody. Bars=10 μm. Dense staining is observed in PSMCs (S) adjacent to adventitia and in endothelial cells (E) and newly formed PSMCs (NSM) in remodeled arteries.

TaqMan RT-PCR

Relative expression of the human 5-HTT in the lung was ≈50% of the expression of the native mouse 5-HTT (Figure 4). However, mouse 5-HTT expression was equal in the wild-type and 5-HTT+ mice. Both human and mouse 5-HTT expression were reduced after exposure to hypoxia (Figure 4).

Discussion

Ubiquitous 5-HTT overactivity and/or transcription occurs in patients with both fPAH and secondary PAH, and this is observed in pulmonary arteries and platelets.^{9,16} Here, we

TABLE 2 K_D and B_{max} for [³H]Citalopram Binding in Lung Preparations From 5-HTT+ and Wild-Type Mice After Exposure for 2 Weeks to Hypoxia or Room Air (Normoxia)

	Normoxia		Hypoxia	
	5-HTT+ (n=3)	WT (n=3)	5-HTT+ (n=6)	WT (n=6)
K_D , nmol/L	0.31±0.06	0.06±0.05*	0.012±0.01*	NSB
B_{max} , fmol/mg	2687±131	438±86*	372±14*	NSB

WT indicates wild-type; NSB, nonspecific binding; and n, number of assays (carried out in triplicate, each done on preparations from n=8–10 lungs).

* $P < 0.001$ vs normoxic 5-HTT+.

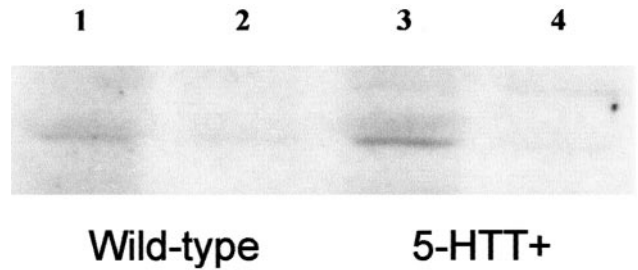


Figure 3. Western blot of 5-HTT in lungs from normoxic (lane 1) and hypoxic (lane 2) wild-type mice and normoxic (lane 3) and hypoxic (lane 4) 5-HTT+ mice.

show that increased 5-HTT expression induced an increase in RVP before any onset of RVH and pulmonary vascular remodeling. Dissociations of pulmonary pressure and remodeling have been reported previously for experimental PAH.^{17–19} To date, it is not clear whether such a phenomenon arises in fPAH patients, because they present to the clinic at an advanced stage. One possible explanation is that overexpression of 5-HTT promotes factors that increase vascular reactivity and resistance while protecting against remodeling. Increased uptake of 5-HT into, eg, platelets may protect the pulmonary and cardiac tissue from the mitogenic effects of circulating 5-HT by reducing free 5-HT. Conversely, inhibition of these protective effects by hypoxia in the face of elevated pulmonary pressure may enhance mitogenic effects by elevating free 5-HT levels. The question of which phenomenon occurs first, the hypertension or the remodeling, has been asked on many occasions. This model has been able to address this and suggests that elevated pulmonary pressures may be the primary phenomenon in some cases of PAH.

PAH has a multifactorial pathobiology, however, and it is unlikely that one factor will explain all cases of PAH. Only

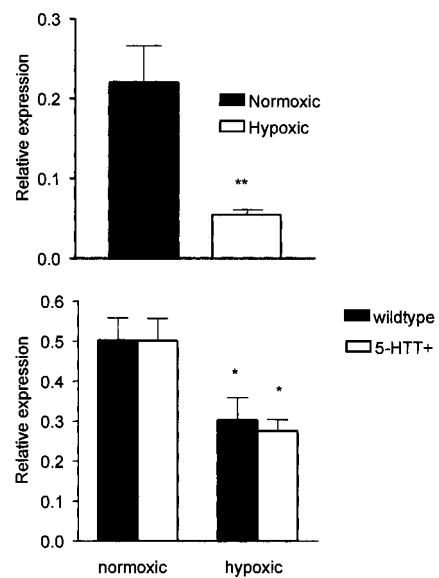


Figure 4. Relative expression of lung 5-HTT mRNA in mouse lungs. A, Human mRNA expression in normoxic (n=6) and hypoxic (n=5) 5-HTT+ mice. B, Mouse mRNA expression in wild-type and 5-HTT+ normoxic and hypoxic mice (n=4 to 6). * $P < 0.05$, ** $P < 0.01$ vs normoxic mice.

≈60% of fPAH patients, 32% of iPAH patients, and 15% of patients with PAH secondary to fenfluramine ingestion have the BMRP2 gene mutation.^{11,12,20} The L-allelic variant of the 5-HTT gene was found in only 65% of patients with PAH.⁹ Therefore, modifying genes or factors, such as hypoxia, must be involved in the pathobiology of PAH. We therefore examined the effect of hypoxia on the development of PAH in the 5-HTT+ mice. The effects of hypoxia were more pronounced in the 5-HTT+ mice, and hypoxia induced RVH and pulmonary vascular remodeling, which were increased twice as much in the 5-HTT+ mice as in the wild types.

