

Deficiency of the 5-Hydroxytryptamine Transporter Gene Leads to Cardiac Fibrosis and Valvulopathy in Mice

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Background—Serotonin (5-hydroxytryptamine; 5-HT) overproduction is responsible for cardiac valvular disease in patients with carcinoid tumors. Reduced 5-HT inactivation is one proposed mechanism of the valvulopathy observed in individuals treated with the appetite suppressants fenfluramine and phentermine. One key protein limiting systemic availability of 5-HT is the 5-HT transporter (5-HTT) expressed by platelets and pulmonary vascular cells; 5-HTT is responsible for 5-HT uptake and subsequent inactivation of the amine passing through the lung. Here we investigated whether 5-HTT-deficient (5-HTT-KO) mice developed structural and/or functional cardiac abnormalities and valvulopathy.

Methods and Results—Cardiac endothelial cells expressed large amounts of 5-HTT in wild-type mice. 5-HTT deficiency appeared to be associated with marked interstitial, perivascular, and valvular fibrosis as evidenced by staining of cardiac collagen in 5-HTT-KO mice. Histological analysis provided evidence for valvulopathy characterized by valvular hyperplasia and prominent fibrosis at the attachment site and base of the leaflets. Echocardiography revealed an increase in left ventricular lumen diameter and a decrease in left ventricular diameter fractional shortening. Although 5-HT_{1B} receptors mediated the 5-HT-induced collagen secretion by human cardiac myofibroblasts, the contribution of this receptor type to valvulopathy was ruled out because double-KO mice deficient in both 5-HTT and 5-HT_{1B} receptors showed the same cardiac alterations as 5-HTT-KO mice.

Conclusions—The present results establish a link between 5-HTT and the development of cardiac fibrosis and valvulopathy in vivo. 5-HTT-KO mice represent an especially relevant model for studying the mechanisms by which 5-HT induces valvulopathy. (*Circulation*. 2006;113:81-89.)

Key Words: heart diseases ■ pathology ■ valves ■ fibrosis ■ serotonin

Serotonin (5-hydroxytryptamine; 5-HT) controls a wide range of biological functions. In the brain, its role as a neurotransmitter has been abundantly documented. At the periphery, 5-HT originating from the gastrointestinal tract is stored in blood platelets and participates in coagulation and blood pressure homeostasis. 5-HT has been shown to play an important role in various cardiovascular disorders, most notably in the pathogenesis of valvular heart disease. The carcinoid syndrome, which is caused by release of 5-HT and other vasoactive substances from the tumor, results in endocardial damage followed by right-sided valvular dysfunction.¹⁻³ Long-term treatment with drugs acting on 5-HT receptors, such as ergotamine or methysergide, is also known to induce valvular changes similar to those observed in carcinoid heart disease.⁴

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More recently, the occurrence of heart valve dysfunction in patients treated with the appetite suppressant combination fenfluramine plus phentermine has been postulated to involve 5-HT.⁵ This hypothesis is based on the well-established fact that dexfenfluramine is a potent platelet 5-HT releaser and 5-HT reuptake inhibitor, whereas phentermine inhibits monoamine oxidase activity and thereby 5-HT degradation within vascular cells. In patients with carcinoid tumors, the cardiac lesions are generally confined to the right side of the heart, whereas fenfluramine/phentermine-induced lesions affect both the left and right sides. One hypothesis is that, at the periphery, 5-HT secreted from enterochromaffin cells is

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partially removed from the circulation by the liver and stored in platelets, but that complete removal of free circulating 5-HT is normally achieved by the lung, thus sparing the left-sided valves from exposure to high 5-HT levels under basal physiological conditions. 5-HT therefore appears as the most likely candidate mediator in the development of valvular heart disease associated with fenfluramine/phentermine treatment. Recent experimental studies showing that long-term 5-HT administration induces heart valve disease in rats is consistent with this hypothesis.⁶

One key protein involved in 5-HT clearance is the membrane-bound 5-HT transporter (5-HTT), which is responsible for cellular internalization of the bioamine.^{7,8} 5-HTT is highly expressed in platelets and in the lung, notably in pulmonary artery endothelial and smooth muscle cells.^{9,10} It is presently unknown whether lung and/or platelet 5-HTT activity exerts a critical protective role against the potential deleterious effects of circulating 5-HT on the heart. Although treatment with selective 5-HTT inhibitors does not seem to be associated with valvular heart disease,¹¹ it is not known whether 5-HTT deficiency reproduces some of the cardiac or valvular alterations observed in patients with carcinoid syndrome and in individuals undergoing long-term fenfluramine/phentermine treatment. In the present study, we reasoned that if 5-HTT were directly involved in the pathogenesis of cardiac fibrosis, mice with targeted disruption of the 5-HTT gene (5-HTT-KO)^{12,13} would be prone to develop structural and functional cardiac abnormalities. We therefore performed hemodynamic and histological investigations aimed at assessing whether abnormal fibrosis and functional alterations occur in the hearts of 5-HTT-deficient mice. Because one of the potential mechanisms of 5-HT-induced valvular heart disease may involve 5-HT_{1B} receptors, similar investigations were also performed in 5-HT_{1B} receptor-deficient mice (5-HT_{1B}-KO) and in double mutants devoid of both 5-HTT and 5-HT_{1B} receptors (5-HTT-KO×5-HT_{1B}-KO). In addition, *in vitro* experiments with human cardiac myofibroblasts allowed direct evaluation of 5-HT_{1B} receptor involvement in collagen synthesis associated with fibrosis.

