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### ► **To cite this version:**

Canan G Nebigil, D.-S. Choi, André Dierich, Pierre Hickel, Marianne Le Meur, et al.. Serotonin 2B receptor is required for heart development. *Proceedings of the National Academy of Sciences of the United States of America*, 2000, 97 (17), pp.9508-9513. 10.1073/pnas.97.17.9508 . hal-02926424

**HAL Id: hal-02926424**

**<https://hal.science/hal-02926424>**

Submitted on 31 Aug 2020

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## Serotonin 2B receptor is required for heart development

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Classification: Biological Sciences, Developmental Biology

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Running title: Serotonin 2B receptor mutant mice

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Key words: neuregulin/ heart/ knockout/ proliferation/ serotonin/ transactivation/

### Abstract

Several lines of evidence suggest that the serotonin (5-hydroxytryptamine, 5-HT) regulates cardiovascular functions during embryogenesis and adulthood. 5-HT binds to numerous cognate receptors to initiate its biological effects. However, none of the 5-HT receptor disruptions in mice have yet resulted in embryonic defects. Here we show that 5-HT<sub>2B</sub> receptor is an important regulator of cardiac development. We found that inactivation of 5-HT<sub>2B</sub> gene leads to embryonic and neonatal death due to heart defects. 5-HT<sub>2B</sub> mutant embryos exhibit a lack of trabeculae in the heart and a specific reduction in the expression levels of a tyrosine kinase receptor, ErbB-2, leading to mid-gestation lethality. These *in vivo* data suggest that the Gq-coupled receptor 5-HT<sub>2B</sub> uses the signalling pathway of tyrosine kinase receptor ErbB-2 for cardiac differentiation. All surviving newborn mice display a severe ventricular hypoplasia due to impaired proliferative capacity of myocytes. In adult mutant mice, cardiac histopathological changes including myocyte disarray and ventricular dilation were consistently observed. Our results constitute the first genetic evidence that 5-HT via 5-HT<sub>2B</sub> receptor regulates differentiation and proliferation of developing and adult heart. This mutation provides a new genetic model for cardiopathy and should facilitate studies of both the pathogenesis and the therapy of cardiac disorders in humans.

**Abbreviations:** central nervous system (CNS); day post coitum (dpc); embryonic stem (ES); Myosin heavy chain (MHC); platelet derived growth factor receptor (PDGFR); platelet endothelial cell adhesion molecule (PECAM); receptor tyrosine kinase (RTK); serotonin (5-hydroxytryptamine, 5-HT); transmission electron microscopy (TEM);

Serotonin (5-hydroxytryptamine) (5-HT) first isolated as a vasoconstrictor from blood was later identified in the central nervous system (CNS). It is found in three main areas of the body: the intestinal wall, the platelets, and the CNS. The functions of 5-HT in CNS as a neurotransmitter are numerous and appear to involve control of appetite, sleep, memory and learning, temperature regulation, mood, behaviour (including sexual and hallucinogenic behaviour), and endocrine regulation (1). Peripherally, 5-HT, which is stored in platelets, appears to play a major role in homeostasis, blood pressure regulation, cardiovascular functions (2), motility of the gastrointestinal tract (3), and carcinoid tumour pathology (4). Independently of its location in adults, 5-HT has also been detected during zygotic cleavage divisions, gastrulation and neurulation in embryos of sea urchins, frogs, chicken and *Drosophila*. The presence of 5-HT and of its receptors in early embryogenesis and the ability of 5-HT-specific pharmacological agents (5) to interfere with embryonic development suggested that early embryos use 5-HT prior to the onset of neurogenesis to regulate cell proliferation and/or morphogenetic movements (6, 7). Furthermore, 5-HT has been suspected for years to regulate craniofacial and cardiovascular morphogenesis: In embryos grown in the presence of either a high concentration of 5-HT or of 5-HT specific reuptake inhibitors, a decreased proliferation of myocardium, cardiac mesenchyme and endothelium has been reported, indicating that 5-HT may regulate proliferation in the embryonic heart (8).

These biological actions of 5-HT are mediated by numerous cognate receptors. It now appears that there are at least 15 receptors subtypes that belongs to four populations: 5-HT<sub>1/5</sub>, 5-HT<sub>2</sub>, 5-HT<sub>3</sub>, and 5-HT<sub>4/6/7</sub> subtypes (9). This diversity has limited the use of a pharmacological approach toward the understanding of the specificity of these various receptors. An alternative way of investigation is genetic inactivation of genes encoding these receptors. Until now the phenotype obtained by homologous recombination of 5-HT receptor genes (5-HT<sub>1A</sub>, 5-HT<sub>1B</sub>, or 5-HT<sub>2C</sub>) has led exclusively to behavioural abnormalities without reported morphological defects in CNS (10). The receptors required for the peripheral 5-HT functions remain to be identified.

