Ablation of Serotonin 5-HT\textsubscript{2B} Receptors in Mice Leads to Abnormal Cardiac Structure and Function

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Background—Identification of factors regulating myocardial structure and function is important to understand the pathogenesis of heart disease. Because little is known about the molecular mechanism of cardiac functions triggered by serotonin, the link between downstream signaling circuitry of its receptors and the heart physiology is of widespread interest. None of the serotonin receptor (5-HT\textsubscript{1A}, 5-HT\textsubscript{1B}, or 5-HT\textsubscript{2C}) disruptions in mice have resulted in cardiovascular defects. In this study, we examined 5-HT\textsubscript{2B} receptor–mutant mice to assess the putative role of serotonin in heart structure and function.

Methods and Results—We have generated \( G_{\text{q}} \)-coupled 5-HT\textsubscript{2B} receptor–null mice by homologous recombination. Surviving 5-HT\textsubscript{2B} receptor–mutant mice exhibit cardiomyopathy with a loss of ventricular mass due to a reduction in number and size of cardiomyocytes. This phenotype is intrinsic to cardiac myocytes. 5-HT\textsubscript{2B} receptor–mutant ventricles exhibit dilation and abnormal organization of contractile elements, including Z-stripe enlargement and N-cadherin downregulation. Echocardiography and ECG both confirm the presence of left ventricular dilatation and decreased systolic function in the adult 5-HT\textsubscript{2B} receptor–mutant mice.

Conclusions—Mutation of 5-HT\textsubscript{2B} receptor leads to a cardiomyopathy without hypertrophy and a disruption of intercalated disks. 5-HT\textsubscript{2B} receptor is required for cytoskeleton assembly to membrane structures by its regulation of N-cadherin expression. These results constitute, for the first time, strong genetic evidence that serotonin, via the 5-HT\textsubscript{2B} receptor, regulates cardiac structure and function. (Circulation. 2001;103:2973-2979.)

Key Words: cardiomyopathy \& cell adhesion molecules \& genetics \& serotonin

Cardiomyopathy is an important risk factor for subsequent cardiac morbidity and mortality. Relatively little is known about the molecular mechanism underlying cardiomyopathy and heart failure. The neurohormone serotonin (5-hydroxytryptamine, 5-HT) is involved in blood pressure regulation and cardiac function in adults. 5-HT plays an important role in hemodynamic stability. 5-HT–specific reuptake inhibitors (by increasing the availability of 5-HT) produce arrhythmia, including atrial fibrillation, bradycardia, and heart block. The mitogenic action of 5-HT triggers the valvular fibrosplasia observed in carcinoid patients and in obese people taking the 5-HT uptake inhibitor/5-HT\textsubscript{2B} receptor ligand fenfluramine as an appetite suppressant.

The various biological actions of 5-HT are mediated by numerous cognate receptors. There are at least 15 receptor subtypes that belong to 4 classes: 5-HT\textsubscript{1A}, 5-HT\textsubscript{1B}, 5-HT\textsubscript{2A}, and 5-HT\textsubscript{2C}. 5-HT binding to 5-HT\textsubscript{2A}, 5-HT\textsubscript{2B}, or 5-HT\textsubscript{2C} receptors activates phospholipase C, releases inositol trisphosphate, and increases intracellular calcium levels. 5-HT\textsubscript{2B} receptor is involved in 5-HT–induced mitogenesis in which c-Src is required for cell cycle progression via the mitogen-activated protein kinase pathway. Stimulation of the 5-HT\textsubscript{2B} receptor results in cross talk with the 5-HT\textsubscript{1B/1D} receptor subtype via activation of phospholipase A\textsubscript{2}. The 5-HT\textsubscript{2B} receptor also activates nitric oxide synthesis through a PDZ domain.