Methods

Animals

Mice lacking 5-HTT (5-HTT-KO)¹³ and the 5-HT_{1B} receptor (5-HT_{1B}-KO)¹⁴ were generated by homologous recombination on a mixed genetic background (129/Sv×C57BL/6). Double 5-HTT-KO×5-HT_{1B}-KO mutants were obtained by crossing 5-HT_{1B}-KO with 5-HTT-KO heterozygous mice. Pups were typed by Southern blot analysis of tail biopsy samples as described previously.^{13,14} The anatomic, histological, and functional studies were performed on 8-to-10-week-old male mice. All animal care and procedures were in accordance with institutional guidelines.

Detection of mRNAs Encoding Tryptophan Hydroxylases, 5-HT Receptors, and the 5-HTT in the Mouse Heart

RNA was extracted from the entire heart with Trizol reagent (Gibco BRL). Total RNA was systemically treated with DNase. RNA concentration and quality were determined by agarose gel electrophoresis and spectrophotometry.¹⁵ mRNAs encoding 5-HT receptors, 5-HTT, and tryptophan hydroxylases 1 (Tph1) and 2 (Tph2) were detected by reverse transcription followed by polymerase chain

reaction (RT-PCR). In brief, 1 μ g of total RNA was reverse-transcribed (45 minutes at 48°C) and amplified with use of the access RT-PCR kit (Promega) with specific primers (Table 1) in the presence of 2.5 mmol/L MgCl₂. Cycle amplifications were performed at 94°C, 54°C, and 72°C (1 minute each, 28 cycles). PCR products were electrophoresed in 1% agarose gels stained with ethidium bromide.

Cardiac 5-HTT Immunoblotting

Immediately after removal, the hearts were quickly frozen in LN₂ and stored at −80°C. After being thawed at 0°C, tissues were sonicated in 0.1 mmol/L phosphate-buffered saline containing anti-proteases (1 μ mol/L leupeptin and 1 μ mol/L pepstatin A). Homogenates were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and proteins in the gel were transferred to a nitrocellulose membrane by electroblotting with use of a transblot Bio-Rad transfer apparatus (Marnes La Coquette). The 5-HTT protein was detected by incubating the nitrocellulose membrane with goat polyclonal anti-5-HTT antibodies (Santa Cruz Biotechnology) at 1:1000 dilution, as described in detail elsewhere.¹²

Immunohistochemical Labeling

Paraffin sections (5 μ m thick) were mounted on Superfrost Plus slides (Fisher Scientific). 5-HTT immunostaining was performed with the same polyclonal anti-5-HTT antibodies as those used for immunoblotting experiments but diluted to 1:2000, as previously described.¹²

Hemodynamic Measurements

Mice aged 8 to 10 weeks were anesthetized with ketamine (60 mg/kg IP) and xylazine (10 mg/kg IP). The right carotid artery was exposed and a polyethylene catheter (PE10) was inserted. Arterial pressure was measured with a Gould P10 EZ pressure transducer connected to pressure modules and a Gould TA 550 recorder. The heart rate under these conditions was between 300 and 500 bpm. If the heart rate fell to <300 bpm, measurements were excluded from analysis (this happened twice). Finally, animals were deeply anesthetized with sodium pentobarbital (40 mg/kg IP) and exsanguinated. The thorax was opened and the heart was removed and weighed.

Echocardiography

Mice were anesthetized with ketamine (60 mg/kg IP) and xylazine (10 mg/kg IP). The left side of the thorax was shaved. Echocardiograms were recorded with a Technos echo device (ESAOTE Biomedica) equipped with a 13.8-MHz probe. Echocardiography data (2D, M-mode, and Doppler) were collected on an optical disk and stored for off-line analysis. Left ventricle wall thickness and lumen diameters at end diastole and end systole were measured from the M-mode images, and fractional shortening was calculated. Five consecutive beats were analyzed in triplicate and averaged.

Histological Analysis and Assessment of Cardiac Fibrosis

The hearts were fixed in a bath of 4% aqueous buffered formalin for 1 week and processed for paraffin embedding. Longitudinal sections (5 μ m thick) containing both right and left ventricles were stained with the collagen-specific Sirius red and Masson's trichrome dye.¹⁶ Image analysis was performed with a charge-coupled device Iris camera (CDD Iris, Sony) coupled with a light microscope (Laborlux). Total collagen volume fraction (ie, the ratio of collagen surface area to total cardiac surface area, as a percentage) of the stained tissue, was determined separately in the valve area and myocardium with Perfect Image software (Clara Vision).

Culture of Human Cardiac Valve Myofibroblasts

Human aortic and mitral heart valves were collected from recipients during valve replacement surgery for nonrheumatic aortic stenosis or mitral insufficiency. Explants were cut into small pieces (3 to 5 mm), which were transferred to cell-culture flasks (Nunc). To allow the

TABLE 1. Primers for RT-PCR Analysis of mRNAs Encoding Mouse 5-HT Receptors, 5-HTT, Tph1, and Tph2

Receptor	Primer	Position	Δ , °C	Length, bp	GenBank Accession No.
5-HT _{1A}	TGCTCATGCTGGTCTCTAT	1118–1137	59.4	180	NM_008308
	TCTCAGCACTGCGCCTGC	1297–1280			
5-HT _{1B}	tcggattttgaacagacacc	699–719	58.2	200	NM_010482
	gtttctctttccagcaggg	898–878			
5-HT _{1D}	tcaaccaggtgaaatcaagc	1783–1803	59.4	277	NM_008309
	gcttgctgaaagtcttcgttg	2059–2039			
5-HT _{2A}	AAGCCTCGAACTGGACAATTGATG	1215–1238	60	470	NM_172812
	AATGATTTTCAGGAAGGCTTTGGTT	1684–1660			
5-HT _{2B}	CTCTTTTCACTGCCTCCATC	441–461	56.5	123	NM_008311
	TGTAATCTTGATGAATGCAGTAGCC	563–539			
5-HT _{2C}	ATTGGCTATGTGTTCAGGC	1759–1779	60	640	NM_008312
	AGGAAGAAAGCAACGAAGGAG	2398–2378			
5-HT _{3A}	GGAAGTCTCCGAACATTCCTT	458–478	62	1122	NM_013561
	CCCCATACTTATCCTAACCA	1579–1559			
5-HT ₄	taatgtgagttccaacgaggg	20–40	60	483	NM_008313
	actatgcgatgtgttccag	502–482			
5-HT ₆	agtctgctgacagtagcgctc	777–797	56.5	104	AF134158
	CAGCCAGGTGACAAAGAACAT	880–860			
5-HTT	ACATCTGGCGTTTCCCTACAT	488–509	60	525	NM_010484
	TTTTGACTCCTTTCCAGATGCT	1012–991			
TPH1	tgatggttccagtgcatatcc	1775–1797	62	241	NM_009414
	cgtggcacgtgaactatattcc	1993–2016			
TPH2	CTACAGTAGACTTGTCAGATGTC	1604–1627	62	935	NM_173391
		2516–2539			

