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Serotonergic 5-HT_{2B} receptors in mitral valvulopathy: bone marrow mobilization of endothelial progenitors

Short title: Serotonin, cardiac valve remodelling, mobilization of progenitors.

Estelle Ayme-Dietrich[1†*], Roland Lawson[1†], Francine Côté[2,3], Claudia de Tapia[1], Sylvia Da Silva[1], Claudine Ebel[4], Béatrice Hechler[5], Christian Gachet[5], Jérome Guyonnet[6], Hélène Rouillard[7], Jordane Stoltz[7], Emily Quentin[8], Sophie Banas[8], François Daubeuf[9], Nelly Frossard[9], Bernard Gasser[7], Jean-Philippe Mazzucotelli[10], Olivier Hermine[2,3], Luc Maroteaux[8], and Laurent Monassier[1]

[1]Laboratoire de Neurobiologie et Pharmacologie Cardiovasculaire (EA7296), Faculté de Médecine, Fédération de Médecine Translationnelle, Université et Centre Hospitalier de Strasbourg, Strasbourg, France.

[2]Department of hematology, Université Paris Descartes-Sorbonne Paris Cité, Institut *Imagine*, INSERM U1183 CNRS ERL 8254, Hôpital Universitaire Necker Enfants Malades, Paris, France.

[3]Laboratory of Excellence GR-Ex.

[4]Department Flow Cytometry, Institut de Génétique et de Biologie Moléculaire et Cellulaire, Illkirch, France.

[5]Etablissement Français du sang (EFS) Alsace, Inserm U949, Strasbourg, France.

[6]Pharmaceutical ResearchDepartment. CEVA Santé Animale, Libourne, France.

[7]Laboratoire de Pathologie, Centre Hospitalier Emile Muller, Mulhouse, France.

[8]INSERM UMR-S 839, F75005, Paris, France; Sorbonne Université, UPMC Univ Paris 06, F75005, Paris, France; Institut du Fer à Moulin, Paris, France.

[9]Laboratoire d'Innovation Thérapeutique, UMR7200 CNRS/Université de Strasbourg, LabExMedalis, Faculté de Pharmacie, Illkirch, France.

[10]Service de Chirurgie Cardiaque, Centre Hospitalier de Strasbourg, Strasbourg, France.

[*]Address for correspondence : Estelle Ayme-Dietrich, Laboratoire de Neurobiologie et Pharmacologie Cardiovasculaire (EA7296), Faculté de Médecine, 11 rue Humann, 67085 Strasbourg Cedex, France. Phone: +33368853083; Fax: +33368853388; E-mail: eayme@unistra.fr

[†]Equal contributors

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Abstract:

Background: Valvular heart disease is highly prevalent in industrialized countries. Chronic use of anorexigens, amphetamine or ergot derivatives targeting the serotonin system has been associated with valvular heart disease.

Purpose and experimental approach: Here, we investigated the contribution of serotonin receptors in a mouse model of valve degeneration induced by nordexfenfluramine the main metabolite of the anorexigens dexfenfluramine and benfluorex.

Key results: Chronically activated 5-HT_{2B} receptors by nordexfenfluramine in mice mimicked early steps of mitral valve remodelling attested by increased valve thickness, and cell density in a thick extracellular matrix. Lesions were totally prevented by inhibition of both 5-HT_{2A} and 5-HT_{2B} receptors by antagonists, in transgenic $Htr_{2B}^{-/-}$, or $Htr_{2A/2B}^{-/-}$ mice. Surprisingly, we found that valve lesions are mainly formed by numerous non-proliferative $CD34^+$ endothelial progenitors. We show that these progenitors originate from bone marrow as revealed by bone marrow transplantation. Initial steps of mitral valve remodelling involve bone-marrow derived $CD34^+CD31^+$ cells mobilization by 5-HT_{2B} receptor stimulation. Moreover, the analysis of human mitral valve prolapse, showing spontaneous degenerative lesions highlights the presence of non-proliferating $CD34^+/CD309^+/NOS3^+$ endothelial progenitors expressing 5-HT_{2B} receptor. Conclusions and implications: This work reveals a crucial contribution of bone-marrow derived endothelial progenitor cells in valve tissue remodelling and highlights the contribution of this new mechanism involved in human valvular heart disease.

Keywords: serotonin, 5-HT_{2A} and 5-HT_{2B} receptors, cardiac valve degeneration, endothelial progenitor cells.

Abbreviations definition list:

5-HIAA: 5-hydroxyindoleacetic acid

5-HT: serotonin

BM: bone-marrow

CVI: cardiac valve injury

NOS3: endothelial nitric oxide synthase

MR: mitral regurgitation

NdF: nordexfenfluramine

pCPA: para-chlorophenylalanine

SERT: serotonin transporter

VHD: valvular heart disease

WT: wild-type

Introduction:

