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Ablation of serotonin 5-HT_{2B} receptors in mice leads to abnormal cardiac structure and function

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Short title. 5-HT_{2B} receptor in murine heart

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Abstract

Background- Identification of factors regulating myocardial structure and function is important to understand the pathogenesis of heart disease. Since 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_{1A}, 5-HT_{1B}, or 5-HT_{2C}) disruptions in mice have resulted in cardiovascular defects. In this study, we examined 5-HT_{2B} receptor mutant mice to assess the putative role of serotonin in heart structure and function.

Methods and Results- We have generated Gq-coupled 5-HT_{2B} receptor null mice by homologous recombination. Surviving 5-HT_{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_{2B} receptor mutant ventricles exhibit dilation and abnormal organization of contractile elements, including Z-stripe enlargement and N-cadherin down regulation. Echocardiography and electrocardiography both confirm the presence of left ventricular dilatation and decreased systolic function in the adult 5-HT_{2B} receptor mutant mice.

Conclusions- Mutation of 5-HT_{2B} receptor leads to a cardiomyopathy without hypertrophy and a disruption of intercalated discs. 5-HT_{2B} receptor is required for cytoskeleton assembly to membrane structures by regulating the N-cadherin expression. These results constitute, for the first time, strong genetic evidence that serotonin, via 5-HT_{2B} receptor, regulates cardiac structure and function.

Short Abstract

Background- Little is known about the molecular mechanism of cardiac functions triggered by serotonin.

Methods and Results- We have generated Gq-coupled 5-HT_{2B} receptor null mice by homologous recombination. Surviving 5-HT_{2B} receptor mutant mice exhibit cardiomyopathy with a loss of ventricular mass, dilation and abnormal organization of contractile elements. Echocardiography and electrocardiography both confirm the presence of left ventricular dysfunction.

Conclusions- Lack of the 5-HT_{2B} receptor leads to cardiomyopathy with downregulation of N-cadherin expression.

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 ¹. The mitogenic action of 5-HT ² triggers the valvular fibroplasia observed in carcinoid patients ³ and in obese people taking the 5-HT uptake inhibitor/5-HT_{2B} receptor ligand, fenfluramine, as an appetite suppressant ^{4,5}.

The various biological actions of 5-HT are mediated by numerous cognate receptors. There are at least 15 receptor subtypes that belong to four classes: 5-HT_{1/5}, 5-HT₂, 5-HT₃, and 5-HT_{4/6/7} ⁶. 5-HT binding to 5-HT_{2A}, 5-HT_{2B} or 5-HT_{2C} receptors activates phospholipase C, releases inositol trisphosphate and increases intracellular calcium levels. 5-HT_{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 ⁷. Stimulation of the 5-HT_{2B} receptor results in cross talk with the 5-HT_{1B/1D} receptor subtype via activation of phospholipase A₂ ⁸. The 5-HT_{2B} receptor also activates nitric oxide synthesis through a PDZ domain ⁹.

To understand the specificity of each receptor subtypes, the genetic inactivation approach in mice has been used. Mutation of 5-HT receptors 5-HT_{1A}, 5-HT_{1B}, or 5-HT_{2C} in mice leads to behavioral abnormalities ¹⁰. We have recently shown that 5-HT_{2B} receptor inactivation in mice leads to trabeculation defects in embryonic heart causing a 30% lethality at mid-gestation ¹¹. Now, we investigated cardiopathy in surviving 5-HT_{2B} receptor mutant mice. This study reveals that 5-HT via 5-HT_{2B} receptor is involved in the regulation of cardiomyocyte cytoarchitecture and function. 5-HT_{2B} receptor ablation in mice leads to cardiomyopathy, including left ventricular dysfunction without hypertrophy.

Methods

Generation of 5-HT_{2B} receptor knockout mice. Targeted mutagenesis by homologous recombination has been previously described¹¹. All animal experimentation was performed in accordance with institutional guidelines, and protocols were approved by the French Animal Care Committee in accordance with European regulations.

Morphological analysis of mouse embryos. Transmission electron microscopy (TEM) and histological techniques were performed as previously described¹². Immunohistochemistry was performed on heart cryosections with the anti-sarcomeric myosin heavy chain (MHC) antibody (MF-20). Anti-tropomyosine and N-cadherin antibody reactions were performed on paraffin sections as described¹¹. Signal intensity was quantified using a fluoroimager (Typhoon, molecular Dynamics) and calculated as the product of averaged pixel intensity per area.

Cardiomyocyte density determination and confocal microscopic analysis. Confocal microscope images of the sections were taken on a Leica TCS4D. Calculation of total number of nuclei per field was obtained by counting propidium iodide-stained nuclei. Nonmyocytes were tabulated by counting the number of nuclei not surrounded by cytoplasmic myosin and this number was used to calculate total myocytes as described¹³.