Δ Indicates hybridization temperature.

myofibroblasts to grow out, the valve tissues were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 15% fetal calf serum (FCS), 2 mmol/L L-glutamine, and antibiotics (100 U/mL penicillin and 0.1 mg/mL streptomycin). After 2 weeks, the cells collected in the culture medium and the valve tissues were transferred to new cell-culture flasks. Cell phenotype was determined with monoclonal antibodies against desmin, vimentin, and factor VIII. Cells positive for vimentin and negative for desmin and factor VIII were labeled as myofibroblasts. We found that >95% of cells were myofibroblasts in these experiments, whether the cells were derived from the mitral or the aortic valve. Cells were used for the study between passages 1 and 3.

Measurement of [³H]Proline Incorporation

Myofibroblasts in DMEM supplemented with 15% FCS were seeded into 24-well plates at a density of 2.5×10^4 cells/well and allowed to adhere. The cells were subjected to 48 hours of growth arrest in medium containing only 0.2% FCS and then incubated in DMEM supplemented with 0.2% FCS, 0.6 mmol/L ascorbic acid, 0.1 mmol/L iproniazid (a monoamine oxidase inhibitor), and 5 μ Ci/mL [³H]proline, with (10^{-8} to 10^{-6} mol/L) or without 5-HT. The effect of 5-HT was also examined in the presence of 10^{-6} mol/L fluoxetine (a specific 5-HTT inhibitor), GR127935 (a 5-HT_{1B/1D} receptor antagonist), ketanserin (a 5-HT_{2A} receptor antagonist), or SB206553 (a 5-HT_{2B/2C} receptor antagonist).¹⁷ Each of these drugs was added 20 minutes before 5-HT. After incubation for 48 hours, the cells were washed with phosphate-buffered saline, treated with ice-cold 10% trichloroacetic acid, and dissolved in 0.3 N NaOH/0.1% sodium dodecyl sulfate. The incorporated radioactivity was

counted. [³H]proline incorporation is reported as counts per minute per well.

Blood 5-HT and 5-HIAA Concentrations

5-HT and its metabolite 5-hydroxyindoleacetic acid (5-HIAA) were determined in whole blood by high-performance liquid chromatography coupled to electrochemical detection, as described in detail elsewhere.¹⁸

Chemicals

[³H]proline was from Amersham Pharmacia Biotech. Iproniazid, ascorbic acid, 5-HT, and SB206553 (*N*-3-pyridinyl-3,5-dihydro-5-methylbenzo[1,2-b:4,5-b']dipyrrole-1[³H]-carboxamide hydrochloride) were from Sigma. The other compounds were fluoxetine (Eli Lilly), ketanserin (Janssen), and GR127935 {2'-methyl-4'-(5-methyl-(1,2,4)oxadiazol-3-yl)-biphenyl-4-carboxylic acid [4-methoxy-3-(4-methyl-piperazine-1-yl)-phenyl]amide; GlaxoSmithKline}.

Statistical Analysis

All results are reported as mean \pm SEM. The nonparametric Mann-Whitney test was used to compare wild-type (WT) and 5-HTT-KO mice. The Kruskal-Wallis test was used to assess the in vitro effects of 5-HT on fibroblasts and for comparisons among WT, 5-HT_{1B}, and 5-HTT mutant mice. When the Kruskal-Wallis test showed a significant difference, the groups were further compared with a nonparametric Student-Newman-Keuls test. Probability values <0.05 were considered statistically significant.

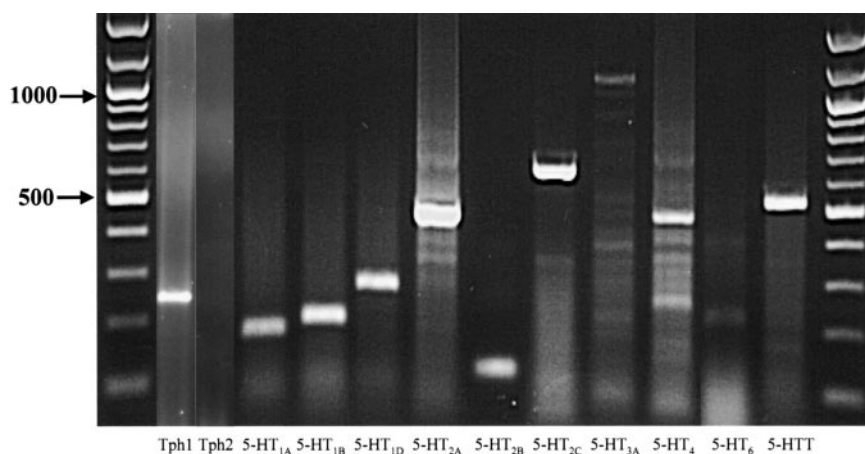


Figure 1. Identification of mRNAs encoding Tph1 and Tph2, 5-HT receptor types, and 5-HTT in cardiac tissues from WT mice. RT-PCR products showed expression of various 5-HT receptor types, notably 5-HT_{1A}, 5-HT_{1B}, 5-HT_{1D}, 5-HT_{2A}, 5-HT_{2B}, 5-HT_{2C}, and 5-HT_{3A}. Cardiac tissues also expressed 5-HT₄ but not 5-HT₆ receptors. The last lane shows the RT-PCR product corresponding to cardiac 5-HTT mRNA. Tph1 mRNAs but not Tph2 mRNAs were detected in cardiac tissues.