To understand the specificity of each receptor subtype, the genetic inactivation approach in mice was used. Mutation of 5-HT receptors 5-HT\textsubscript{1A}, 5-HT\textsubscript{1B}, or 5-HT\textsubscript{2C} in mice leads to behavioral abnormalities. We have recently shown that 5-HT\textsubscript{2B} receptor inactivation in mice leads to trabeculation defects in embryonic heart, causing a 30% lethality at midgestation. Now, we investigated cardiopathy in surviving 5-HT\textsubscript{2B} receptor–mutant mice. This study reveals that 5-HT via the 5-HT\textsubscript{2B} receptor is involved in the regulation of cardiomyocyte cytoarchitecture and function. 5-HT\textsubscript{2B} receptor ablation in mice leads to cardiomyopathy, including left ventricular (LV) dysfunction without hypertrophy.
Methods

Generation of 5-HT$_{2B}$ Receptor–Knockout Mice

Targeted mutagenesis by homologous recombination was described previously.

Morphological Analysis of Mouse Embryos

Transmission electron microscopy and histological techniques were performed as previously described. Immunochemistry was performed on heart cryosections with the anti–sarcomeric myosin heavy chain (MHC) antibody (MF-20). Anti-tropomyosin and N-cadherin antibody reactions were performed on paraffin sections as described. Signal intensity was quantified with a fluoroimager (Typhoon, Molecular Dynamics) and calculated as the product of averaged pixel intensity per area.

Cardiomyocyte Isolation and Video Imaging

Ventricular cardiomyocytes from newborn mice were isolated as previously described. Beating rate in response to dobutamine was determined by recording video of isolated cardiomyocytes. The analysis was performed on the stage of an inverted microscope (Leica DMIRB) with software developed by J-LV.

Echocardiographic Methods

Animals (19-week-old mice) anesthetized with sodium pentobarbital (30 mg/kg IP) were observed with 2D-guided M-mode echocardiograms with a short-focal-length, 12-MHz (Hewlett-Packard Medical Systems) transducer. LV end-systolic and end-diastolic diameters (LVESD and LVEDD, respectively) were measured. The percentage of LV fractional shortening was then calculated.

Blood Pressure Measurements

Systolic arterial pressure and heart rate were recorded by the tail-cuff technique with the LE5002 Storage Pressure Meter (Letica) in awake 19-week-old mutant and control mice.

Electrocardiograph

Nineteen-week-old mice anesthetized with tribromoethanol (2.5% solution, 13 μL/g body wt SC) were recorded with the 4 arms of the ECG leads attached at the origin of each papillary and bipolar lead derivations. The signal was recorded by an ECG (EKG-Burdick, Siemens) connected to a data acquisition system (MP100 and Acknowledge Software, Biopac Systems Inc).

Isolated Perfused Heart Preparation

Hearts from mice (12 to 19 weeks old, 23 to 25 g) anesthetized with sodium pentobarbital (60 mg/kg IP) and heparinized (500 U/kg IP) were cannulated and perfused according to Langendorff at 37°C and pH 7.4 with modified Krebs-Henseleit solution containing (mmol/L) NaCl 118, NaHCO$_3$ 24, KCl 4.7, KH$_2$PO$_4$ 1.2, MgSO$_4$ 1.2, CaCl$_2$ 2.5, disodium EDTA 0.5, and glucose 10, gassed with 95% O$_2$/5% CO$_2$. Perfusion pressure was constant and equivalent to 100 cm H$_2$O. The diastolic tension of the suture was adjusted to 1 g during the stabilization period of the heart.

Measurement of Markers for Cardiac Failure and Myocardial Damage

Enzyme immunoassay for creatine kinase-MB isoenzyme and for cardiac troponin I was determined from samples of serum from adult mice.

Data Analysis and Statistics

All values represent the average of independent experiments±SEM (n=number of experiments as indicated in the text). Comparisons between groups were performed with Student’s unpaired t test or ANOVA and a Fischer test. Significance was set at P<0.05.