Analysis of hypertrophic cardiac genes by RT-PCR. Semi-quantitative RT-PCR was performed on 1 µg of total RNA extracted from age matched control and knockout mice using the ribosomal elongation factor 1A as an internal control as previously described¹¹. The following primers were used: atrial natriuretic factor (ANF) 5'-ccaggccatattggagcaaa and 5'-gaagctgttgacgcctagtc; GATA4 5'-cactatgggcacagcagctcc and 5'-ttggagctggcctgcatgctc; alpha-MHC 5'-ctgctggagagggtattcctcg and 5'-ggaagagtgagcggcgcatcaagg; and beta-MHC 5'-tgcaaaggctccaggctctgagggc and 5'-gccaacaccacctgtccaagttc. The PCR products were quantified using an image analyzer (Bio-Rad, GS-700) and calculated as arbitrary units.

Cardiomyocyte isolation and video imaging. Ventricular cardiomyocytes from newborn mice were isolated as previously described¹⁴. Beating rate in response to dobutamine was determined by video recording of isolated cardiomyocytes. The analysis was performed on the stage of an inverted microscope (Leica DMRiB) using software developed by J.-L. V.

Echocardiography methods. Animals (19 week old mice) anesthetized with sodium pentobarbital (30 mg/kg intraperitoneally) were observed with two-dimensionally guided M-mode echocardiograms using a short focal length 12-MHz (Hewlett Packard Medical systems, USA) transducer. LV end systolic and diastolic diameters (respectively LVESD and LVEDD) were measured. The percentage of LV fraction shortening was then calculated.

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

Electrocardiogram (ECG). 19 week old mice, anesthetized with tribromoethanol (13 μ l of 2.5% solution per g of body weight subcutaneously), were recorded with the four arms of ECG leads attached at the origin of each paw by uni and bipolar lead derivations. The signal was recorded by an electrocardiograph (EKG-Burdick, Siemens) connected to a data acquisition system (MP100 and Acknowledge Software, Biopac Systems Inc, USA).

Isolated perfused heart preparation. Hearts from mice (12-19 weeks, 23-25 g) anesthetized with sodium pentobarbital (60 mg/kg intraperitoneally), and heparinized (500 U/kg intraperitoneally) were cannulated and perfused according to Langendorff, at 37°C and pH 7.4, with modified Krebs-Henseleit solution containing (mM) NaCl 118, NaHCO₃ 24, KCl 4.7, KH₂PO₄ 1.2, MgSO₄ 1.2, CaCl₂ 2.5, di-sodium EDTA 0.5 and glucose 10, gassed with 95 %O₂/ 5% CO₂. Perfusion pressure was constant and equivalent to 1 meter H₂O. 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 the cardiac troponin I was determined from samples of serum from adult mice ¹⁵.

Data analysis and statistics. All values represent average of independent experiments \pm SEM (n=number of experiments as indicated in the text). Comparisons between groups were performed using Student 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 mid-gestation lethality (30% n= 120)¹¹. The 5-HT_{2B} receptor mutant mice that reached birth displayed no obvious defects, although 30% (n=120)¹¹ of newborn mice developed signs of fatigue and dyspnea between postnatal days 2-5 and died within 24 h from the onset of these symptoms. A likely cause of neonatal death is inadequate cardiac output due to hypoplasia of the left ventricle, despite the lack of evidence for pulmonary edema. All 5-HT_{2B} receptor mutant mice surviving 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 heart to body weight ratio (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 PI respectively) revealed 15% fewer cardiomyocytes in the newborn mutants as shown in Fig. 1B. Isolated mutant cardiomyocytes are 12% smaller in length compared to wild-type (n>15) (Fig. 1C). The decrease in ventricular mass observed in 5-HT_{2B} receptor mutant mice results, therefore, from decreases 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 markers¹⁶, ANF, alpha-MHC, beta-MHC and GATA4 expression was evaluated in 12 week old mutant hearts. Semi-quantitative RT-PCR analysis of mutant heart mRNA demonstrated that none of these mRNAs showed significant variation in expression level (less than 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_{2B} receptor mutant mice was cell intrinsic, the function of spontaneously beating isolated cardiomyocytes from newborn was investigated. Beta-adrenergic receptor agonist, dobutamine increased the beating rate of wild-type cardiomyocytes in a dose-dependent manner. However, mutant cardiomyocytes exhibited impaired response to dobutamine in the absence of sympathetic innervation (Fig. 2), indicating cell autonomous defects.