Several drugs as ergot derivatives (pergolide, cabergoline, ergotamine) (Van Camp et al., 2004), (Zanettini et al., 2007) and anorectic compounds (fenfluramine, dexfenfluramine) (Connolly et al., 1997) have been reported to associate with left cardiac (mitral and aortic) valve remodelling and interact with the serotonergic system. More precisely these drugs target the 5-HT_2 receptor sub-types (5-HT_{2B} and 5-HT_{2A}) (Fitzgerald et al., 2000), (Rothman et al., 2000), which belong to the superfamily of Gq-protein-coupled receptors that activate phospholipase C. Numerous observations implicated 5-HT_2 receptors in drug-induced valvular heart disease. Stimulation of 5-HT_2 receptors leads to upregulation of target genes involved in proliferation and stimulation of valvular interstitial cells (VICs) through activation of protein kinase C, Src-protein, phosphorylation of ERK1/2 and TGF-β receptor activation (Jian et al., 2002)(Xu et al., 2002)(Hutcheson et al., 2012). Phosphorylated ERK is thought to induce TGF-β signaling and transcription of effector genes mediating myxomatous pathology (Disatian and Orton, 2009). Overexpression of TGF-β1 has also been implicated in cardiac diseases where fibrosis is a prominent feature. The activation of 5-HT_{2B} receptors in human VICs is mitogenic, resulting in ERK1/2 phosphorylation and [^{3}H]-deoxythymidine incorporation (Setola et al., 2003) and has also been implicated in 5-HT induced valvulopathy in experimental animals (Elangbam et al., 2008). The serotonergic 5-HT_{2B} receptor appears enriched in heart valves from various species including dog (Oyama and Chittur, 2006), (Cremer et al., 2015a), (Lu et al., 2015), rat (Elangbam et al., 2005), pig (Fitzgerald et al., 2000), (Cremer et al., 2015b) and human (Fitzgerald et al., 2000), mainly in healthy tissues. Nevertheless, other serotonergic receptors than the 5-HT_{2B} subtype have been reported in valve tissue (Cremer et al., 2015b), (Lu et al., 2015), and the implication of 5-HT transporter (SERT) in valvular heart disease (VHD) has been suggested by the development of valvular fibrosis in SERT KO mice (Mekontso-Dessap et al., 2006). Moreover, the well known "carcinoid heart", a valvulopathy associated with excess of plasma (free) 5-HT secreted by neuroendocrine tumors highlights the important role of this biogenic amine in VHD (Lundin et al., 1988). Despite previous works linking 5-HT_2 receptors with VHD, some limits have to be reported: 1) many experiments were performed on healthy tissues (Fitzgerald et al., 2000)(Setola et al., 2003), making difficult the extrapolation to pathological mechanisms, 2) 5-HT stimulation was performed with high concentrations ($\geq 10^6$ M) during long time (Jian et al., 2002)(Xu et al., 2002)(Elangbam et al., 2008) and 3) 5-HT_2 receptor agonists applied *in vitro* to valve tissues induced relatively small effects (Barzilla et al., 2010). Taking all these limitations into consideration, we hypothesized that the 5-HT_2 receptors stimulation involved

in VHD could take place in other sites than the valve itself and that other(s) receptor(s) than the 5-HT_{2B} subtype could also be involved.

Cardiac valves are made of valvular endothelial (VECs) and interstitial cells (VICs). Both cell types are required for the homeostatic maintenance of valve tissue, which is exposed to high hemodynamic constraints. These two cell populations are heterogeneous. VICs can be found quiescent with a fibroblast phenotype or activated with various markers typical of myofibroblasts, stem cells and osteoblasts (Liu et al., 2007). Progenitors VICs were also observed in response to injury, but their origin and the mechanism implied to repair are not yet discovered (Liu et al., 2007). Some VECs are able to synthesize extracellular cell matrix (ECM) and to populate and assemble all three-valve layers. Valve endothelium is absolutely required to renew the VICs population from endothelial progenitors (Paruchuri et al., 2006). Nevertheless, the origin of these last cells and the mechanism(s) by which they reach the valve were not yet solved. Here, we hypothesized that 5-HT stimulation could be involved in VHD through the mobilization of endothelial progenitors in cardiac valve tissue. In fact, some authors described rapid migration of bone-marrow (BM)-derived cells in mitral valve tissue after fluorescent cells engraftment in wild-type (WT) mice (Visconti et al., 2006) showing, in basal homeostatic conditions, the recruitment of precursor cells to the valvular tissue. These cells could constitute part of a so-called “VECs reservoir” of endothelial progenitors that would then transdifferentiate via the general process of endothelial to mesenchymal transition (Paranya et al., 2001), (Paruchuri et al., 2006). In the first approach of this study, using pharmacological and transgenic mice experiments, we investigated the contribution of 5-HT_{2B} receptors in drug-induced valvulopathy, through endothelial progenitors mobilization. In the second part, the association between mitral valve prolapse and platelets activation (Walsh et al., 1981)(Martini et al., 1996), main cellular stockage of 5-HT in periphery, lead us to investigate if similar mechanisms could be involved in human VHD.

Materials and Methods:

Animals

Studies were performed in 12 week-old male mice. Wild-type 129S2/SvPasCrl (WT) were purchased from Charles River Laboratories (L'Arbresle, France). $Htr_{2A}^{-/-}$ (González-Maeso et al., 2003) were obtained from Dr. René Hen's laboratory, $Htr_{2B}^{-/-}$ (Nebigil et al., 2000) and $Htr_{2A/2B}^{-/-}$ mice were obtained from Dr. Luc Maroteaux's laboratory and left on a pure 129S2/SvPasCrl background.

All procedures were performed in accordance with the guidelines for animal experimentation of the European Communities Council Directive EU/63/2010 and obtained the authorization number AL/81/88/02/13.

Experimental procedures

To induce valvulopathy, mice were submitted to a 28 day-long infusion of nordexfenfluramine (NdF, 1mg/kg/day) delivered by micro-osmotic pumps (Alzet, model 1004, USA), implanted subcutaneously during an isoflurane anesthesia (Aerrane, Baxter, France). All drugs were purchased from Sigma-Aldrich, France.