Results

Expression of Tph, 5-HT Receptors, and 5-HTT in the Mouse Heart

RT-PCR analysis showed that mouse cardiac tissues contained mRNAs for Tph1, 5-HTT, and various 5-HT receptor types, including 5-HT₁ (a, b, and d), 5-HT₂ (a, b, and c), 5-HT_{3A}, and 5-HT₄ (Figure 1). In contrast, mRNAs encoding 5-HT₆ receptor or Tph2 were not detected in the mouse heart. Significant amounts of Tph1 mRNAs were found in human valvular tissues (data not shown). 5-HTT expression in the heart was further confirmed by immunoblotting experiments, which showed high levels of 5-HTT protein in cardiac tissues from WT mice. Immunohistochemical analyses showed predominant localization of 5-HTT in the endocardium and endothelium of coronary arteries and capillaries (Figure 2a). In contrast, 5-HTT protein was not detected in tissues from 5-HTT-KO mice (Figure 2b) or in isolated cardiomyocytes from WT animals (data not shown).

Blood 5-HT and 5-HIAA Concentrations in 5-HTT-KO Versus WT Mice

Whole-blood 5-HT levels were dramatically reduced in 5-HTT-KO mice (n=6) compared with WT mice (n=6), from 29.25 ± 1.10 to 0.35 ± 0.02 nmol/mL ($P=0.004$), whereas blood 5-HIAA levels did not differ between the 2

groups (1.60 ± 0.30 nmol/mL in 5-HTT-KO mice and 1.15 ± 0.20 nmol/mL in WT mice; n=5 in each group).

Anatomic and Hemodynamic Characteristics of the Heart in 5-HTT-KO Versus WT Mice

As shown in Table 2, 5-HTT-KO and WT mice did not differ with regard to body weight, heart weight, heart weight to body weight ratio, systemic arterial pressure, or heart rate. Moreover, cardiac function evaluated by noninvasive transthoracic Doppler echocardiography revealed both dilated cardiopathy (increased left ventricular lumen diameter) and myocardial hypokinesis (decreased fractional shortening) in 5-HTT-KO mutants compared with paired WT mice (Table 3).

Histological Evidence of Cardiac Fibrosis in 5-HTT-KO Versus WT Mice

Histological analysis showed that 5-HTT-KO mice developed cardiac fibrosis compared with WT mice. Collagen staining predominated in valvular tissues (Figure 3a and 3d) but was also seen in interstitial (Figure 3b and 3e) and perivascular (Figure 3c and 3f) regions in both ventricles. Quantification of collagen staining revealed significantly more collagen in both myocardial and valvular regions in 5-HTT-KO mice compared with littermate controls (Figure 4). Myocardial fibrotic lesions did not appear to affect sections diffusely but instead seemed to occur focally within the myocardium. In

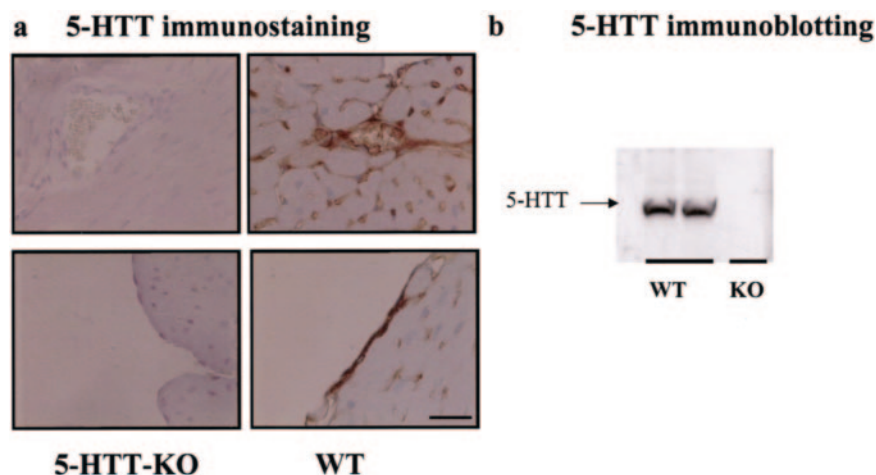


Figure 2. a, Immunohistochemical labeling of 5-HTT in cardiac sections from a WT and a 5-HTT-KO mouse. Dense immunostaining was observed in the endocardium and vessels from the WT mouse. No immunostaining was detected in the 5-HTT-KO mouse. Scale bar=100 μ m. b, Western blot of 5-HTT protein in cardiac tissues from WT and 5-HTT-KO mice. The specific antibodies recognized a protein with an apparent molecular weight of 64 kDa.