Ultrastructural analysis

A loss of myocardial organization, scattered area of degenerated cardiomyocytes and myofibrillar disarray were apparent in newborn mutant hearts. Wavy myofibrils were

identified by anti-tropomyosine staining (Fig. 3A). In this area, myofilaments appeared misaligned, I bands were not detectable, abnormally wide Z bands were seen, and mitochondria were rounded and irregular (Fig. 3B). Sarcomeric length of mutants is 33% smaller compared to that of 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_{2B} receptor mutant cardiomyocytes had reduced numbers of adherens junctions (Table 1) and the intercalated discs were consistently disorganized (Fig. 4A). Z line-associated protein expression was investigated. Vinculin staining in mutant newborn ventricles was unaltered (not shown). However, N-cadherin expression was reduced by 38.8% in mutant myocardium (Fig. 4B- Table 1).

Hemodynamic Measurements

Trans-thoracic echocardiograms (Fig. 5A, Table 2) show LV dilation and reduced systolic performance of the adult mutant mice. In male mutants, the LV end-diastolic diameter (LVEDD) was 25% higher than wild type. Extreme LV dilation (increased LVEDD) was observed, and the LV end-systolic diameter (LVESD) was increased by 50% in male 5-HT_{2B} receptor mutants (n>4). The percent of LV fraction shortening, as an indicator of systolic cardiac function, was significantly decreased in male (20%) (Fig. 5A, Table 2) but not in female mutants (not shown). However, when myocardial function was measured by Langendorff's heart preparation *in vitro*, 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 atrio-ventricular 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-HT_{2B} receptor mutants (both females and males) was dramatically elevated T wave amplitude, which is an indicator of abnormalities in repolarization of ventricles (Fig. 5C, Table). However, serum potassium levels 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 cardiac specific biochemical markers, troponin-I and creatine kinase-MB¹⁵. Striking elevated markers were observed in the serum of 5-HT_{2B} receptor mutants (6 week old), (Fig. 5D). Interestingly, male 5-HT_{2B} receptor mutants exhibited more pronounced biological changes than females.

Discussion

In this study, we provide the first evidence that Gq-coupled 5-HT_{2B} receptor ablation in mice leads to cardiomyopathy with LV dysfunction, dilation and an abnormal structure within Z band correlated with a deficiency in N-cadherin expression.

5-HT_{2B} receptor mutant mice exhibit thinning of the ventricular wall and the reduction in ventricular mass that appears due to two complementary mechanisms: loss of myocardial cells and decrease in cell size. The primary loss of myocardial cells could be due to apoptosis and/or impaired proliferation of cardiomyocytes. No apoptotic bodies were observed by TEM yet mitogen activated protein kinase (MAPK/ERK) activation in response to 5-HT was strongly reduced in newborn mutant hearts (unpublished observation). Together these data suggest that ventricular hypoplasia is mainly due to impaired proliferation but not to apoptosis. Mutation of the thin filament protein, troponin T in mice, also results in cardiomyopathy, due to a primary loss of cardiomyocytes and decrease in cell size¹³. Myofibril loss is the most obvious structural change in human cardiomyopathy¹⁷ and sarcomeric disarray is characteristic of failing hearts¹⁸. Actually, the decrease in cardiomyocyte size could be due to impaired growth during postnatal development. The loss of ventricular mass creates biomechanical stress on the remaining viable heart muscle, which typically triggers a hypertrophic response by inducing embryonic gene re-expression. However, in the 5-HT_{2B} receptor mutant heart, despite increased preload conditions (increased LVESD), the expression of hypertrophic markers was not elevated and there were no morphological signs of hypertrophy. Unlike the 5-HT_{2B} receptor mutant mice, alpha-MHC and myf5 mutant mice develop a hypertrophy and interstitial fibrosis accompanied cardiomyopathy¹⁹. Why 5-HT_{2B} receptor mutants fail to have a hypertrophic response remains to be investigated. Combined myofibrillar breakdown and inhibited myofibrillogenesis may account for loss of ventricular mass without substantial hypertrophy. Mice overexpressing tropomodulin²⁰ or mutated troponin T are models of dilated cardiomyopathy with inhibited myofibrillogenesis without a hypertrophic response¹³.

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 B₂²¹, endothelin 1, norepinephrine and prostaglandin F_{2A}, not only in stimulation of cardiac hypertrophy, but also in decompensation of the hypertrophied heart through induction of cardiomyocyte apoptosis²². Targeted expression of the carboxy-terminus of the alpha subunit of Gq or overexpression of the Gq protein in the heart causes cardiomyopathy. However, the regulation of cardiomyocyte cytoarchitecture through Gq-coupled pathway is poorly understood.