Seven groups of WT mice were designed: (1) vehicle, (2) NdF, (3) NdF + the 5-HT_2 receptors antagonist, ritanserin (2mg/kg/day), (4) NdF + the selective 5-HT_{2A} receptors antagonist, sarpogrelate (2 mg/kg/day), (5) NdF + the selective 5-HT_{2B} receptors antagonist, SB204741 (1mg/kg/day), (6) NdF + the 5-$HT_{2B/2C}$ receptors antagonist, SB206553 (1mg/kg/day) and (7) NdF + the non-selective tryptophan hydroxylase inhibitor, para-chlorophenylalanine (100mg/kg/day). Ritanserin dose and administration route were validated by a platelet aggregation test (Supplemental material and Figure S1). Ritanserin and para-chlorophenylalanine were added to food pellets. Sarpogrelate, SB204741 and SB206553 were infused by the mean of a second osmotic pump. Food and water intake were controlled together with body weight to adjust orally delivered drugs (Figure S2).

Six groups of transgenic mice were studied: (1) $Htr_{2A}^{-/-}$ vehicle, (2) $Htr_{2A}^{-/-}$ NdF, (3) $Htr_{2B}^{-/-}$ vehicle, (4) $Htr_{2B}^{-/-}$ NdF, (5) $Htr_{2A/2B}^{-/-}$ vehicle and (6) $Htr_{2A/2B}^{-/-}$ NdF. All groups were monitored weekly for systolic blood pressure and heart rate by tail-cuff photoplethysmography (Visitech, BP-2000, USA) (Figure S4). Transthoracic echocardiography was performed one day before the beginning of the experiments (day -1) and after 28 days of treatment (in WT and NdF mice groups, Table 1). At day 28, at the end of the last echocardiography, euthanasia was performed by a lethal intraperitoneal injection of pentobarbital (150mg/kg, CEVA Santé Animale, France). Hearts harvested from mice,

were immediately rinsed, weighted (Figure S3) and fixed with 10% formalin solution before being processed.

Biochemical measurement

Whole blood 5-HT and urinary 5-HIAA concentrations were obtained by high-performance liquid chromatography (HPLC) (Plateau Technique de Biologie, Nouvel Hôpital Civil, Strasbourg).

Echocardiography

Animals were analysed for cardiac anatomy and function on a Sonos 5500 (Hewlett Packard, USA) with a 15MHz linear transducer (15L6). All the examinations were performed in mice anesthetized with 1-1.5% isoflurane. The heart was first imaged in the two-dimensional (2D) mode in the parasternal long-axis view to obtain the aortic root dimensions. The aortic flow velocity and the heart rate (HR) were measured with pulsed-wave Doppler on the same section. The cardiac output (CO) was calculated from the following equation: $CO = 0.785xD^2xVTIxHR$ where D is the diameter of the aortic root and VTI is the velocity-time integral of the Doppler aortic spectrum. Left ventricular cross sectional internal diameters in end-diastole (EDLVD) and end-systole (ESLVD) were obtained by an M-mode analysis of a 2D-short axis view at the papillary muscle level. The shortening fraction was calculated as SF = (EDLVD-ESLVD/EDLVD) x 100. From this view, the diastolic septum (S) and posterior wall (PW) thicknesses were measured. The left ventricular mass (LVM) was calculated with the following formula: $LVM = 1.055x[(S+PW+EDLVD)^3-(EDLVD)^3]$. All the measurements were performed on, at least three beats, according to the guidelines of the American Society of Echocardiography.

Histology and quantification of mitral valve lesions

Fixed mice hearts were paraffin embedded, sectioned at 4µm (using HM 355s Automatic microtome) and stained with hematoxylin and eosin. Sections were performed in a way to obtain four chamber views of the heart with long axis section of the tricuspid, mitral and aortic leaflets. A single operator (R.L), blinded to the experimental groups, performed valve morphometric analysis with a microscope (Carl Zeiss, Germany) equipped with a 40x calibrated objective giving, at this magnification, a large square of 250µm x 250µm divided in 100 equal parts of 25µm x 25µm each. NdF induced non-reproducible lesions of the aortic valve and no alteration of the tricuspid one in our conditions. Therefore, we decided to focus on the mitral valve. For quantification, the whole mitral valve leaflet section was divided in three equal segments: proximal (near the insertion), medial and distal (including the tip). The thickness of each segment is the mean value obtained in 3 distinct sites. The surface cells

density was determined on the whole segment (mean value of 3 distinct sites) and the result expressed in number of cells per 0.01 mm^2.

Immunohistochemistry was performed on paraffin-embedded samples with anti-Ki-67 (Mib1, USBiological, 1/100) and anti-CD34 (EP373Y, Genetex, 1/100) antibodies.

Bone-marrow (BM) transplantation

As previously described, thirteen 8-week-old WT males were subjected to 9.5 Gray lethal total body irradiation (Potteaux et al., 2006). The day after, mouse BM was reconstituted by direct intravenous injection with 2.5 $x10^6$ cells of freshly isolated BM from femurs and tibias of age and sex matched WT or $Htr_{2B}^{-/-}$ mice. All lethally irradiated and transplanted mice survived, revealing the efficiency of BM reconstitution by either WT (n=6) or $Htr_{2B}^{-/-}$ (n=7) BM. After 4 weeks of recovery, transplanted mice were then exposed to NdF and followed the same protocol applied to other groups.

Blood mobilization of progenitors and bone-marrow analysis

To analyze the effects of NdF on the mobilization of progenitor cells in the blood and BM, WT control and $Htr_{2B}^{-/-}$ mice received a single 3mg/kg NdF subcutaneous injection. They were compared to vehicle-treated animals. Blood was collected, by intracardiac puncture in animals anesthetized with sodium pentobarbital (40mg/kg i.p.), for flow cytometric analysis (LSRII, H48700015; software BD FACSDiva 6.1.2) with the following antibodies: CD31 (130-102-571/Miltenyi), and CD34 (553930/BD Pharmingen). Blood was treated with a solution of 0.83% NH_4Cl to induce erythrocytes lysis and then washed before labeling. BM cells were isolated from tibias and femurs, filtered, treated with a solution of 0.83% NH_4Cl to induce erythrocytes lysis and labeled with the following antibodies: CD31 (130-102-571/Miltenyi), CD34 (553930/BD Pharmingen) and phalloidin (P1951, Sigma), for flow cytometric analysis (LSRII, H48700015; software BD FACSDiva 6.1.2). Phalloidin analysis was evaluated by FACS analysis on a CANTOII BD. Analysis was performed on the FlowJo® software.