The 5-HT<sub>2</sub>-receptor family comprises three subtypes, the 5-HT<sub>2A</sub>, 5-HT<sub>2B</sub>, and 5-HT<sub>2C</sub> subtypes. The 5-HT<sub>2B</sub> receptor belongs to the G-protein-coupled seven-transmembrane spanning receptor family. The 5-HT<sub>2B</sub> receptor is expressed in embryonic (11) and adult (12) cardiovascular tissues, gut and brain from the rat, mouse and human species. Binding of 5-HT to this receptor activates G $\alpha_q$ /11 protein, thereby activating phospholipase C, which initiates a rapid release of inositol trisphosphate, resulting in a rise in intracellular calcium levels in transfected cells. Recently, this receptor has been shown to activate the phospholipase A<sub>2</sub> (13) and nitric oxide synthases (14) in transfected and endogenously 5-HT<sub>2B</sub> expressing cells. Agonist stimulation of 5-HT<sub>2B</sub> receptor subtype also causes a rapid and transient activation of the proto-oncogene product p21ras (15) and of p42mapk/p44mapk mitogen-activated protein kinases and transduces a mitogenic signal by activating the non-receptor tyrosine kinase (RTK) c-Src and the RTK platelet derived growth factor receptor (PDGFR) (16) in mouse fibroblast cells. 5-HT<sub>2B</sub> receptor expression has been shown to induce tumours in nude mice and has been detected at a high level in human carcinoid tumours (15). By using a mouse embryo culture technique, treatment with

broad spectrum 5-HT<sub>2</sub> receptor antagonists resulted in severe morphological defects of neuroectodermal and mesodermal derivatives including ventricular myocardium (11). These differentiation processes may be controlled, at least in part, by the mitogenic properties of 5-HT<sub>2B</sub> receptor and by integration of RTK signalling pathways. However, the molecular mechanism by which 5-HT regulates embryonic development is currently unknown since none of the inactivated 5-HT receptors have yet been reported to induce obvious developmental defects other than behavioural defects (10).

Here it is shown that targeted inactivation of the 5-HT<sub>2B</sub> receptor gene leads to disturbed heart development resulting in embryonic lethality and death in neonatal mice. Our results identify a 5-HT<sub>2B</sub> receptor-mediated pathway by which 5-HT interacts with ErbB-2 to control differentiation in developing heart. Together, these results reveal 5-HT via 5-HT<sub>2B</sub> receptor as an important regulator of cardiac myocyte differentiation and proliferation.

## Materials and Methods

**5-HT<sub>2B</sub> receptor gene targeting and genotype analysis.** The targeting vector was constructed with the selective bacterial neo gene introduced into the exon 2 sequence of the 5-HT<sub>2B</sub> receptor locus. This resulted in a non-functional allele of 5-HT<sub>2B</sub> as it interrupts the protein reading frame. One recombination event was obtained after screening of about 4000 embryonic stem (ES) cells clones. Southern blot analysis on the recombined clone using a probe derived from neo sequence revealed that it contained a single expected 16 Kbp fragment in an HpaI restriction enzyme digestion excluding multiple insertion of the targeting vector (not shown). From this recombined 5-HT<sub>2B</sub> ES cells strain, 13 chimeric animals were created by injection into C57/BL6 blastocyst embryos. These chimeras were bred to either C57/BL6 or 129/Sv mouse strain to obtain germ-line transmission. After transfection of the 5-HT<sub>2B</sub> targeting vector into 129/Sv ES cells, recombined clones were analysed by Southern blot using a probe (P) located 3' to the sequence present in the targeting vector. Tail DNAs from newborn mice derived from intercrosses between heterozygous mice were digested with BglII and analysed by Southern blot with probe P. All data have been obtained with mice bearing the mutation in a pure 129/Sv background, but similar defects have been observed in a mixed C57/BL6- 129/Sv background.

**Analysis of mouse 5-HT<sub>2</sub> receptor expression.** Membrane proteins prepared from 6 weeks old mice heart ventricles (n=4) or stomach (n=5) were analysed by binding studies with specific tritiated antagonists of the 5-HT<sub>2B</sub> (3H-LY266070), 5-HT<sub>2A</sub> (3H-MDL100907) or 5-HT<sub>2C</sub> (3H-Mesulergine) receptors as previously described (11).

**Morphological analysis of mouse embryos.** Electron microscopy and histological experiments were performed as previously described (11). The hearts were fixed in paraformaldehyde, dehydrated and paraffin embedded, then sectioned (7 mm) transversely from the apex, and sections were examined after Masson's trichrome staining. The hearts immersed in a cardioplegic solution (25 mM KCl, 5% dextrose in PBS) to ensure complete myocardial relaxation before the hearts were fixed for transmission electron microscopy (TEM). Immunocytochemical labelling of embryos with a 5-HT<sub>2B</sub> specific antibody on cryosections or with anti ErbB-2 (SC-284) specific antibody (Santa-Cruz) on paraffin sections were performed as described (11). Whole mount immunohistochemical staining of embryos for developing blood vessels using anti- platelet endothelial cell adhesion molecule (PECAM) (PharMingen 01951D) antibody were performed as described (11). Western blot analysis for ErbB-2 and PDGFR-b (SC-432) were performed in the

whole embryo or cell lysate as previously described (16).

**Analysis of ErbB-2 mRNA expression.** Semi quantitative RT-PCR was performed on 1 mg of total RNA for 30 cycles at an annealing temperature of 55°C. Normalisation was performed using RNA for elongation factor 1a (EF1a). The oligonucleotides used for ErbB-2 are 5'-cgtgctagtcaagagtccaacc and 5'-tactcttcagcatcgaccagctc; for EF1a 5'-acaactgaagctgagcgtg and 5'-gtgcattccacagacttgac; for ErbB-4 5'-tgaacaatgtgatggcaggtgc and 5'-cacgaagttatgaggacatttc.