Results

Heart Morphology

5-HT$_{2B}$ receptor inactivation leads to partial embryonic death due to trabecular defects in the heart leading to midgestation lethality (30%, n=120). The 5-HT$_{2B}$ receptor–mutant mice that reached birth displayed no obvious defects, although 30% (n=120) of newborn mice developed signs of fatigue and dyspnea between postnatal days 2 and 5 and died within 24 hours from the onset of these symptoms. A likely cause of neonatal death is inadequate cardiac output due to hypoplasia of the LV, despite the lack of evidence for pulmonary edema. All 5-HT$_{2B}$ receptor–mutant mice that survived the first postnatal week developed to adulthood with cardiac problems. This variation in severity of the phenotype could not be attributed to variability in the genetic background of the mice (all the findings were obtained from 129/PAS pure background mice, and similar mortality was also observed on a C57/Black6J-129/PAS mixed background).

Newborn 5-HT$_{2B}$ receptor–mutant hearts display a striking decrease in the ratio of heart to body weight (28%). This difference was 24% in 6-week-old mutants (Table 1). Histological analysis demonstrated that the decrease in heart mass was restricted to the ventricles (as shown in Figure 1A).

Cardiomyocyte Number and Size

The ratio of cardiomyocytes to total cells (stained with MF-20 antibody, myocyte-specific MHC, and propidium iodide, respectively) revealed 15% fewer cardiomyocytes in the newborn mutants, as shown in Figure 1B. Isolated mutant cardiomyocytes are 12% shorter than wild-type (n>15) (Figure 1C). The decrease in ventricular mass observed in 5-HT$_{2B}$ receptor–mutant mice results, therefore, from decrease in both cell density and size of cardiomyocytes.
Hypertrophic Gene Expression in Heart

To determine whether the loss of ventricular mass creates compensatory hypertrophic growth associated with altered expression of hypertrophic markers, ANF, α-MHC, β-MHC, and GATA4 expression was evaluated in 12-week-old mutant hearts. Semiquantitative RT-PCR analysis of mutant heart mRNA demonstrated that none of these mRNAs showed significant variation in expression level (≤5% variation compared with control, n = 5 different individuals). Similar results were obtained in newborn mutants (data not shown).

Cardiomyocyte Function

To determine whether the cardiac phenotype of 5-HT\textsubscript{2B} receptor–mutant mice was cell-intrinsic, the function of spontaneously beating isolated cardiomyocytes from newborns was investigated. The β-adrenergic receptor agonist dobutamine increased the beating rate of wild-type cardiomyocytes in a dose-dependent manner. Mutant cardiomyocytes, however, exhibited an impaired response to dobutamine in the absence of sympathetic innervation (Figure 2), indicating cell autonomous defects.

Ultrastructural Analysis

A loss of myocardial organization, a scattered area of degenerated cardiomyocytes, and myofibrillar disarray were apparent in newborn mutant hearts. Wave myofibrils were identified by anti-tropomyosin staining (Figure 3A). In this area, myofilaments appeared misaligned, I bands were not detectable, abnormally wide Z bands were seen, and mitochondria were rounded and irregular (Figure 3B). The sarcomere length in mutants is 33% smaller than that in control mice (n = 25). Notably, no evidence for myocardial apoptosis, fibrosis, or significant inflammatory cell infiltrates was found. Nearly identical histopathological findings were observed in all adult mutant hearts.

Furthermore, 5-HT\textsubscript{2B} receptor–mutant cardiomyocytes had reduced numbers of adherens junctions (Table 1), and the intercalated disks were consistently disorganized (Figure 4A). Z line–associated protein expression was investigated. Vinculin staining in mutant newborn ventricles was unaltered (not shown). N-cadherin expression, however, was reduced by 38.8% in mutant myocardium (Figure 4B, Table 1).