Our data suggest that alteration in cardiomyocyte cytoarchitecture results from 5-HT_{2B} receptor mutation. How does 5-HT_{2B} receptor affect the organization of myofibrils and related cardiomyocytes cytoarchitecture? 5-HT_{2B} receptor mutant cardiomyocytes exhibit abnormal organization of contractile elements, including Z-stripe enlargement (Fig. 5). Interestingly, most of the mutations leading to dilated cardiomyopathy in humans affect structural proteins involved in cytoskeleton-extracellular matrix interaction at the Z-stripe²³. The altered intercalated disc structures observed in the 5-HT_{2B} receptor mutant mice heart 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-HT_{2B} receptor mutant mice exhibit decreased N-cadherin levels. N-cadherin plays an important role in maintaining myofibril integrity²⁴, in cardiomyocyte interaction and in myofibrillogenesis²⁵. Down regulation of N-cadherin and disruption of intercellular adhesion have also been reported in failing guinea pig hearts²⁶. Addition of antibodies against N-cadherin to cardiomyocyte cultures also induces myofibrillar and cytosolic disorganization²⁷. Furthermore, mutation of the *Drosophila* 5-HT_{2Dro} receptor (pharmacological ortholog to 5-HT_{2B} receptor) results in embryos which do not gastrulate properly due to changes in E-cadherin-dependent cell adhesiveness²⁸. Our data suggest that 5-HT_{2B} receptor, in mammals, is required for proper myofibril integrity and myofibrillogenesis by regulating N-cadherin expression.

The 5-HT_{2B} receptor mutant mice phenotype has similarity with the natural history of patients with dilated cardiomyopathy. LV dilatation and depressed LV systolic performance in the mutant mice are typical features used to diagnose dilated cardiomyopathy in human. Moreover, serum biochemical indicators of myocardial infarction are increased in the 5-HT_{2B} receptor mutant mice. No apparent changes in basal blood pressure and heart rate are detected (Table), suggesting that either 5-HT_{2B} receptor is not involved in basal blood pressure control or that systemic vascular flow redistribution compensates at least partially this impaired contractility.

5-HT_{2B} receptor mutant mice exhibit gender 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 the steady-state condition. Similar gender differences occur in other cardiomyopathy models such as in the beta-MHC mutant mouse²⁹. In X-linked cardiomyopathy in humans, heart failure occurs rapidly after onset of symptoms in male, but is delayed in its onset and progression in females³⁰. Cardioprotective effects in females have been attributed to estrogen action.

5-HT_{2B} receptor specific agonist, norfenfluramine, ergot drugs, and 5-HT released from carcinoid tumors contribute to valvular fibroplasia in humans^{4,5}. However, the lack of detectable valvular defects in mutant mice, indicates that the 5-HT_{2B} receptor is not required for heart valve development.

Mutation of a non-cytoskeletal molecule, the 5-HT_{2B} receptor, provides the first genetic evidence that 5-HT via this receptor regulates cardiomyocyte function and structure. These findings should facilitate a genetic approach and new avenues of drug design in fighting cardiovascular disease.