**Analysis of proliferation rate.** Ventricular cardiomyocytes were prepared from the hearts of newborn and 9,5 dpc embryos from 5-HT2B mutant and wild-type mice according to Adams et al (17). The hearts were excised and kept in the buffer containing (113 mM NaCl, 4.7 mM KCl, 0.6 mM NaH<sub>2</sub>PO<sub>4</sub>, 0.6 mM Na<sub>2</sub>HPO<sub>4</sub>, 1.2 mM MgSO<sub>4</sub>, 12 mM NaHCO<sub>3</sub>, 20 mM Glucose, 10 mM HEPES). The atrium was removed and the ventricle was minced and then cells were dispersed by gentle agitation or gentle pipetting (for embryos). After 15 min of enzymatic digestion with collagenase type II and pancreatin at 37°C, myocytes were pelleted and washed, and differentially plated for 1h to eliminate remaining non myocytes. Cells were then briefly pelleted at low speed (800g), and rinsed twice in buffer. Rod-shaped cardiac myocytes were plated in culture medium on gelatine (2%) pre-coated 8 well chamber slides in the presence of antimetabolic (cytosine arabinoside) overnight. Cardiomyocytes were kept in DMEM with 10% fetal calf serum and antibiotics at a density of 10<sup>3</sup> cells/well. Approximately 95% of the cells displayed spontaneously contractile activity in culture and were verified by myosin heavy chain (MHC) staining.

Thymidine incorporation was detected in cardiomyocytes as previously described (16). Briefly, quiescent cells (24 h serum starved) were treated with 5-HT (1mM) or neuregulin (heregulin) (25 mg/ml) for 16h, and 0.5 mCi of (3H)-thymidine was added to the culture during the last 4 hours of incubation. The free radioactivity was washed in 5% trichloroacetic acid, the incorporated radioactive thymidine was quantified by scintillation counting. BrdU incorporation was performed using a BrdU-labelling and staining kit (Boehringer Mannheim). Newborn mice were intraperitoneally injected with BrdU. 4h after injection, hearts were dissected and cardiac cryosections were stained with BrdU antibody. The sections were washed with water and mounted in DAPI containing medium. Slides were kept at 4°C in the dark.

## Results and Discussion

To generate 5-HT2B mutant mice, we produced ES cells in which one 5-HT2B allele was disrupted by homologous recombination (Fig. 1a). After injection into blastocyst of the recombined ES cells, we obtained chimeras that transmitted the mutation. The genotype of 5-HT2B recombinant mice was determined by Southern blot analysis of tail DNA (Fig. 1b). The lack of functional 5-HT2B receptors was confirmed at the RNA level by RT-PCR amplification (data not shown) and at the protein level by binding experiments using receptor subtype-specific labelled antagonists. In addition to a loss of 5-HT2B receptor expression, no compensatory overexpression of either 5-HT2A or 5-HT2C receptors in heart and stomach tissues from 5-HT2B homozygous mice was detected (Fig. 1c). Heterozygous 5-HT2B mutant intercrosses resulted in a frequency of homozygous pups in newborns of only 16.7%, significantly different from the expected 25% ( $p < 0.05$ ,  $n = 120$ , according to an unpaired t test), suggesting some embryonic lethality (Fig. 1d). When embryos were examined, a Mendelian ratio of homozygous mutant was seen up to 10.5 days post-coitum (dpc). The surviving curve for mutants shows the periods of lethality before 11.5 dpc and during the first week of life (Fig. 1e). Nevertheless, the growth of some mutant embryos was

clearly retarded at 9.5 and 10.5 dpc, exhibiting enlarged hearts and a pooling of blood in the abdominal area. Closer examination revealed that this was due to blood leakage into the pericardial cavity (Fig. 2a). Surviving homozygous embryos at 12.5 dpc exhibited developmental delays and paler colour than wild-type littermates (Fig. 2b). Since the 5-HT<sub>2B</sub> receptor is expressed in embryonic (11) and adult (12) cardiovascular tissues, including myocardial, endothelial, and vascular smooth muscle cells, we reasoned that defects observed in the mutant embryos might reflect problems in either vascular organisation or cardiac morphogenesis. Whole-mount immunohistochemical staining of 10.5 dpc mutant embryos using a monoclonal antibody against PECAM as a marker of developing blood vessels revealed no gross defects in vascular patterning (Fig. 2c). Histological analysis of hearts from 5-HT<sub>2B</sub> mutant embryos revealed defects in the subepicardial layer, and a lack of trabecular cells in the ventricle (Fig. 2d-e). However, the endocardial cushion appeared to be normal or only slightly reduced in size in some mutant embryos. The atrium and the outflow tract of the mutants appeared normal. The myocardial abnormalities in the 5-HT<sub>2B</sub> mutants correlate with the pattern of 5-HT<sub>2B</sub> receptor expression in the compact zone and in the myocardial trabeculae (see fig. 3c). TEM studies revealed that all observed mutant hearts (n= 6) at 9.5 dpc display premature differentiation of sarcomeres (the contractile unit found in differentiated myocardial cells) within the compact zone (Fig. 2f), even within regions of the heart showing otherwise nearly normal trabeculation. The most severe reduction in the thickness of the myocardium is likely to induce myocardial rupture resulting in escape of blood into the pericardium and death. Since we reported that 5-HT<sub>2B</sub> receptor transduces mitogenic signals in fibroblasts (15, 16), impaired differentiation, migration, and/or proliferation in the heart of 5-HT<sub>2B</sub>-mutant the embryos probably accounts for the mid-gestation lethality. 5-HT developmental action has been suspected to trigger embryopathies including cardiac pathology observed in embryos from phenylketonuria patients who exhibit low level of 5-HT in their blood (18).