Hemodynamic Measurements

Transsthoracic echocardiograms (Figure 5A, Table 2) show LV dilation and reduced systolic performance of the adult mutant mice. In male mutants, the LVEDD was 25% higher than wild-type. Extreme LV dilation (increased LVEDD) was observed, and the LVESD was increased by 50% in male 5-HT\textsubscript{2B} receptor mutants (n = 4). The percent of LV fractional

| TABLE 1. Morphometry of 5-HT\textsubscript{2B} Receptor–Mutant Mouse Cardiac Parameters |
|-----------------|-----------------|-----------------|
|                  | +/+             | −/−             |
| Heart-to–body weight ratio |
| Newborn          | 1.06 ± 0.12     | 0.76 ± 0.07     |
| 6 weeks          | 0.67 ± 0.40     | 0.51 ± 0.03     |
| Sarcomere length |
| Newborn          | 2.04 ± 0.02     | 1.38 ± 0.05     |
| N-Cadherin expression |
| Newborn          | 73.4 ± 12.4     | 44.0 ± 8.2      |
| Intercalated disk size |
| Newborn          | 2.54 ± 0.08     | 0.64 ± 0.09     |

Heart-to–body weight ratio is in % (n = 5 per group); sarcomere length in μm (n = 25 per group, 2 individuals each); N-cadherin expression in arbitrary units (n = 10 per group, 4 individuals each; intercalated disk size in μm assessed by direct measurement on electron micrograph per unit picture (n = 5 per group, 2 individuals each). Values are expressed as mean ± SEM.

*P < 0.05: difference between mutant (−/−) and wild-type (+/+).
shortening, as an indicator of systolic cardiac function, was significantly decreased in male (20%) (Figure 5A, Table 2) but not in female mutants (not shown). When myocardial function was measured by Langendorff’s heart preparation in vitro, however, the developed force in response to adrenergic stimuli (isoproterenol) was also significantly reduced in female mutants (Table 2, Figure 5B). A slight decrease in mutant female coronary flow was also observed, whereas no apparent change in basal blood pressure or heart rate was detected (Table 2).

ECG Analysis
ECG analysis in mutants revealed neither atrioventricular nor intraventricular conduction defects (similar PR intervals, QRS duration, and amplitude). The resting heart rate was significantly decreased in the anesthetized female mutants. The P duration, but not P amplitude, was significantly increased in female (47%) and to a lesser extent in male (17%) mutants. The most striking difference between wild-type and 5-HT2B receptor mutants (both female and male) was dramatically elevated T-wave amplitude, which is an indicator of abnormalities in repolarization of ventricles (Figure 5C, Table 2). Serum potassium levels, however, were not altered (not shown).

Biochemical Markers of Heart Failure
Clinical indications of human acute myocardial infarction and injury are revealed by serum levels of the cardiac-specific biochemical markers troponin I and creatine kinase-MB. Strikingly elevated markers were observed in the serum of 5-HT2B receptor mutants (6 weeks old) (Figure 5D). Interestingly, male 5-HT2B receptor mutants exhibited more pronounced biological changes than females.

Discussion
In this study, we provide the first evidence that Gq-coupled 5-HT2B receptor ablation in mice leads to cardiomyopathy with LV dysfunction, dilation, and an abnormal structure within the Z band correlated with a deficiency in N-cadherin expression. 5-HT2B receptor–mutant mice exhibit thinning of the ventricular wall and a reduction in ventricular mass that appears
Combined myofibrillar breakdown and inhibited myofibrillogenesis may account for loss of ventricular mass without substantial hypertrophy. Mice overexpressing tropomodulin or mutated troponin T are models of dilated cardiomyopathy with inhibited myofibrillogenesis without a hypertrophic response.

Other neurotransmitters and hormones that use Gq protein signaling are also involved in cardiomyopathies. In vitro and in vivo studies have indicated a role for hormones such as angiotensin II, bradykinin B2, endothelin 1, norepinephrine, and prostaglandin F2α, not only in stimulation of cardiac hypertrophy but also in compensation of the hypertrophied heart through induction of cardiomyocyte apoptosis. Targeted expression of the carboxy-terminus of the α-subunit of Gq or overexpression of the Gq protein in the heart causes cardiomyopathy. The regulation of cardiomyocyte cytoarchitecture through the Gq-coupled pathway, however, is poorly understood.