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Nebigil et al Figure 2

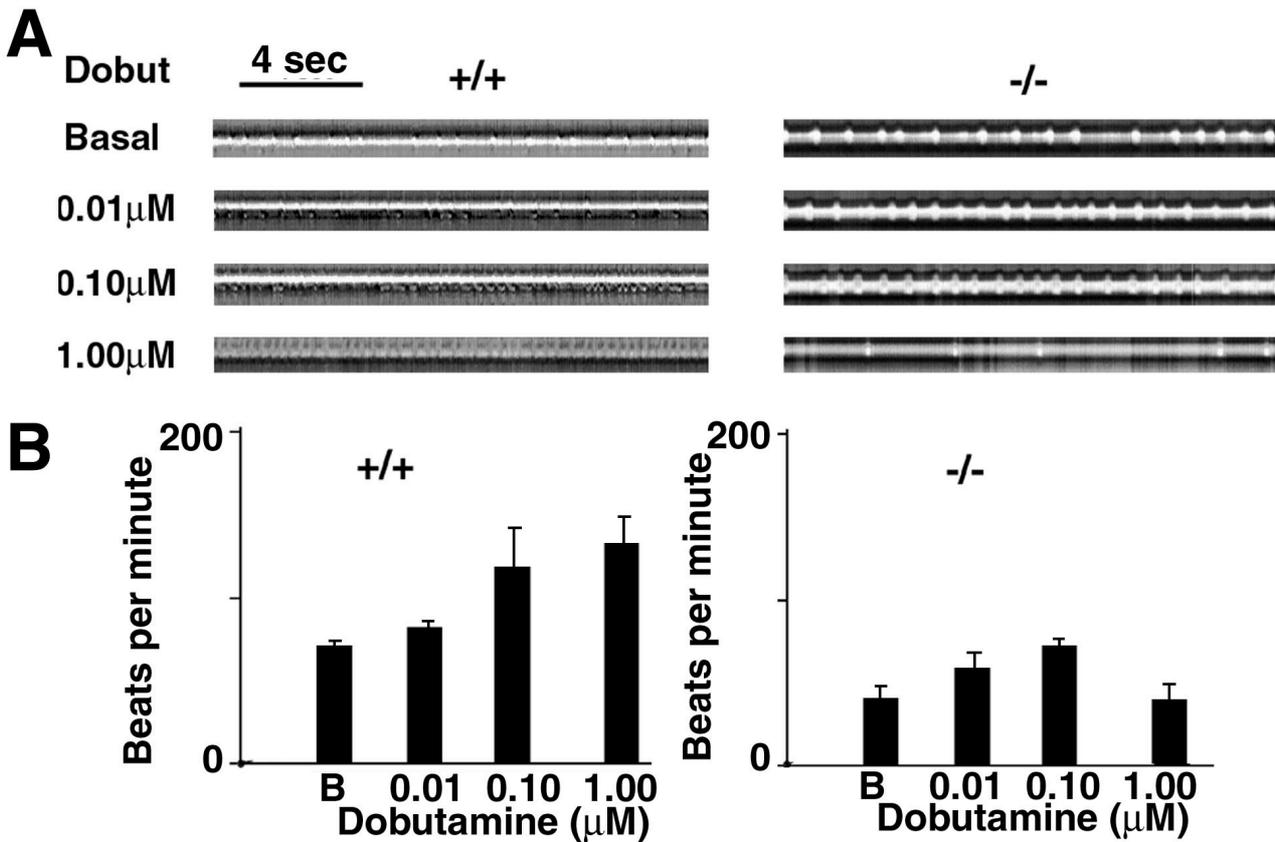


Figure 2. Cardiac phenotype in 5-HT_{2B} receptor mutant is intrinsic to cardiomyocytes. A) The beating rate of isolated cardiomyocytes from newborn hearts in response to different concentrations of dobutamine is shown. B) The beating rate in isolated single cardiomyocytes was deduced from these digital video recordings. The beating rate is expressed as mean myocyte contractions per minute \pm SEM, * $p < 0.05$: difference between mutant (-/-, n=5) and wild-type (+/+, n=3) mice.