Identification of factors controlling myocardial differentiation and proliferation is of great importance for understanding the pathogenesis of congenital heart diseases. Remarkably, embryonic heart defects reported in the mice lacking neuregulin or its receptors, ErbB-2 (HER-2/neu) and ErbB-4 are similar to those noted in 5-HT<sub>2B</sub> embryos (19): Expression studies showed that neuregulin is expressed in the endothelial cells of the endocardium and both ErbB-2 and ErbB-4 are localised into the ventricular wall of the myocardium (20). In ErbB-2 or ErbB-4 mutant mice, strong defects in ventricular trabeculation that are nearly identical to that seen in most severely affected 5-HT<sub>2B</sub> mutant embryos, trigger death at 10.5 dpc. This similarity suggested possible interaction between these two signalling pathways. To this purpose, we analysed the ErbB-2 and ErbB-4 expression in 5-HT<sub>2B</sub> mutants. Interestingly, at 9.5 dpc mutant embryos exhibit a significant reduction in ErbB-2 mRNA, whereas ErbB-4 mRNA expression is not altered. Immunohistochemical analysis revealed that the pattern of ErbB-2 expression in the wild-type heart (Fig. 3a, 3b right) is overlapping that of 5-HT<sub>2B</sub> receptor expression (Fig. 3c) but is significantly reduced in 5-HT<sub>2B</sub> mutant embryo hearts (Fig. 3a, 3b left). Moreover, Western blot analysis revealed a significant reduction in ErbB-2 protein levels in the mutant embryo extracts at 9.5 dpc (Fig. 3d, 3e). However, another growth factor receptor expressed in heart at this period, such as PDGFR protein remains unchanged (Fig. 3e). Reduction in ErbB-2 protein expression becomes less significant at 11.5 dpc, i.e. after the period of lethality, since the most affected 5-HT<sub>2B</sub> mutant embryos are resorbed at 11 dpc (Fig. 3e). Since ErbB-2 can be phosphorylated (transactivated) by activation of G-protein-coupled receptors (21), this cardiac phenotype could result from a lack of ErbB-2 transactivation by 5-HT<sub>2B</sub>. However, an ErbB-2 regulation at transcriptional level seems more likely because both mRNA and protein levels of ErbB-2 are reduced in 5-HT<sub>2B</sub> mutant

embryos. Moreover, in mouse LMTK- cells transfected by the 5-HT2B cDNA, 5-HT raises the endogenous expression but not the phosphorylation levels of ErbB-2 protein (data not shown). Furthermore, the 5-HT2B receptor expression remains unchanged in ErbB-2 knockout mice (22). We also investigated if ablation of 5-HT2B receptor selectively reduced expression of other genes that are involved in heart development. We performed RT-PCR as a quantitative approach for the expression of genes whose ablation resulted in defects in the heart, such as N-myc or for chamber specific markers such as MLC2v. No significant changes in either marker (N-myc or MLC2v) mRNA expression were detected in the 5-HT2B mutant embryos (data not shown). The mechanism whereby 5-HT2B activation regulates ErbB-2 expression is currently under investigation in our lab. Since ErbB-2 but not ErbB-4 receptor levels are impaired in the 5-HT2B mutant mice, our data suggest that Gq-coupled 5-HT2B receptor specifically uses ErbB-2 RTK pathways in the heart.

Interestingly, mice lacking both  $G\alpha_q$  and  $G\alpha_{11}$  also die at embryonic day 11 due to hypoplasia in the ventricular wall. Two active alleles of these genes are required for extra uterine life, and at least one intact  $G\alpha_q$  allele is needed to bring the embryo to term (23).  $G\alpha_{11}$  seems to be preferentially required for 5-HT2B-dependent heart developmental events, since none of the skeletal abnormalities observed in  $G\alpha_q^{-/-}G\alpha_{11}^{+/-}$  embryos could be detected in the 5-HT2B mutants (data not shown).

The less affected 5-HT2B mutant embryos survived into the neonatal period and appeared normal in their gross morphology although a second period of lethality appears during the first week of life (Fig. 1e). Furthermore, 5-HT2B mutant mice fertility was consistently lower than the control 129/Sv strain, and their body weight was slightly reduced by 9%. Moreover, their percent of heart weight to body weight was significantly reduced by 28% ( $1.06\pm 0.12$  vs.  $0.76\pm 0.07$ ,  $p < 0.05$  by a Fisher test,  $n > 5$ ). Investigation of newborn mutants heart at histopathological level revealed a strong decrease in ventricular mass and abnormal compact layer in all observed newborn mutant hearts ( $n=6$ ) (Fig. 4a). Shortly after birth, cardiac myocytes lose their proliferative capacity, and growth of the myocardium occurs through enlargement of existing myocardial cells. Previous report indicated that 5-HT may regulate proliferation in the embryonic heart (8). We determined the in vivo proliferative capacity of myocardium in 5-HT2B newborn heart by measuring BrdU incorporation. Essentially no BrdU incorporation was detected in the heart of 5-HT2B mutant newborn (Fig. 4c, left), whereas BrdU incorporation was clearly observed in the compact zone of the wild-type newborn heart (Fig. 4c, right). The hypoplasia in the 5-HT2B mutant heart is probably due to impaired proliferation.

Accordingly, in isolated newborn ventricular myocytes from 5-HT2B mutant, thymidine incorporation in response to 5-HT was also reduced (Fig. 4b). These in vitro data indicate that the 5-HT2B mutant cardiac phenotype is intrinsic to myocytes and is cell autonomous. These data suggest that 5-HT indeed acts as a cardiac mitogenic factor via 5-HT2B receptor-mediated pathway. Furthermore, the requirement for the neuregulin-ErbB-2 signalling pathways is demonstrated by a thymidine incorporation assay in response to neuregulin (heregulin) in the isolated cardiomyocytes from embryo (9.5 dpc) and newborn mice. Figure 4b shows that 5-HT2B deficient cardiomyocytes obtained from newborn hearts also have defective proliferative responses to neuregulin. These data strongly suggest that Gq coupled-5-HT2B receptor specifically uses the receptor tyrosine kinase ErbB-2 signalling pathways in cardiac development.