Human mitral valve tissues

Eighteen human degenerative heart valves were obtained from patients referred to the Department of Cardiovascular Surgery, University Hospitals of Strasbourg, France for a cardiac valve replacement (mitral valve prolapsed diagnosis). All patients authorized the subsequent use of their valve tissues for research purpose (protocol approved by the Ethics Committee of the Faculty of Medicine of Strasbourg (6th of December 2011)).

Valve histology and immunohistochemistry analysis

Human mitral valve tissues (n=4) were fixed with formalin solution for 2 days and embedded in paraffin; 4 µm-slices were stained with blue alcian and hematoxylin/eosin to identify valve lesions and evaluate cellular density. Immunostaining was performed to localize 5-HT_{2B} receptors (A72-1 BD Pharmingen, BD Biosciences, France) andCD34 progenitors (QBEnd 10, Dako®, France). Finally, Ki-67 was used as a marker of cell proliferation (MIB-1, Dako®, France). Immunostaining was performed using a biotin-labeled peroxidase-conjugated secondary antibody incubated with diaminobenzidinetetrahydrochloride as a final chromogen (Ultraview DAB Detection Kit, Roche®, France). Labeling control was performed by incubating only the secondary antibody. Cell count was performed with a microscope (Carl Zeiss, Germany) equipped with a 40x calibrated objective giving, at this magnification, a large square of 250µm x 250µm divided in 100 equal parts of 25µm x 25µm each. The surface cell density was determined at the endothelium, sub endothelium and spongiosa levels and the results expressed in number of cells per 0.01 mm^2. To quantify immunostained cells, results were expressed as a percentage of positive cells compared to total cells per 0.01 mm^2 (5-HT_{2B} receptor, CD34 and Ki-67). The number of total or labeled cells in valve lesions is the mean value obtained in 3 distinct sites per valve.

Mitral valvular cells isolation and Flow Cytometry and Magnetic Cell Sorting

To remove cells from mitral valve tissues, specimens were immersed in a collagenase solution (NB8 Broad range 1mg/ml; Serva, Coger, France) for 2 hours at 37°C. Tissue pieces were scrubbed through a 70µm strainer, cell suspension was filtered to remove debris. In a first set of experiments, using 8 mitral valves, single-cell suspensions were labeled with CD34-Alexa Fluor 647-conjugated antibody (ICO115, Santa Cruz; CliniSciences, France) and CD31-FITC-conjugated antibody (MEM-05, Exbio), before cell sorting on a BD FACSAria® II flow cytometer. In a second set of experiments, single-cell suspension was stained with a CD34 antibody conjugated to magnetic beads (MACS) and passed on a midi column (CD34 MicroBead Kit UltraPure human, MiltenyiBiotec, France) according to the manufacturer's protocol (3 mitral valves were used). This technology was used to select and purify $CD34^+$ cells only, representing more than 90% of the whole cell population after MACS procedure. These cells were characterized by FACS using a double labeling with a CD34-FITC-conjugated (CD34, MiltenyiBiotec) and a CD309-PE-conjugated (VEGFR2, BD Pharmingen) antibodies. Acquisition was carried out on a BD LSR flow cytometer (BD Biosciences). Percentages shown in all figures are percent of single cells fell within gates determined using a negative control (isotype specific IgG) stained cell population. Analysis was performed on the FlowJo® software.

RNA extraction and Gene expression by RT-qPCR on selected valve cell populations

Total mRNA was extracted using TriReagent (Life Technologies) according to the manufacturer's recommended protocol. All RNA samples were initially quantified via spectrophotometer. RNA samples were converted to cDNAs using Kit iScript cDNA Synthesis BioRad®. Semi-quantitative reverse transcription–polymerase chain reaction (RT-PCR) was produced on an amplification system (PCR Light Cycler®Caroussel) with the kit Light Cycler Fast Start DNA Master Plus SYBR Green I® (Roche, France). Forward and reverse primer sequences for all analyzed genes were obtained by Qiagen® (Hs_HTR2A_1_SG/ Hs_HTR2B_1_SG/Hs_NOS3_1_SG, Hs_RRN18S_1_SG, Qiagen®).

Immunocytochemical Staining

To identify the expression of 5-HT_{2B} receptor in endothelial cells, the whole cell suspension obtained after human mitral valve collagenase treatment was stained with antibodies against the serotonergic receptor and some other surface markers (n=3 mitral valves). Briefly, cells were plated and cultured overnight at 37°C, 5% CO_2 with DMEM/F12 (1:1) medium containing antibiotics (100U/ml penicillin and 0,1 mg/ml streptomycin). The cells were then fixed with 4% paraformaldehyde, and incubated with a rabbit anti-human CD34 antibody (PA1334, Boster Biological Technology, VWR, France), and a mouse anti-human 5-HT_{2B} receptor antibody (BD Pharmingen, BD Bioscience, France) in 1% BSA solution. Cells were then incubated with fluorescent-tagged secondary antibodies, goat anti-rabbit antibody-Alexa Fluor® 488 (AP132JA4, Millipore) and goat anti-mouse antibody-Cy3 (AP124C, Millipore), and finally covered with a Dako Fluorescence mounting medium (S3023, Dako). Nuclei were stained with the Hoechst 33342 dye (H1399, Invitrogen). Double positive cells (CD34-5-HT_{2B} receptor) (average of 5 fields per valve) were counted by bright-field and UV illumination.