TABLE 2. Anatomic and Hemodynamic Parameters in WT and 5-HTT KO Mice

	WT (n=6)	5-HTT KO (n=5)	P
Body weight, g	33.8±0.6	34.4±1.3	0.60
Heart weight, mg	173.7±13.6	164.0±11.0	0.92
Heart weight/body wt, mg/g	5.1±0.3	4.8±0.3	0.75
Systolic arterial pressure, mm Hg	105±8	99±11	0.52
Diastolic arterial pressure, mm Hg	75±3	69±8	0.30
Mean arterial pressure, mm Hg	88±4	84±10	0.37
Heart rate, bpm	327±18	312±15	1.00

valvular regions, histological examination demonstrated leaflet thickening and marked collagen accumulation at the attachment site and in the leaflet itself. These lesions were homogeneously distributed in the mitral, tricuspid, aortic, and pulmonary valves, as illustrated by Figure 5c, showing similar collagen staining of the mitral and aortic valves in the same cardiac section. Interestingly, several areas of chondroid metaplasia were identified within the valvular tissue in 4 of 6 5-HTT-KO mice; these areas were more prominent at the attachment site and base of the leaflets (Figure 5e).

5-HT_{1B} Receptor-Mediated Increase in [³H]Proline Incorporation in Human Cardiac Valve Myofibroblasts

In myofibroblasts cultured in serum-free medium, 5-HT produced a concentration-dependent increase in [³H]proline incorporation. Pretreatment of the cells with GR127935 (10⁻⁶ mol/L), a 5-HT_{1B/1D} receptor antagonist, significantly inhibited the effect of 5-HT (Figure 6). In contrast, the 5-HT-induced increase in [³H]proline incorporation was not affected by cell exposure to 10⁻⁶ mol/L of the 5-HT_{2A} and 5-HT_{2B/2C} receptor antagonists ketanserin and SB206553, respectively. Similarly, the effect of 5-HT was unchanged by cell treatment with the 5-HTT inhibitor fluoxetine (Figure 6).

Anatomic, Hemodynamic, and Histological Characteristics of the Heart in 5-HT_{1B}-KO and Double 5-HTT-KO×5-HT_{1B}-KO Mice Compared With WT Mice

In contrast to 5-HTT-KO mutants, 5-HT_{1B}-KO mice were not significantly different from the relevant WT controls with regard to anatomic, histological, and functional characteris-

tics of the heart (data not shown). However, double 5-HTT-KO×5-HT_{1B}-KO mutants exhibited marked alterations in cardiac parameters. Thus, echocardiography revealed similar changes in left ventricular lumen diameter and fractional shortening in double mutants as in 5-HTT-KO mice. Moreover, the extent of cardiac fibrosis was similar in these 2 groups (Figure 7).

Discussion

The present results show that 5-HTT gene deficiency in 5-HTT-KO mice led to the development of cardiac myocardial and valvular fibrosis, together with left ventricular dysfunction and dilation, supporting a role for 5-HT in the induction of valvular heart disease. Thus, 5-HTT-deficient mice seem highly relevant for studying the mechanisms by which 5-HT may induce valvulopathy. Although 5-HT_{1B} receptors were found to mediate the stimulatory effect of 5-HT on cardiac fibroblast-induced collagen production, double-mutant mice deficient in 5-HTT- and 5-HT_{1B}-encoding genes exhibited the same cardiac alterations as 5-HTT-KO mice, indicating that 5-HT_{1B} receptors do not contribute to 5-HT-induced cardiac fibrosis.

The potential role of 5-HT in cardiac function, as well as its involvement in various cardiovascular disorders, has generated considerable interest in recent years. Thus, disruption of the nonneuronal *Tph1* gene encoding tryptophan hydroxylase, the rate-limiting enzyme of 5-HT synthesis in peripheral tissues, was recently shown to induce cardiomyopathy, demonstrating the importance of peripheral 5-HT for normal heart development and function.¹⁹ As shown in the present study, several 5-HT receptor types are expressed by cardiac cells, suggesting that 5-HT may exert various direct effects on the heart. Although 5-HT₄ receptors have been implicated in the control of cardiac electrical activity,²⁰ previous studies in mice lacking or overexpressing 5-HT_{2B} receptors revealed important roles for this receptor in heart morphogenesis and cardiomyocyte hypertrophy.²¹ However, the roles for other 5-HT receptor types expressed by the mouse heart, including 5-HT_{1A}, 5-HT_{1B}, 5-HT_{1D}, 5-HT_{2A}, and 5-HT_{2C}, remain to be determined. In addition, we found that cardiac tissues expressed large amounts of 5-HTT. Although 5-HTT has been shown to be expressed by fetal cardiac myocytes and to mediate their proliferation,²² we found, in accordance with other studies,²² that 5-HTT was not expressed by adult cardiomyocytes. This suggests a transient effect of 5-HTT on cardiomyocytes confined to the developmental period. In adult WT mice, cardiac 5-HTT expression predominated in vascular and endocardial endothelial cells, suggesting a high capacity of these cells to internalize 5-HT and presumably to achieve its degradation. This observation therefore suggests that, in addition to pulmonary vascular cells, cardiac endothelial cells may be well equipped to clear 5-HT from the bloodstream, thereby controlling the potential cardiac effects of indoleamine released at remote peripheral sites.

We used 5-HTT-KO mice, which have been well characterized in terms of locomotor activity and behavior.¹³ As reported previously, we found that blood 5-HT concentrations were dramatically reduced in the mutants compared with WT mice, an expected finding, because platelet 5-HT is known to

TABLE 3. Echocardiographic Parameters in WT and 5-HTT-KO Mice

	WT (n=5)	5-HTT-KO (n=6)	P
LVD-ED, mm	3.02±0.10	3.86±0.05	<0.01
LVD-ES, mm	1.48±0.09	2.46±0.18	0.02
FS, %	51±2	39±4	<0.01
IVS-ED, mm	0.80±0.03	0.90±0.09	0.34
PW-ED, mm	0.82±0.03	0.87±0.09	0.33

LVD indicates left ventricular lumen diameter; ED, end diastole; ES, end systole; IVS, interventricular septum thickness; PW, left ventricular posterior wall thickness; and FS, left ventricular diameter fractional shortening.