Adult hearts from surviving 5-HT2B mutants (6 weeks old) also exhibited dilated chambers and a decreased ventricular mass, their percent of heart weight to body weight was significantly reduced by 24% ( $0.67\pm 0.4$  vs.  $0.51\pm 0.03$ ,  $p < 0.05$  by a Fisher test,  $n=10$ ). On histological sections, 6 weeks old adult hearts from 5-HT2B mutants showed a consistent pattern of myocellular

disorganisation, and scattered areas of myocellular disarray unlike wild-type hearts (Fig. 4d top). At TEM level, the mutant myocardium display an irregular array of sarcomeric myofibrils including abnormally wide Z bands relative to controls (Fig. 4d bottom). In particular, the well defined sarcomeric pattern is disrupted, the sarcomere lengths are greatly reduced and M bands are indistinct. There were no obvious apoptotic bodies observed at TEM level in the mutant heart either at 9.5 dpc, newborn or adult stage. These cardiac sarcomeric defects are similar to those observed in mice heterozygous for the alpha MHC knockout (24) with numerous degenerating fibers. Loss of myofibrils is the most obvious structural change in many cardiomyopathies (25) and sarcomeric disarray is characteristic of failing hearts (26). Several studies have suggested a role for Gq-coupled receptors in various heart compartments (27) and Gq has been shown to regulate cardiomyopathy development. For example, both the Angiotensin II receptor and  $\alpha 1B$ -adrenergic receptors signal via Gq: The Angiotensin II transgenic mice died in utero or shortly after birth with grossly enlarged atria (28), but with no changes in ventricular morphology seen in  $\alpha 1B$  transgenic mice (29), indicating a specificity of downstream pathways. Parallel to the previous observations, targeted inhibition of Gq signalling by overexpression of a carboxy-terminal peptide of Gq in the heart reduces ventricular hypertrophy in response to pressure overload (30), whereas D'Angelo et al (31) found that a fourfold overexpression of the Gq protein in the heart generates cardiac hypertrophy and heart failure.

We show that the 5-HT<sub>2B</sub> receptor is required for 5-HT to regulate cardiovascular functions. Previously we have shown that treatment of embryos with selective antagonists of 5-HT<sub>2B</sub> receptor caused trabeculation defects in the heart along with defects in neural crest cell derivatives. 5-HT<sub>2B</sub> mutant mice exhibit obvious cardiac defect but gross morphology of the CNS appears unaffected. These preliminary observations suggest that the morphological effects of antagonists on neural crest cells derivatives in embryo cultures, are probably masked in the 5-HT<sub>2B</sub> mutant mice, but could be reflected at functional levels. Further investigations are necessary to assess if CNS functions are affected by 5-HT<sub>2B</sub> receptor elimination. The present data strongly suggest that the Gq coupled-5-HT<sub>2B</sub> receptor is involved in cardiac proliferation and differentiation. These findings should facilitate a genetic approach and a new drug design in the cardiovascular disease.

### Acknowledgements

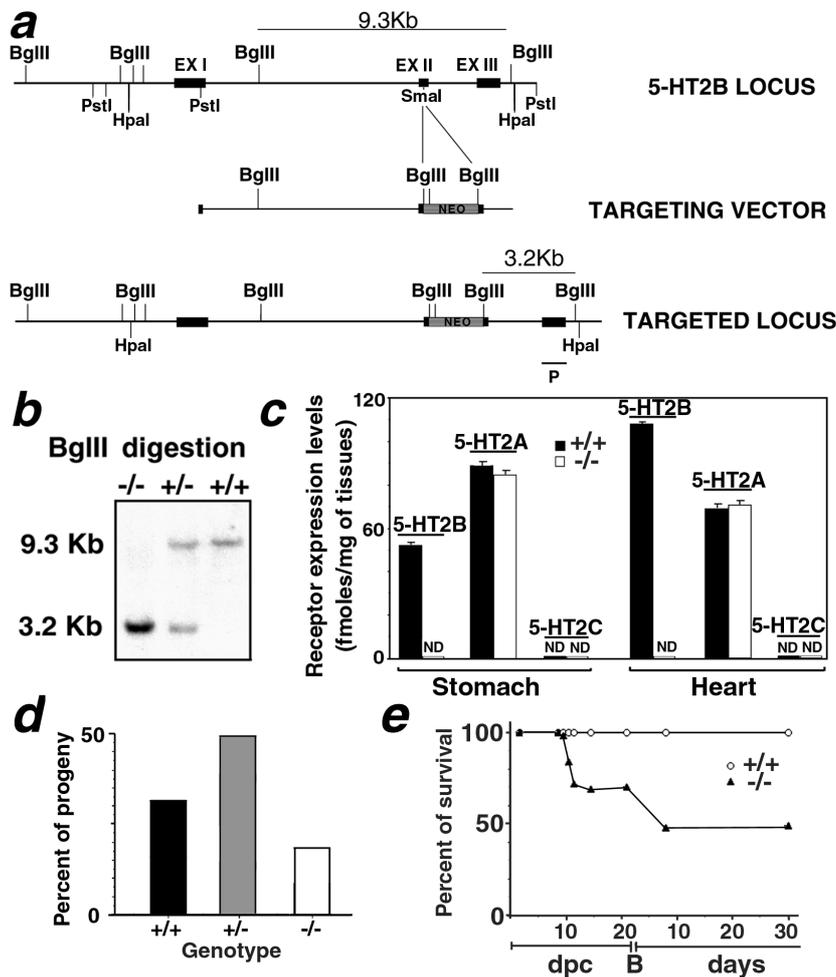
We thank Drs. K. Niederreither, F. Guillemot and Pr. M. Mark for stimulating discussions and critical reading of the manuscript. This work has been supported by funds from the Centre National de la Recherche Scientifique, the Institut National de la Santé et de la Recherche Médicale, the Hôpital Universitaire de Strasbourg, the Université Louis Pasteur, and by grants from the Association Française contre les Myopathies, from the Association pour la Recherche contre le Cancer, the Ligue Nationale contre le Cancer and C. G. N. is supported by a Lilly-ULP fellowship.