Data analysis and statistics

Mice: Values are expressed as mean ± SEM. Statistical comparisons between 2 or more groups were performed when appropriate using Student's unpaired t tests or ordinary one-way ANOVA followed by post hoc analysis with Dunnett's or Fisher's LSD test (GraphPad Software version 6.0).

$P<0.05$ was considered statistically significant.

Humans: Values are expressed as mean ± SEM. Statistical comparisons between two or more groups were performed when appropriate using Student's unpaired t-tests or non-parametric tests (Kruskall Wallis) (GraphPad Software version 6.0).

Results:

Chronic nordexfenfluramine administration induces mitral valve remodelling

Twenty-eight day-long NdF infusions did not modify body weight, systolic arterial blood pressure, heart rate and echocardiographic parameters (Table 1and Supplemental Figures S2-S4). In particular, no cardiac remodelling and/or systolic dysfunction were observed. Whole blood 5-HT and 5-HIAA urinary dosage revealed an increase in 5-HT turnover with a two-fold augmentation of 5-HIAA, while blood 5-HT concentration remained unchanged (Table 2). WT-C mice presented a homogenous and thin valve architecture bordered by regular endothelial $CD31^+$ lining (data not shown) and only few $CD34^+$ cells (Figure 1F). Conversely, we observed segmental thickened **(84%)** and retracted leaflets in WT-NdF animals (Figures 1A/B) with increased endothelial cell number bordering the valves (Figures1A/C). Concerning interstitial cell density, no significant change was observed between WT-NdF and WT-C (Figures 1A/D). Finally, clusters of rounded shape cells positive for the CD34 antigen were observed (Figure 1F). However, similarly to the WT-C group, in WT-NdF mitral valves only few nuclei were Ki-67 positive indicating a low rate of proliferation (Figure 1E).

Involvement of serotonergic 5-HT_{2A} and 5-HT_{2B} receptors but not 5-HT in NdF-induced valvulopathy

Two sets of experiments using pharmacological antagonists and knockout mice were used to investigate the role of serotonergic 5-HT_{2A} and 5-HT_{2B} receptors. Hemodynamic and physical parameters of these experimental groups are presented in supplemental data (Supplemental Figures S2-S4). To test the contribution of the 5-HT_{2A} receptor, we evaluated NdF effects in WT mice co-treated with sarpogrelate, a selective 5-HT_{2A} receptor antagonist, or in $Htr_{2A}^{-/-}$ mice. Sarpogrelate treatment prevented NdF-induced urine 5-HIAA and blood 5-HT increase (Table 2). No difference was observed regarding blood 5-HT and urine 5-HIAA in NdF-treated $Htr_{2A}^{-/-}$ mice (Table 3). Sarpogrelate treatment prevented valve injuries induced by NdF (Figure 2). However, we detected a significant increase thickness with $Htr_{2A}^{-/-}$ mice under NdF treatment **(31%)** (Figures 3B/G). To test the contribution of 5-HT_{2B} receptors, we evaluated NdF effects in WT mice co-treated with SB204741 or SB206553, two 5-HT_{2B} receptor antagonists or in $Htr_{2B}^{-/-}$ mice. In WT mice, NdF-induced urine 5-HIAA increase was prevented by SB204741 co-treatment, but not blocked by SB206553. Blood 5-HT concentration was not statistically different from WT-NdF group (Table 2). $Htr_{2B}^{-/-}$ mice had higher basal urine 5-HIAA, which was not affected by NdF treatment (Table 3). SB204741

and SB206553 treatments prevented valve injuries induced by NdF (Figure 2). Furthermore, no lesion was detectable in $Htr_{2B}^{-/-}$ mice under NdF treatment (Figures 3C/D/H/K/N). To test the contribution to lesions of both 5-HT_{2B} and 5-HT_{2A} receptor blockade, we evaluated NdF effects in WT mice co-treated with ritanserin, a non-selective murine 5-HT_2 receptor antagonist or in $Htr_{2A/2B}^{-/-}$ double mutants. NdF-induced urine 5-HIAA increase was blocked by the ritanserin co-treatment. Peripheral blood 5-HT concentration was not statistically different (Table 2). $Htr_{2A/2B}^{-/-}$ double mutant mice had a two-fold increase of urine 5-HIAA under NdF treatment, and significantly lower blood 5-HT compared to their control group (Table 3). Histological lesions were fully prevented by ritanserin (Figure 2). Histological and morphometric analysis of $Htr_{2A/2B}^{-/-}$ mice mitral valve did not reveal any lesion (Figures 3E/F/I/L/O). These results confirm the role of both 5-HT_{2A} and 5-HT_{2B} receptors in valve remodelling with a major contribution of the 5-HT_{2B} receptor subtype according to a persistent valve increased thickness observed in $Htr_{2A}^{-/-}$ mice.

Taking into account that NdF induces 5-HT release by platelets through its interaction with the 5-HT transporter, SERT, we investigated whether 5-HT contributes to NdF-induced valve remodelling. We induced a peripheral 5-HT depletion by treating mice with 100 mg/kg/day of para-chlorophenylalanine (pCPA), a tryptophan hydroxylase (Tph) inhibitor. Chronic pCPA markedly decreased blood 5-HT (3x) and urinary 5-HIAA (Table 2). Nevertheless, this massive depletion failed to prevent NdF-induced valve leaflets remodelling (Figure 2). This result indicates that peripheral 5-HT is not necessary for NdF-induced mitral valve injuries, and that the direct 5-HT_{2B} receptor stimulation by NdF is sufficient to trigger valve remodelling.