The results suggest that early intervention with a 5-HTT inhibitor may ameliorate some cases of fPAH, but perhaps a combination of therapies will be required if environmental factors, such as hypoxia, are also a consideration. Fluoxetine has been shown to inhibit hypoxia-induced PAH in mice.²¹ The mice studied, however, exhibit normal 5-HTT activity before hypoxic exposure. Furthermore, 3 variants of the murine 5-HTT are known to exist,²² and allelic variations in humans result in differential effects of selective 5-HTT inhibitors.²³ Therefore, careful clinical studies on patients with and without the L-allelic variation will be necessary to determine fully the usefulness of the 5-HTT inhibitors.

Previous studies in rats have suggested that hypoxia can either decrease or increase 5-HTT activity/transcription.^{4–6} In mice, hypoxia decreases 5-HT uptake.⁷ The variation in the results of these studies may be due to age, sex, or strain differences combined with differences in hypoxic exposure and/or resident atmospheric pressures. In addition, it has been shown that there are marked differences in peripheral and central 5-HTT expression and function between rat strains in commercially obtained and homebred rats.²⁴ In the present study, hypoxia induced a decrease in 5-HTT transcription, receptor number, and protein. Several intracellular signal transduction pathways converge on the 5-HTT transcriptional apparatus, and expression can be regulated by cAMP, protein kinase C, and tyrosine kinase-dependent mechanisms.²⁵ Hypoxia can induce cell-specific changes in intracellular signaling mechanisms. For example, hypoxia can decrease cAMP in pulmonary arteries and endothelial cells,^{26,27} which may provide one putative mechanism for decreasing 5-HTT. The proximal promoter regions of both mouse and human 5-HTT genes contain sequence elements conferring responsiveness to cAMP. cAMP is considered an important upregulator of 5-HTT transcription.^{10,25} Hence, the effect of hypoxia on 5-HTT expression will be a result of the net effect of such inhibitory mechanisms and any positive influence that factors such as hypoxia-inducible factor-1 may exert.⁸ This, in turn, may well be species, sex, and age dependent and influenced by the duration and degree of hypoxia.

An increase in 5-HTT expression is clearly not a requirement for the development of hypoxia-induced PAH, which can occur despite decreased 5-HTT expression. This is supported by the observations that hypoxia has species-dependent effects on 5-HTT expression, because the mechanism of chronic hypoxic PAH is unlikely to be different between species. The observation that 5-HTT overexpression alone can have an isolated effect on pulmonary pressures, whereas the effect of hypoxia causes RV and pulmonary

vascular remodeling simultaneously, suggests that 2 independent mechanisms are at play.

The effects of 5-HTT overexpression and hypoxia were specific to the pulmonary circulation. This is most likely because of the unique ability of PASMCs to proliferate to 5-HT via a 5-HTT-dependent mechanism. Indeed, there are no reports of 5-HT-induced proliferation being mediated by the 5-HTT in the systemic vascular SMCs, in which 5-HT-induced proliferation is mediated almost exclusively by 5-HT_{2A} receptors (eg, see Sharma et al²⁸).

There was an increase in 5-HTT immunoreactivity in the pulmonary arteries of the 5-HTT+ mice. This was present in the endothelial cells and the PASMCs, particularly those bordering the adventitia. There was also 5-HTT immunoreactivity in newly formed muscular layers of remodeled small pulmonary arteries. This suggests that there is differential expression of the 5-HTT between different smooth muscle cell phenotypes within the vessel wall. This hypothesis is consistent with previous reports suggesting that different phenotypes of smooth muscle cell respond differentially to contractile and mitogenic stimuli.²⁹ There was a 6-fold increase in [³H]citalopram binding in the 5-HTT+ lungs. The B_{max} observed for the wild-type mice is consistent with that previously reported for rat and mouse central 5-HTT binding sites.^{30,31} The affinity for [³H]citalopram was higher against the native mouse transporter than the human transporter. This is consistent with studies showing that [³H]citalopram binds to rat neuronal membranes with higher affinity than to human.³² There was a decrease in B_{max} for [³H]citalopram in the lungs of hypoxic 5-HTT+, mice although the affinity was increased, which may have compensated, in part, for the reduction in binding sites per se. Hypoxia abolished the binding completely in the wild-type controls, which still developed PAH after hypoxic exposure. This decrease was verified by Western blotting and TaqMan RT-PCR analysis and confirms that overexpression of the 5-HTT receptor site is not required for the development of hypoxia-induced PAH.

In conclusion, we have demonstrated that overexpression of the 5-HTT can induce elevated RVP, in the short term, in the absence of RVH or pulmonary vascular remodeling. Initial overexpression of the 5-HTT does increase hypoxia-induced PAH, but this is not dependent on further increases in 5-HTT activity.

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Overexpression of the 5-Hydroxytryptamine Transporter Gene: Effect on Pulmonary Hemodynamics and Hypoxia-Induced Pulmonary Hypertension

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