Nebigil et al Figure 3

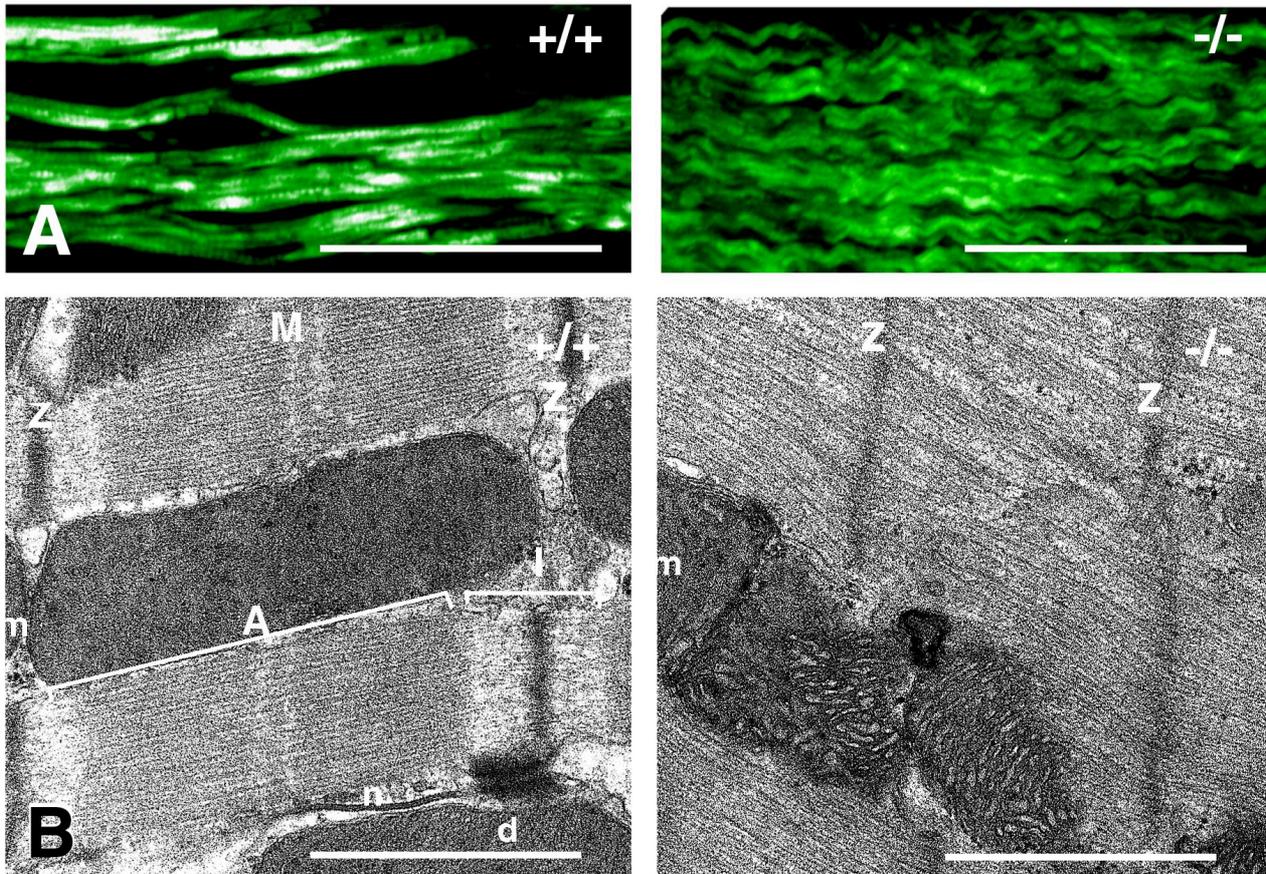


Figure 3. 5-HT_{2B} receptor mutant mice display dramatic disruption of cardiomyocyte cytoarchitecture with myofibrillar disarrays. A) Representative sections from paraffin-embedded adult hearts stained with tropomyosine antibody. B) High magnification of sarcomeres