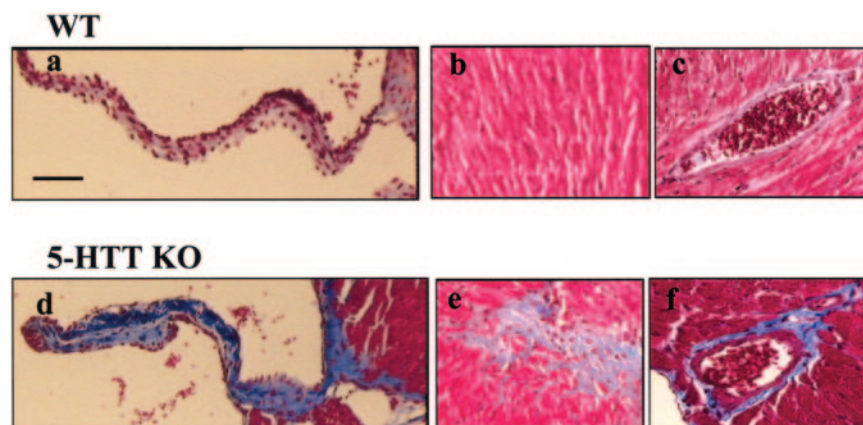


Figure 3. Collagen accumulation visualized by Masson's trichrome staining of heart sections from 5-HTT-KO mice. Compared with WT mice, there was marked collagen accumulation in the aortic leaflets from 5-HTT-KO mice (a, d). In addition, collagen staining was observed in interstitial (b, e) and perivascular (c, f) regions but to a lesser degree than in the valves. Scale bar=100 μ m.

contribute 99% of whole-blood 5-HT.¹² In contrast, blood 5-HIAA levels did not differ between 5-HTT-KO and WT mice, arguing against adaptive alterations in 5-HT synthesis in the KO animals. Suppression of 5-HT uptake by platelets or vascular cells may have resulted in an increase in the plasma 5-HT concentration, which is usually in the nanomolar range and therefore difficult to assess, because platelet activation occurs during blood sampling.⁶ Our results showing Tph1 expression in mouse cardiac tissues and in human valvular tissues also suggest cardiac synthesis of 5-HT. A deficiency in 5-HTT gene activity may therefore result in more indoleamine available for binding to 5-HT receptors on cardiac tissues, either contained in plasma or produced locally within the heart.

In clinical disorders associated with increased 5-HT circulating levels such as carcinoid tumors, cardiac valve dysfunction is common. The pathological characteristics of carcinoid tumor-associated heart disease include diffuse collections of thick white plaques composed of myofibroblasts. These plaques are deposited on the endocardial surfaces of the right heart and valves, where increased extracellular matrix accumulation and increased thickness of the valve leaflets are also found. In 5-HTT-KO mice, we found fibrotic areas to be interstitial and perivascular, with evidence for valvulopathy characterized by hyperplastic valvular lesions. Although we did not observe plaque on the endocardium, the extent and

location of the fibrotic lesions in 5-HTT-KO mice differed strikingly from those commonly observed in heart failure. Indeed, cardiac fibrosis as a marker of heart failure is mainly interstitial and homogeneously distributed throughout the myocardium. In 5-HTT-deficient mice, myocardial fibrosis was heterogeneously distributed and associated with marked valvular fibrosis. An interesting feature in these mutants was the presence of several areas of chondroid metaplasia within the valvular apparatus, which were prominent at the attachment site and base of the leaflets, suggesting a contribution of 5-HT to cell metaplasia and chondrocyte development within valvular tissues.

In 5-HTT-KO mice, fibrotic lesions were found in both the right and left parts of the heart, with no apparent predominance on the right side. It is likely that the left-sided lesions contributed to the development of left ventricular dysfunction, as reflected by the increase in left ventricular lumen diameter and the decrease in left ventricular diameter fractional shortening on echocardiography. In humans, such carcinoid involvement of left-sided heart valves has been reported in patients with patent foramen ovale, carcinoid tumor of the lung, or active carcinoid syndrome with high 5-HT levels. Similar findings, including bilateral cardiac involvement and closely related pathological changes, have been reported in patients exposed to fenfluramine or ergot alkaloids. Ergot alkaloids are considered nonselective 5-HT receptor agonists, and the phentermine/fenfluramine combination is believed to increase the availability of free 5-HT as a result of both increased 5-HT release from platelets and inhibition of pulmonary monoamine oxidase activity. Taken together, these observations have led to the suggestion that increased cardiac 5-HT delivery or pharmacological activation of cardiac 5-HT receptors was the mechanism underlying the development of valvulopathy in patients taking these medications. This issue has also been addressed in patients treated with selective 5-HT reuptake inhibitors, although no relation was found between use of those drugs and development of valvular heart disease.¹¹ A direct relation between 5-HT overload and valvulopathy was recently demonstrated in an *in vivo* rat model.⁶ Both right- and left-sided heart lesions were observed, consisting of thickened valvular cusps and carcinoid-like plaques. The present results obtained in 5-HTT-KO mutants lacking lung, cardiac, and platelet 5-HTT

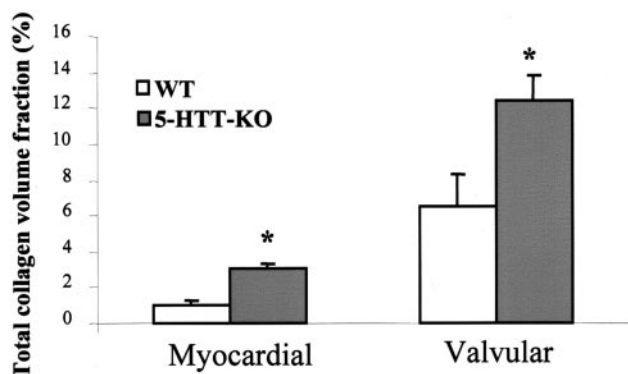


Figure 4. Myocardial and valvular fibrosis quantification in WT and in 5-HTT-KO mice. Each bar is the mean \pm SEM of 6 independent determinations (1 per mouse). * P <0.05 compared with corresponding values in WT mice.