Taking into account that mitral valve lesions show a low rate of proliferation but are highly cellularized ($CD34^+$ cells), we hypothesized that these cells could be recruited at the valve surface from the blood stream after being mobilized from the bone marrow.

The 5-HT_{2B} receptor is involved in the mobilization of endothelial progenitors from the bone-marrow

To test this hypothesis, we compared the effect of a chronic NdF treatment in lethally irradiated WT mice engrafted with a $Htr_{2B}^{-/-}$ BM to WT mice engrafted with a WT BM. Mice transplanted with WT BM exhibited histological lesions with a significant increase of the thickness **(28.4%)** and endothelial cell density in the mitral valves (Figures 4A/C). By contrast, histological lesions were **totally** prevented in mice that received BM from $Htr_{2B}^{-/-}$ mice (Figures 4B/C). Therefore, the restricted ablation of 5-HT_{2B} receptors to BM prevents

the increase in thickness and endothelial cellularity of mitral valve, supporting the contribution of 5-HT_{2B} receptors to the mobilization of BM-derived endothelial progenitors.

Whole body irradiation probably affects the repopulation of the bone marrow in non-hematopoietic cells leading to a reduction of the bone marrow mobilization capabilities of endothelial progenitors.

NdF BM 5-HT_{2B} receptor stimulation mobilizes $CD34^+/CD31^+$ cells into the blood

To investigate whether 5-HT_{2B} receptor stimulation affects the mobilization of BM-derived endothelial progenitors, we measured $CD34^+/CD31^+$ cells by FACS 15 minutes after a single NdF (3 mg/kg) injection. In the BM of WT mice, the single NdF injection induced, a significant increase in the number of free (i.e. released from the extracellular cell matrix) $CD34^+/CD31^+$ cells (Figure 5A), which was associated to an increase in actin polymerization in these cells as attested by phalloidine labeling (Figure 5B). Simultaneously, a significant increase in circulating $CD34^+/CD31^+$ cells was observed in the blood of these animals (Figure 5C). Conversely, NdF did not produce these events in $Htr_{2B}^{-/-}$ mice. **This reduction of migration capabilities is probably at the origin of the higher number of these cells found in the bone marrow in basal conditions.** These data support the idea that NdF induces endothelial progenitors mobilization by a 5-HT_{2B} receptor-dependent mechanism and that NdF triggers the acute mobilization of $CD34^+/CD31^+$ cells from BM into the blood. These cells then home into target tissues that require homeostatic repair.

In a second part of this work, we addressed the question about a similar mechanism triggered by endogenous 5-HT in spontaneous human mitral valve degeneration.

Identification of $CD34^+$ cells sharing the 5-HT_{2B} receptor in human mitral valve lesions

We analyzed mitral valve tissue obtained from 4 patients with a mitral valve prolapse, a common acquired disorder (Figure 6). Mitral valve leaflets show typical fibromyxoid lesions with high cellular density at the valve surface (37.5±6.8 cells/0.01 mm²), sub-endothelium area (25.4±3.4 cells/0.01 mm²), and into the spongiosa layer (26.2±3 cells/0.01 mm²) (Figure 6). Surprisingly, despite the important cellular density, only few nuclei are Ki-67 positive (2.6±0.4% in MV) indicating a low rate of cell proliferation in valve lesions (Figure 6D). Interestingly, the cellular areas of the sub-endothelium surface and the spongiosa layer are densely populated with $CD34^+$ cells (48.0±1%) (Figures 6E/G). Expression of 5-HT_{2B} receptor is found in endothelial cells (at the valve surface) but also inside the valve lesions (40.2±4.2% of 5-HT_{2B}R positive cells) (Figures 6F/G).

We hypothesized that the large number of $CD34^+$ cells identified in myxomatous lesions, could be precursors of the endothelial lineage. To confirm this hypothesis, we used flow cytometry to profile the distribution of the double surface markers: PECAM1 (CD31) and CD34. After collagenase treatment, 59.8% mitral valve cells were $CD34^+$. Among the $CD34^+$cell population, only 12% were $CD31^+$ (Figures 7A/B). The CD34/CD31 labeling identified 3 major cell populations i.e. $CD34^+/CD31^-$, $CD34^+/CD31^+$ and $CD34^-/CD31^-$. In these three populations, we investigated by RT-qPCR the mRNAs expression of the HTR_{2B} and HTR_{2A}, and the endothelial nitric oxide synthase (*NOS3*). Interestingly, *NOS3* mRNA was expressed in all $CD34^+$ but not $CD34^-$ cells, supporting the endothelial lineage commitment of $CD34^+$ cells. Moreover, these $CD34^+$ cells express both HTR_{2A} and HTR_{2B} mRNA subtypes (Figure 7C). In isolated valve cells, using double immunocytochemical staining with anti-5-HT_{2B} receptors and anti-CD34 antibodies, we confirmed at the protein level that all $CD34^+$ cells express 5-HT_{2B} receptors (Figure 7D). To confirm the endothelial commitment of the $CD34^+$ cell population, we used magnetic cell sorting (MACS) with a CD34 antibody conjugated to magnetic beads. We obtained a homogenous cell population with more than 95% $CD34^+$ cells. Among this population, the co-labeling using another anti-CD34 and an anti-CD309 (VEGFR2) antibodies revealed about 80% double positive cells in mitral valves (Figures7E/F). These results confirm the endothelial lineage of the $CD34^+$ valvular cells and support that endothelial progenitor cells, expressing both 5-HT_{2A} and 5-HT_{2B} receptors, contribute to cardiac valve remodelling in human mitral valve prolapse.