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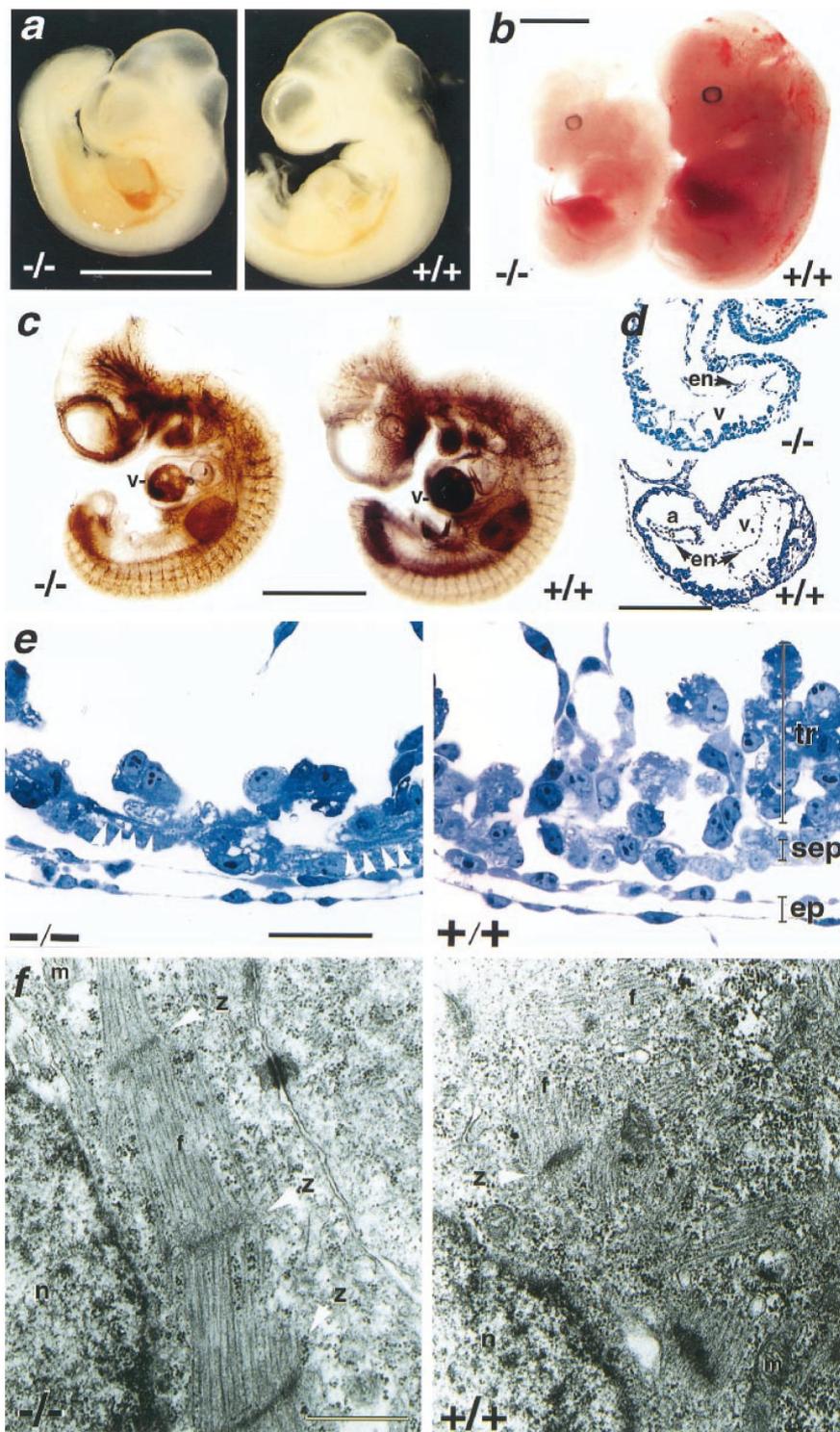
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## Figure Legends