from LVs in hearts. A-bands (A), M-lines (M) and I-bands (I) Z-bands (Z), desmosomes (d), mitochondria (m), nexus (n);. Bars: A 50 μ m, and B 1 μ m. 5-HT_{2B} receptor mutant (-/-), and control (+/+) mice.

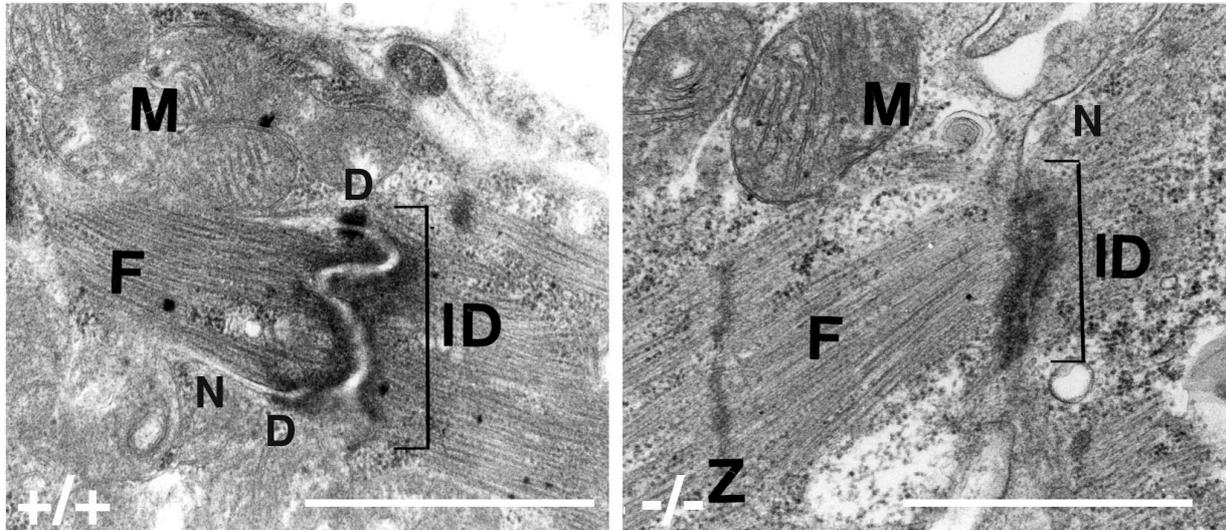
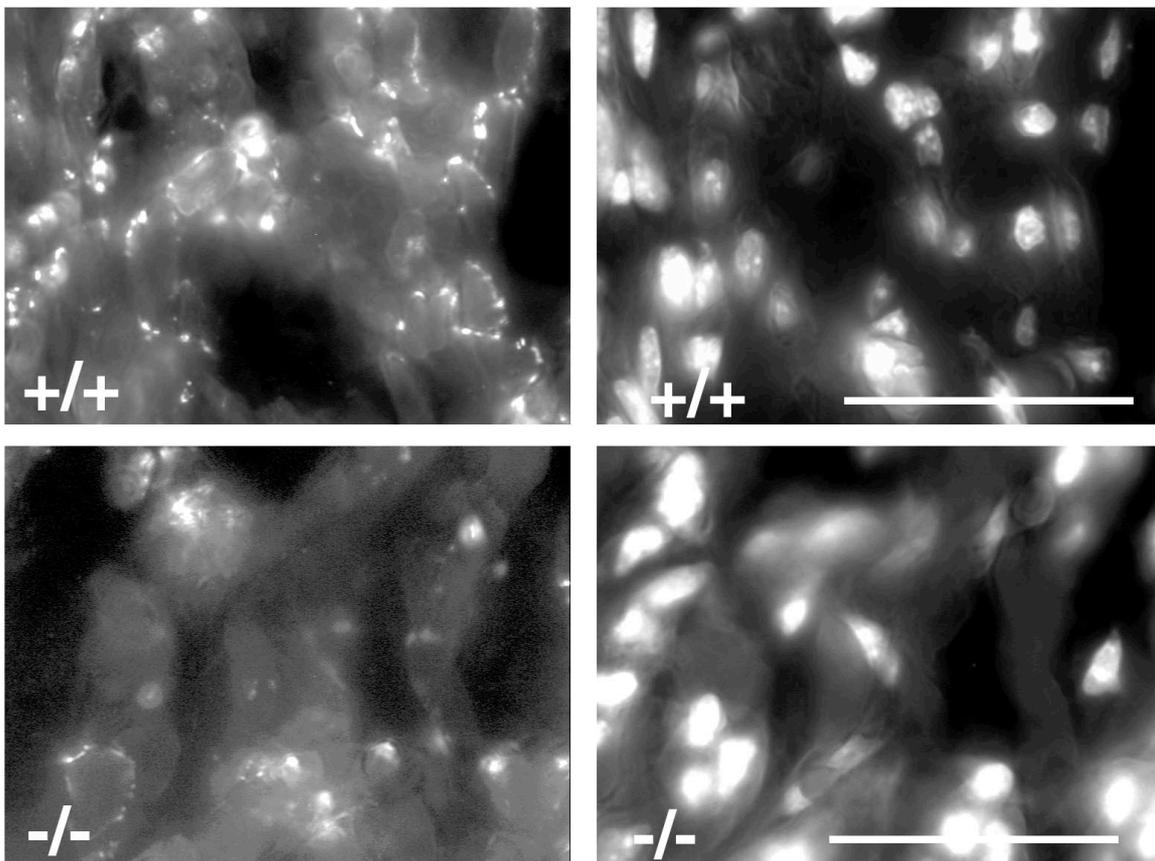
A**B****Anti-N-Cadherin****DAPI**