Discussion:

The present work demonstrates that (i) cardiac mitral valve remodelling occurs after chronic administration of nordexfenfluramine (NdF) and is characterized by segmental increased valve thickness and endothelial cells density with no change in interstitial cell number, (ii) lesions display low proliferation (Ki-67) but numerous $CD34^+$ cells, (iii) both 5-HT_{2A} and 5-HT_{2B} receptor blockade by antagonists or in transgenic $Htr_{2B}^{-/-}$ or $Htr_{2A/2B}^{-/-}$ mice successfully prevent NdF-induced valve lesions, (iv) restricted ablation of 5-HT_{2B} receptors to BM prevents NdF-induced valve lesions, and (v) upon acute NdF exposure, BM mobilization of $CD34^+/CD31^+$ precursors is dependent on 5-HT_{2B} receptors. Moreover, the analysis of human mitral valve prolapsed, showing spontaneous degenerative lesions highlights the presence of non-proliferating $CD34^+/CD309^+/NOS3^+$ endothelial progenitors expressing 5-HT_{2B} receptor.

5-HT_{2B} receptors have been implicated in the fibrosis of various organs (Maroteaux et al., 2017). In the liver, 5-HT_{2B} receptor genetic suppression and antagonists limit CCL4-induced fibrogenesis by blocking TGF-◘ secretion by stellate cells (Ebrahimkhani et al., 2011). Similarly, in the skin, pharmacological inactivation of 5-HT_{2B} receptors reduces dermal fibrosis in models where a contribution of platelet's 5-HT is highly suspected (Dees et al., 2011). Nevertheless, fibrosis side effects induced by 5-HT_{2B} receptors stimulation were more frequently reported in lung and cardiac valve tissues. The hepatic deethylated dexfenfluramine metabolite, NdF, shows a high affinity at the 5-HT_{2B} receptor (Ki= 11.2 ± 4.3nM), 5-HT_{2C} receptor (Ki = 324 ± 7.1nM) and a lower affinity towards the 5-HT_{2A} subtype (Ki = 1516 ± 88nM) (Rothman et al., 2000). Given chronically to mice, this compound induced VHD typical of the ergot derivatives-induced valve lesions. **NdF induced non reproducible lesions of the aortic valve in this model. Species differences, hemodynamics and/or embryologic origin of valve cells could explain some differences related to the response of these two valves to drugs.** Here we show that these lesions appear following mainly 5-HT_{2B} receptors stimulation and partially 5-HT_{2A} receptors as demonstrated by antagonist treatments (SB204741, SB206553, sarpogrelate and ritanserin) and transgenic mice experiments ($Htr_{2B}^{-/-}$ and $Htr_{2A/2B}^{-/-}$). These data confirm previous results showing that cyclic stretch in porcine valve cusp increases proliferation and extracellular matrix remodelling through upregulation of 5-HT_{2A} and 5-HT_{2B} receptors mRNAs (Balachandran et al., 2011) and emphasize that both receptors are required for VHD. A small persisting increased mitral valve thickness was observed in $Htr_{2A}^{-/-}$ transgenic mice. Taking into account that the same endothelial cellularity was measured in

Htr$_{2A}^{-/-}$ transgenic mice treated or not by NdF, it is possible that a local 5-HT$_{2B}$ compensatory overexpression could contribute to collagen synthesis and extracellular matrix remodelling in these particular animals (Balachandran et al., 2012). Some authors suggested that fenfluramine effects could be, at least in part, mediated by peripheral and central 5-HT release following direct activation of SERT (Rothman et al., 2010). This endogenous locally released 5-HT could explain non 5-HT$_{2B}$-mediated effects in valve tissue. Nevertheless, in our study, the massive peripheral 5-HT depletion induced by pCPA failed to prevent NdF-induced valve leaflets remodelling, indicating that peripheral 5-HT is not necessary for NdF-induced mitral valve injuries, and that the direct 5-HT$_{2A/2B}$ receptor stimulation by NdF is sufficient to trigger valve remodelling.

In this study, we show in mice that valve lesions are made by numerous CD34^{+} cells confirming previous studies showing that hematopoietic stem cells contribute to adult valve fibroblast population (Visconti et al., 2006). Serotonin can promote the differentiation of progenitor cells (Hirota et al., 2014). These data support the idea that 5-HT through 5-HT$_{2B}$ receptor stimulation could induce maturation of progenitor cells in the valve tissue and/or their mobilization from the BM followed by their recruitment into the valves. We investigated the last hypothesis in NdF mouse model.

To confirm the BM origin of progenitor cells and the role of 5-HT$_{2B}$ receptors, we performed *Htr$_{2B}^{+/+}$* and *Htr$_{2B}^{-/-}$* BM transplantation in wild-type mice. The absence of 5-HT$_{2B}$ receptors in BM prevented NdF-induced mitral lesions. This result attests of the crucial contribution of BM derived endothelial progenitors in mitral valve tissue remodelling and firmly demonstrates the role of 5-HT$_{2B}$ receptors in this process triggered by serotonergic agonists. In addition, this work makes a parallel with pulmonary hypertension in which a similar mechanism involving BM progenitors was previously demonstrated (Launay et al., 2012). **The contribution of circulating cells originating from the bone marrow to fibrosis has been suggested in tumours (Quante et al., 2011) but also in a non-tumour tissue such as the liver (Castillho-Fernandes et al, 2011; Abe et al., 2001). In this last tissue myofibroblasts originate at least in part from cells coming from the bone marrow (Brenner et al., 2012).**