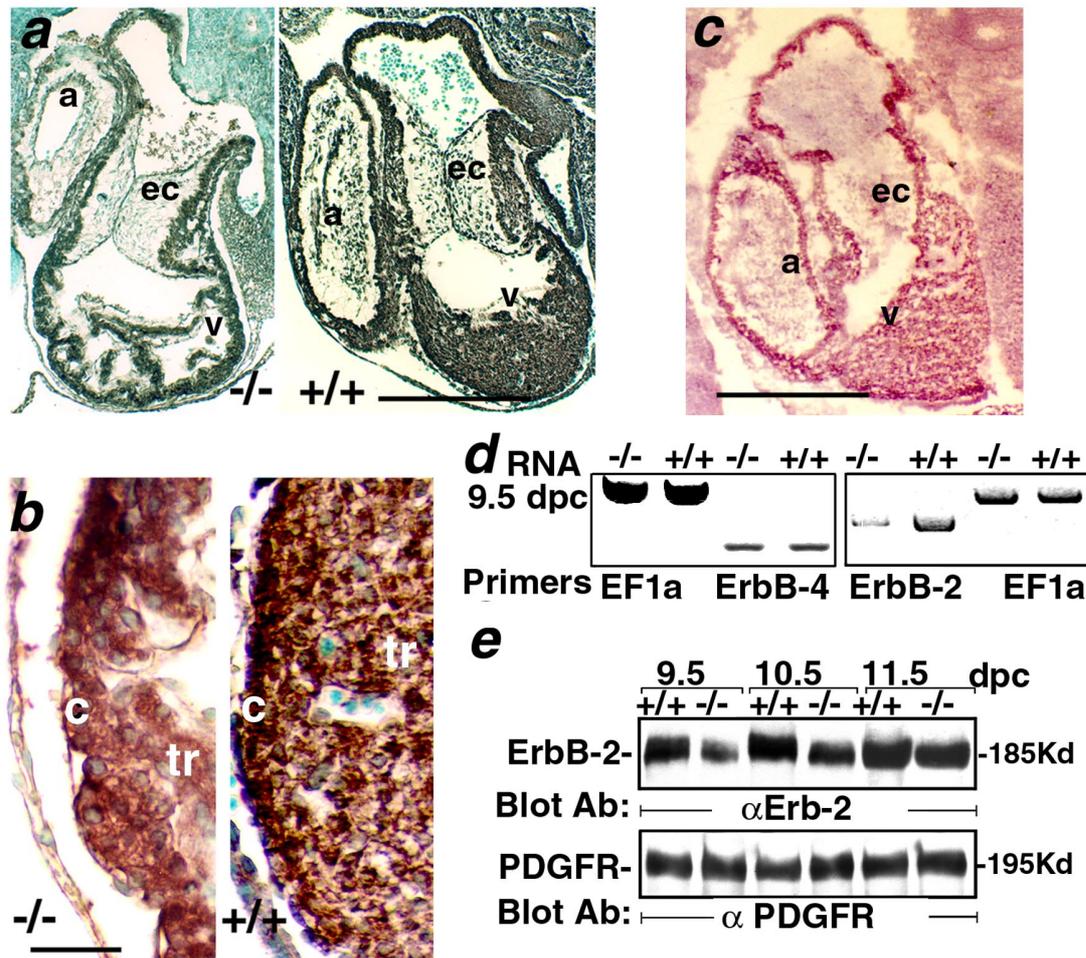
**Figure 1. Targeted disruption of the 5-HT2B receptor gene.** a, The restriction map of the 5-HT2B receptor genomic locus of interest is shown on top, the targeting construct is shown in the center, and the mutated locus after homologous recombination is shown at the bottom. b, The wild-type (+/+), heterozygous (+/-) and homozygous mutant (-/-) alleles were detected by Southern blot analysis. The probe (P) located outside of the construct detects a 9.3 Kbp BglIII fragment from wild-type DNA while it is reduced to 3.2 Kbp in the homologous recombined allele. c, The analysis of receptor membrane proteins by binding assay revealed a complete loss of 5-HT2B -specific sites in stomach and heart tissues of mutant mice. The maximal amount of binding sites is expressed as femtomoles of specific binding per mg of tissues  $\pm$ SEM (n>4). Kbp, kilo base pairs; ND, no specific binding detected. d, Genotyped progeny from 5HT2B +/- intercrosses showed that the homozygous genotype did not exhibit a Mendelian ratio indicating some embryonic lethality among the homozygous. The percent of progeny is calculated from 120 newborn mice from 14 different litters. The homozygous mutant (-/-) (22 out of 120) represented 66% of the expected viable calculated assuming sum of +/+ and +/- is 75%. e, Percentage of homozygous survival before and after the birth in the progeny of heterozygous crosses. Before birth (B), the percent of survival is estimated from more than four litters of heterozygous crosses (n>30), and a Mendelian ratio is observed until 10.5 dpc.