Figure 4. Intracellular junctional structures are perturbed in 5-HT_{2B} receptor mutant mice. A) High magnification of intercalated disc (ID) structures from LVs of newborn mice. Actin fibers (F); Desmosomes (D); Mitochondria (M); Nexus (N); Z-bands (Z). B) Immunohistochemistry for the intercalated disc protein N-cadherin in newborn hearts. The DAPI staining shows the distribution of cells (right). Bars: B 50 μ m, and A 0.5 μ m. 5-HT_{2B} receptor mutant (-/-), and control (+/+) mice.

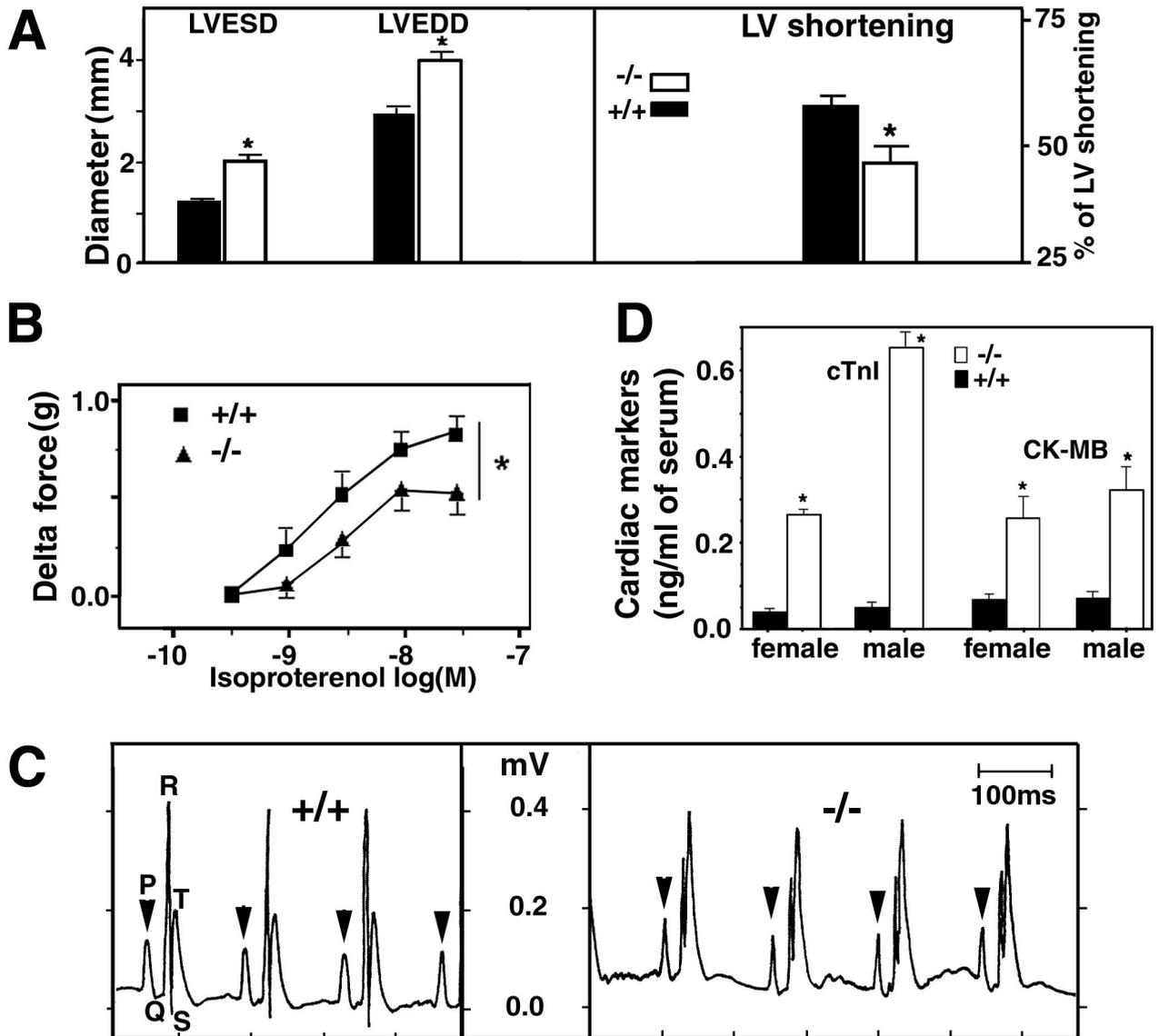


Figure 5. Functional analysis reveals LV dysfunction in 5-HT_{2B} receptor mutant mice. A) Ventricular diameters (left) and LV shortening fraction (right) of adult heart were obtained by two-dimensional echocardiogram. Values are expressed as mean \pm SEM, n=5. FS (LV fraction shortening). B) Langendorff preparation of isolated hearts from adult female perfused and stimulated with adrenergic agonist. Values are given \pm SEM (n=3). C) ECG from wild (left panel) and mutant (right panel) adult male, in the DI two arms bipolar derivation. Letters indicate the P, Q, R S and T waves. Arrows indicate all P waves. D) Classical markers of human heart failure, creatine kinase MB isoenzyme (CK-MB mass measurement) and for the cardiac troponin I (cTnI) were detected in serum of mice by enzyme-immunoassay. Values in ng/ml are given \pm SEM (n>10) and stars indicate difference between mutant (-/-) and wild-type (+/+) mice (P<0.05).

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Table 1 Morphometry of 5-HT_{2B} receptor mutant mice cardiac parameters

Genotype	+/+	-/-
		Heart to body weight ratio [†]
Newborn	1.06±0.12	0.76*±0.07
6 weeks	0.67±0.40	0.51*±0.03
		Sarcomeric length [‡]
Newborn	2.04±0.02	1.38*±0.05
		N-cadherin expression [§]
Newborn	73.4±12.4	44*.0±8.2
		Intercalated disc size
Newborn	2.54±0.08	0.64*±0.09

[†]Heart to body weight ratio in % (n>5 per group). [‡]Sarcomeric length in μm (n=25 per group, 2 individual each); [§]N-cadherin expression in arbitrary units (n=10 per group, 4 individual each); ^{||}Intercalated disc size in μm assessed by direct measurement on electron micrograph per unit picture (n=5 per group, 2 individual each); Values are expressed as mean \pm SEM, * $p < 0.05$: difference between mutant (-/-) and wild-type (+/+) mice.

Table 2 5-HT_{2B} receptor adult mice cardiovascular parameters

Gender	Male		Female	
Genotype	+/+	-/-	+/+	-/-
	Awake animals [†]			
SAP (mm Hg)	119±5	121±3	135±3	132±4
Heart Rate (b/min)	423±18	463±12	446±25	452±16
	Isolated heart [‡]			
Heart Rate (b/min)	299 ± 29	272 ± 20	327 ± 25	259 ± 38
Developed Force (g)	1.94 ± 0.21	1.55 ± 0.33	1.69 ± 0.25	1.41 ± 0.20
Coronary Flow (ml/min)	1.92±0.20	1.68±0.35	1.83±0.16	1.07±0.20*
	ECG [§]			
P (ms)	28±2	33±1	19±1	28±3*
P (μV)	112±17	155±31	71±17	79±15
QRS (ms)	18±1	16±2	19±2	20±3
PR (ms)	56±3	58±2	57±3	64±5
RR (ms)	139±6.1	39±8	123±6	149±7*
QT (ms)	71±8	66±5	56±5	56±2
QTc	6±0	6±0	5±0	5±0
T(μV)	228±28	400±40*	211±79	300±68

[†]SAP (systolic arterial pressure), and heart rate were assessed by tail cuff 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 anaesthetized animals, QTc (QT/√RR) (n=5 per group); Values are expressed as mean ± SEM, * p<0.05: difference between mutant (-/-) and wild-type (+/+) mice.