Our data suggest that alteration in cardiomyocyte cytoarchitecture results from 5-HT2B receptor mutation. How does the 5-HT2B receptor affect the organization of myofibrils and related cardiomyocyte cytoarchitecture? 5-HT2B receptor–mutant cardiomyocytes exhibit abnormal organization of contractile elements, including Z-stripe enlargement (Figure 5). Interestingly, most of the mutations leading to dilated cardiomyopathy in humans affect structural proteins involved in cytoskeleton–extracellular matrix interaction at the Z stripe. The altered intercalated disk structures observed in the hearts of 5-HT2B receptor–mutant mice could be a molecular mechanism leading to impaired contractility and myofibrillar degeneration. Z line–associated structures are responsible for the lateral alignment of myofibrils, and their anchorage is at N-cadherin– and vinculin-containing costameres along the cell membrane. The 5-HT2B receptor–mutant mice exhibit decreased N-cadherin levels. N-Cadherin plays an important role in maintaining myofibril integrity, and in cardiomyocyte interaction, and in myofibrillogenesis. Downregulation of N-cadherin and disruption of intercellular adhesion have also been reported in failing guinea pig hearts. Addition of antibodies against N-cadherin to cardiomyocyte cultures also induces myofibrillar and cytosolic disorganization. Furthermore, mutation of the Drosophila 5-HT2Dro receptor (a pharmacological orthologue to 5-HT2B receptor) results in embryos that do not gastrulate properly or exhibit impaired proliferation cardiomyocytes. No apoptotic bodies were observed by transmission electron microscopy, yet mitogen-activated protein kinase (MAPK/ERK) activation in response to 5-HT was strongly reduced in newborn mutant hearts of mice by enzyme immunoassay. Values are expressed as mean±SEM, n=5–6. SEM, n=6–10; *difference between mutant (−/−) and wild-type (+/+ or +/−) mice (P<0.05).

Figure 5. Functional analysis reveals LV dysfunction in 5-HT2B receptor–mutant mice. A, Ventricular diameters (left) and LV fractional shortening (right) of adult hearts were obtained by 2D echocardiograms. Values are expressed as mean±SEM, n=5–6. B, Langendorff preparation of isolated hearts from adult females perfused and stimulated with adrenergic agonist. Values are ±SEM (n=3). C, ECG from wild-type (left) and mutant (right) adult males, in 2-arm bipolar derivation. Letters indicate P, Q, R, S, and T waves. Arrows indicate all P waves. D, Classic markers of human heart failure, creatine kinase-MB isoenzyme (CK-MB mass measurement) and cardiac troponin I (cTnl), were detected in serum of mice by enzyme immunosassay. Values are ng/mL±SEM (n>10); *difference between mutant (−/−) and wild-type (+/+ or +/−) mice (P<0.05).
tion compensates at least partially for this impaired contractility.

5-HT$_{2B}$ receptor–mutant mice exhibit sex differences: Consistent with the idea that the morphological lesions detected in male mutant mice underlie abnormal functions, female mutant mice with less severe histopathological findings did not reveal significant functional changes under steady-state conditions. Similar sex differences occur in other cardiomyopathy models, such as in the conditions. Similar sex differences occur in other cardiomyopathy models, such as in the

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<th>TABLE 2. 5-HT$_{2B}$ Receptor–Mutant Adult Mouse Cardiovascular Parameters</th>
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<td><strong>Awake animals</strong></td>
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<td>SAP (systolic arterial pressure) and heart rate were assessed by tail-cuff method on awake animals (n=5 per group); basal heart rate and developed force values were obtained from isolated perfused heart (n=8 per group). ECG was performed on anesthetized animals; QTc was performed on anesthetized animals; QTc = QT/RR, n=5 per group. Values are expressed as mean±SEM.</td>
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References


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