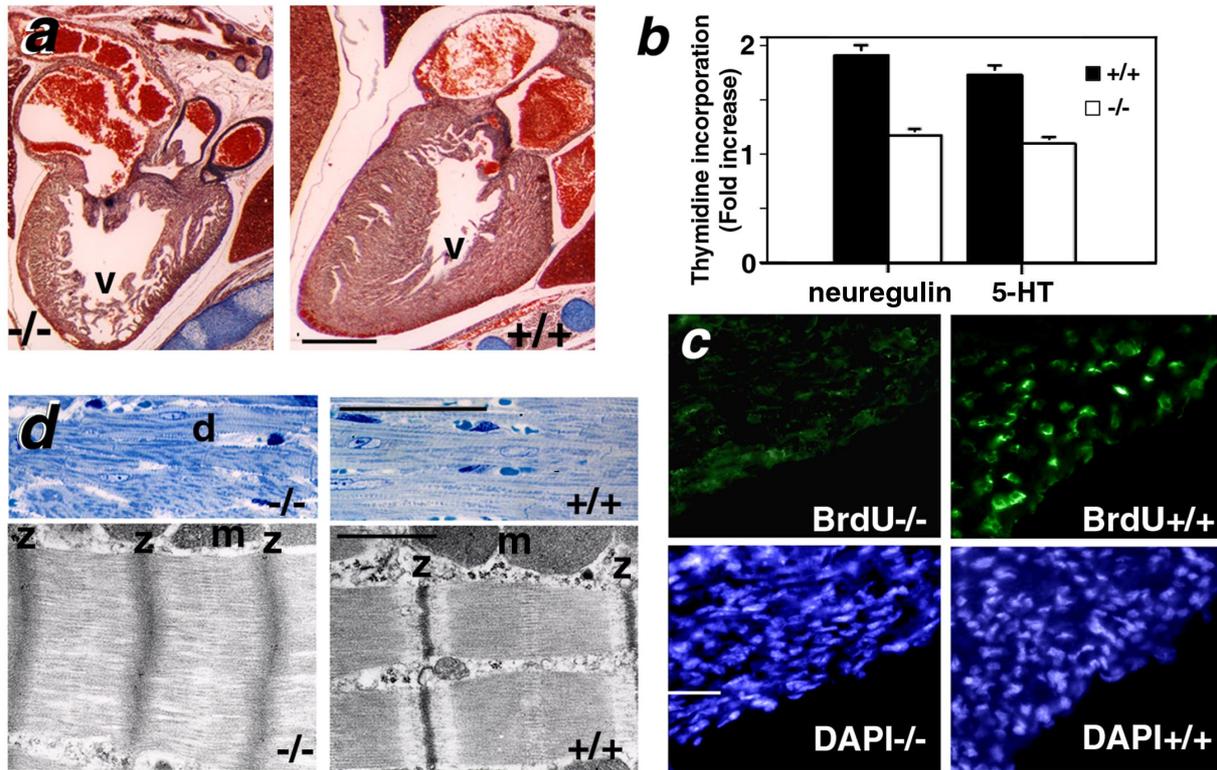
**Figure 2. Morphology of 5-HT2B mutant embryos.** a, 5-HT2B - deficient embryos at 10.5 dpc exhibit a typical bleeding into the pericardial cavity. b, At 12.5 dpc the mutant embryos are smaller and paler than their wild-type littermates. c, Whole mount

immunohistochemical staining of 10.5 dpc embryos for developing blood vessels using anti-PECAM antibody revealed no gross defects in vascular patterning compared to age-matched wild-type littermates but reduced staining in heart ventricle (v). d, Semi-thin sagittal sections of 9.5 dpc embryos demonstrate a severe reduction in the thickness of the ventricle (v) including both the compact zone and the trabeculae in 5-HT2B - deficient embryos. atrium (a); endocardium (en). e, Higher magnification shows a reduction of trabecular cells (tr) in the mutant heart, whereas elongated cells (white arrowhead) are visible in the compact subepicardial layer (sep).

Epicardial cells (ep) are normally developed. f, TEM analysis of these embryos reveals the abnormal sarcomeric differentiation within the subepicardial layer in all observed mutant heart (n=6) but not in wild-type heart. Z band of the sarcomeres (z); actin fibers (f); mitochondria (m) Nucleus (n). Genotype designations are +/+, wild-type; -/-, homozygous mutant. Bars a, b, c 500 mm. d 100 mm, e 5 mm, and f 0.5 mm.



**Figure 3. Reduced ErbB-2 expression in 5-HT2B-mutant embryos.** a. The immunohistochemical staining of paraffin sections with anti-ErbB-2 antibody through the heart region of 10.5 dpc wild-type (right) and mutant embryos (left). b. Enlargement of figure 3a. The ErbB-2 expression is globally reduced, including over the trabeculae (tr) and the compact zone (c) of the mutant ventricle (v). c. Immuno-histochemical labelling with an anti-5-HT2B specific antibody performed on cryostat sections of 10.5 dpc embryos illustrates the wild-type expression of 5-HT2B receptor in the trabecular and the compact zone of the heart but not in the endocardial cushions (ec). d. Semi quantitative RT-PCR shows a strong reduction of ErbB-2 mRNA in 9.5 dpc mutant embryos whereas ErbB-4 and the ribosomal elongation factor EF1a expression in the same RNA preparation remains unchanged. e. A western blot analysis using an anti-ErbB-2 antibody demonstrates a reduction of ErbB-2 expression in total protein extract from 5-HT2B mutant embryos (from 9.5 to 11.5 dpc) (top). The PDGFR immuno-reactivity in the same mutant embryo extracts remains unmodified (bottom). Atrium (a). Bars a, c, 250 μm, and b, 25 μm. a, anti-, M, size markers.



**Figure 4. Heart defects in post-natal 5-HT<sub>2B</sub> mutant mice.** a, Histological analysis of newborn mutant hearts shows an obvious loss of ventricular (v) mass (hypoplasia). b, Thymidine incorporation of isolated cardiomyocytes from newborn heart in response to 5-HT and neuregulin (heregulin) is significantly reduced in the 5-HT<sub>2B</sub> mutant, indicating that myocyte proliferation is impaired and this effect is cell autonomous. The thymidine incorporation rate is expressed as fold increase over basal  $\pm$ SEM (-/-, n=3; +/+, n=3). c, In vivo, BrdU incorporation (green) is almost undetectable in the heart of 5-HT<sub>2B</sub> newborn mutant (left), whereas BrdU incorporation was observed in the compact zone of control newborn heart (right). DAPI staining of the same sections localises the cell nuclei (blue). d top, Semi-thin sections of heart in 6 weeks old mutant show abnormal morphology, including degenerating fiber (d) (left). d bottom, TEM of ultra-thin sections shows that, in mutant heart, the sarcomere lengths are greatly reduced, the M-lines and the I-bands are indistinguishable and the A-bands occupy the entire length between Z-bands (z) which themselves are thickened (left) compared to wild-type age mated heart (right). Mitochondria (m). Bars: a 100  $\mu$ m, c and d (top) 20  $\mu$ m, d (bottom) 0.5  $\mu$ m.