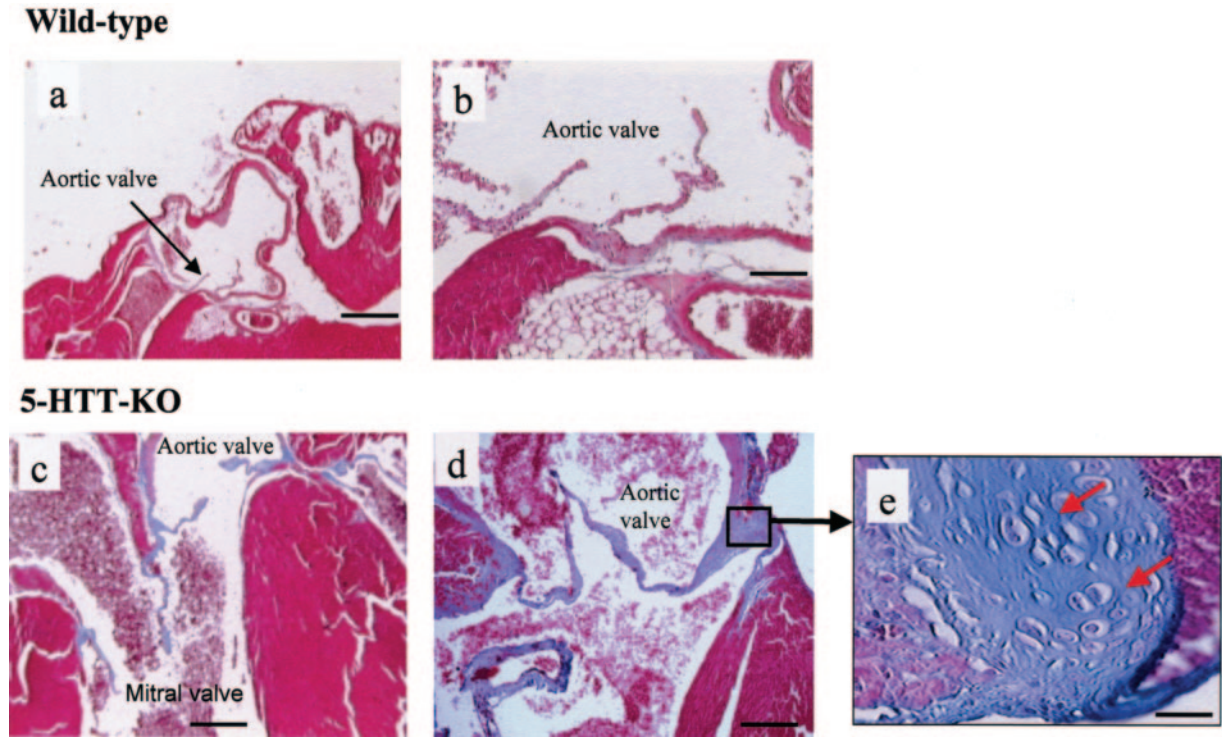


Figure 5. Histological staining (Masson's trichrome) of valvular fibrosis in a WT (a, b) and a 5-HTT-KO (c, d) mouse and chondroid metaplasia in the aortic valve apparatus of a 5-HTT-KO mouse (e). Scale bar=500 μ m in a and c, 125 μ m in b and d, and 25 μ m in e.

provide further support for a direct link between 5-HT and the development of valvulopathy. In addition, they suggest that 5-HTT may play a key role in protecting the heart from the effects of circulating or locally produced 5-HT.

The potential mechanisms leading to the formation of 5-HT-induced carcinoid plaque are still a matter of debate. Although 5-HTT was proposed as a key protein because of its high affinity for fenfluramine derivatives, its involvement seems unlikely, given the present results showing development of cardiac fibrosis in the absence of this transporter. Among potentially involved 5-HT receptors, 5-HT₁ and 5-HT₂ receptor families are good candidates because (1) they are abundantly expressed by interstitial cells in human heart valves and (2) their stimulation leads to fibroblast hyperplasia.

Special attention was given to 5-HT_{1B} receptors, whose stimulation triggers fibroblast proliferation.²³ Furthermore, this receptor type is detected in subendocardial cells, where its stimulation also leads to cell proliferation.²⁴ In the present study, we used cultured human valvular fibroblasts to examine the potential role of these receptors in 5-HT-induced incorporation of proline as an index of collagen production. Interestingly, only GR127935, an antagonist of 5-HT_{1B/1D} receptors,¹⁷ inhibited the effect of 5-HT, whereas selective blockade of either 5-HT_{2A} or 5-HT_{2B} receptors had no effect. However, double-KO mice (5-HTT-KO \times 5-HT_{1B}-KO) deficient in both the transporter and the 5-HT_{1B} receptor type did not differ from 5-HTT-deficient mice with regard to the extent of cardiac fibrosis. Thus, 5-HT_{1B} receptors do not seem

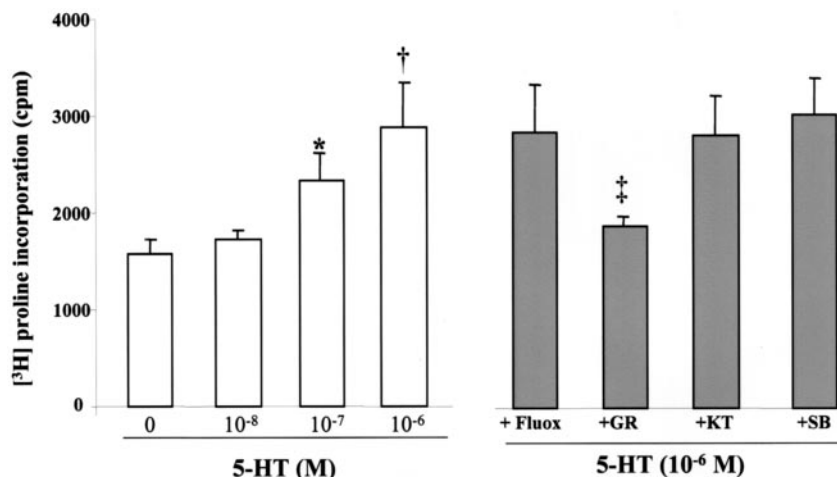


Figure 6. [³H]proline incorporation into cultured human cardiac valve myofibroblasts in response to increasing concentrations of 5-HT (10⁻⁸ to 10⁻⁶ mol/L). The response to 10⁻⁶ mol/L 5-HT was also measured in the presence of ketanserin (KT), SB206553 (SB), GR 127935 (GR), or fluoxetine (Fluox) at 10⁻⁶ mol/L each. Values are mean \pm SEM of 6 independent experiments. **P*<0.05, †*P*<0.01, compared with [³H]proline incorporation with no 5-HT added (0); ‡*P*<0.01 compared with 10⁻⁶ mol/L 5-HT alone.

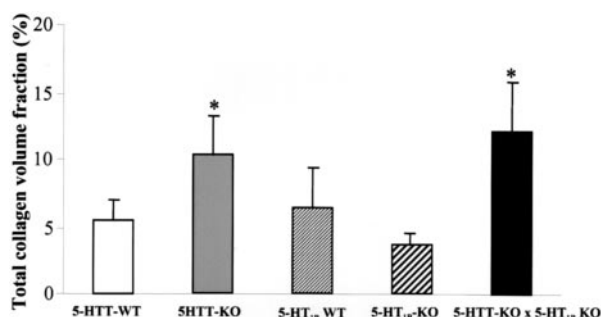


Figure 7. Valvular fibrosis quantification in 5-HTT-KO, 5-HT_{1B}-KO, and double-mutant 5-HTT-KO×5-HT_{1B}-KO mice and the respective WT controls with the same genetic background. Collagen accumulation did not differ significantly between 5-HTT-KO and double-mutant mice. However, collagen accumulation in these 2 groups was significantly greater ($P<0.05$) than in 5-HTT-WT, 5-HT_{1B}-WT, and 5-HT_{1B}-KO mice. Each bar is the mean±SEM of 6 independent determinations (1 determination per mouse).

to play a role in cardiac fibrosis, at least in mice. Until now, most of the studies were performed in vitro to evaluate the response of fibroblasts or cardiac interstitial cells to 5-HT, ergot-related drugs (ergotamine, methysergide), and fenfluramine derivatives. Depending on the studies, activation of 5-HT_{2A} or 5-HT_{2B} receptors was suggested as the potential mechanism.²⁵ Indeed, norfenfluramine, the fenfluramine metabolite, has been shown to exhibit high affinity for 5-HT_{2B} and 5-HT_{2C} but not for 5-HT_{2A} receptors.^{25,26} In contrast, studies of sheep valvular interstitial cells showed that 5-HT acted mainly through 5-HT_{2A} receptors.^{27,28} Interestingly, 5-HT_{2A} receptor activation leads to increased synthesis and function of transforming growth factor- β , suggesting that 5-HT may also have indirect fibrotic effects mediated by transforming growth factor- β . Because the present studies were performed on cells from diseased human valves, whereas the aforementioned studies investigated cells from normal animal heart valves, we cannot exclude the possibility that the action of 5-HT may vary according to the species or the presence of underlying valve disease. It is also possible that no direct relation exists between in vitro results obtained with cultured cells and in vivo results in 5-HTT-KO mice. The data reported herein suggest that the experimental model of 5-HTT-KO mice may prove useful for identifying the 5-HT receptors involved in the cardiac alterations caused by 5-HT or ergot and fenfluramine derivatives and for elucidating the underlying mechanisms.

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Disclosures

None.

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CLINICAL PERSPECTIVE

At present, a role for 5-HT in valvulopathy has been established in only a small number of conditions, including carcinoid disease and long-term fenfluramine/phentermine treatment. Here, we show that a 5-HTT deficiency leads to the development of valvulopathy in mice, probably because of increased bioavailability of 5-HT, which is no longer internalized by vascular cells or platelets and cannot be degraded. Because 5-HTT is expressed by cardiac and lung tissues, these findings suggest a key role for this transporter in protecting the cardiac valves against the toxic effects of 5-HT released from platelets. This possibility is of considerable clinical relevance because 5-HTT expression is governed by many factors, including polymorphism of its gene promoter, hypoxia, inflammatory cytokines, and drugs. Although treatment with selective 5-HTT inhibitors given for mood disorders does not seem to induce valvular heart disease, some individuals may be more susceptible to these drugs because of a 5-HTT genotype associated with low expression of the protein. Whether 5-HT contributes to the pathogenesis of aortic sclerosis is also an open question. Examining 5-HTT gene polymorphism as a risk factor for aortic sclerosis is one way to examine this possibility. Another important finding from our study is that tryptophan hydroxylase, the rate-limiting enzyme of 5-HT synthesis, is expressed in the heart. This raises the intriguing possibility that dysregulation in local 5-HT synthesis may also play a role in valvular disorders, in addition to alterations in the activity or expression of 5-HTT.

Deficiency of the 5-Hydroxytryptamine Transporter Gene Leads to Cardiac Fibrosis and Valvulopathy in Mice

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