Migration of BM-derived endothelial progenitors can be suspected from previous studies. Vaturi et al (Vaturi et al., 2011) observed a reduction of blood endothelial progenitors in patients with aortic stenosis that was interpreted as a reparation defect but that could be due to a massive homing of these cells in the injured tissue. Similarly, in severe aortic stenosis, blood endothelial progenitors decrease under the control level (Matsumoto et

al., 2009). Serotonergic stimulation could favour mobilization and/or recruitment of endothelial progenitors as reported in an accelerated mitral valve bioprosthesis degeneration induced by benfluorex, a NdF precursor (Ayme-Dietrich et al., 2012). The non-cellularized collagen matrix was rapidly colonized and developed typical myxomatous lesions. In our work, the acute NdF administration rapidly triggered the increase of $CD34^+/CD31^+$ cells in BM and blood. These cells were released from the BM matrix and migrated after cytoskeleton activation. The NdF-induced blood mobilization from BM was prevented in $Htr_{2B}^{-/-}$ mice. Both of these effects require 5-HT_{2B} receptor since they were fully prevented in Htr_{2B} knockout animals. Interestingly, BMPR2, a TGF-β1 high affinity receptor, mutations are associated with heritable pulmonary arterial hypertension (International PPH Consortium et al., 2000)(Deng et al., 2000). Mice bearing the R899X mutation in the *BMPR2* gene (loss of function) develop spontaneous pulmonary hypertension that is prevented by the serotonin 5-HT_{2B} receptor antagonist, SB204741 (West et al., 2016) or by transplantation of WT BM (Yan et al., 2016). Similarly, animals lacking BPMR2 in endothelial progenitors develop a valvulopathy that closely mimics the one we obtained following NdF infusion (Beppu et al., 2009). This common mechanism of 5-HT_{2B}R mediated bone-marrow mobilization of endothelial progenitors could explain why valvulopathies are frequently associated with pulmonary hypertension. Therefore, we propose that 5-HT_{2B} receptor by regulating BMPR2/Src signaling regulates cytoskeletal genes and function in endothelial progenitor cells, leading to their mobilization from the BM and their subsequent recruitment in pulmonary vessels and cardiac valves.

In human degenerated valves, 40% of all cells are expressing the glycoprotein CD34, a progenitor cell marker. Stem cells ($CD34^+$, $CD133^+$ and $CD45^+$) residing in the BM niche give rise to a population of cells that differentiate into a progenitor subtype ($CD34^+$, $CD133^+$ and $VEGFR2^+$) considered as endothelial (Balaji et al., 2013) or haematopoietic because it lacks vessel forming activity (Case et al., 2007). The CD34 transmembrane molecule is expressed by precursors of both endothelial cells and fibroblasts (Lanza et al., 2001). Few reports described a $CD34^+$ labeling in the spongiosa and fibrosa layers of the valves (Barth et al., 2005) and assumed that it corresponds to mesenchymal cells proliferating in the tissue. **In the same paper, these authors also analysed 10 so-called normal mitral valves obtained from 41 to 85-year-old patients deceased from non-cardiovascular causes. Immunohistochemistry identified CD34+ cells in the *spongiosa*. Their photomicrographs is also showing a positive staining at the endothelial surface arguing in favour of a recruitment followed by migration in the *spongiosa*. So, it seems that**

CD34+ cells are present in the "normal" mitral tissue and their number increased in the pathological state." The low rate of cell proliferation supported that the high cell content was due to migration instead of local proliferation. Based on CD34 expression, we identified two main valve cell populations, from collagenase-treated mitral samples, i.e. $CD34^+/CD31^-$ and $CD34^-/CD31^-$. The $CD34^+/CD31^+$ cells probably correspond to differentiating endothelial cells (Murohara, 2001). The most prevalent $CD34^+/CD31^-$ population was made by 60-85% of cells that co-express CD309/VEGFR2 and NOS3 supporting that they are endothelial cell progenitors (Schatteman et al., 2007). Nevertheless, the definition of an endothelial progenitor is still matter of debate and is used to describe cell types included in the "proangiogenic hematopoietic progenitor cells" family (Asahara et al., 1997). Some of these cells are involved in neoangiogenesis but others lack proangiogenic capabilities (Wara et al., 2011) and would be involved in tissue repair. Our immunohistochemistry experiments revealed α-SMA^+ cells around cushions made of $CD34^+$ cells at sites of myxomatous lesions. In these $CD34^+$ cells, we found expression of 5-HT_{2B} receptors both at mRNA and protein levels, leading to the conclusion that human degenerated mitral valves are made of numerous endothelial progenitors sharing 5-HT_{2B} receptor expression. These cells could transdifferentiate to α-SMA^+ myofibroblasts (Wylie-Sears et al., 2011).

In conclusion, the present work demonstrates that 5-HT and 5-HT_{2B} receptors are involved in early processes of valve remodelling. The cellular mechanism involves BM mobilization of endothelial progenitors expressing the 5-HT_{2B} receptor. These cells are then recruited in the valve where they later undergo differentiation to myofibroblast or other cell types to propagate valve lesions to an irreversible step. This work opens new fields with potential clinical impacts. **First, it drives to the idea that the screening of drugs and their metabolites acting on BM-derived endothelial progenitors via 5-HT_{2B} receptors or other targets could be useful to predict valve and pulmonary side effects. Such screening would be done in preclinical models (Whitebread et al., 2016).** Second, it opens the way to search for serotonergic antagonists acting at 5-HT_{2A} and/or 5-HT_{2B} receptors to protect, reverse or slow down early steps of cardiac valve degeneration in patients at risk.

Author contributions

E.A.D. performed and analyzed the molecular and biochemical experiments of the human part of the study, analyzed histological human samples, performed and analyzed study of blood mobilization progenitors in mice; R.L. performed mice experimental procedures and treatment, analyzed histological mice samples, performed and analyzed echocardiography experiments; C.D.T. contributed to mice experimental procedures and treatment; S.D.S analyzed histological graft mice samples; C.E. gave technical support for flow cytometry sorting and analysis; B.H. and C.G. provided advice for ritanserin dose using platelets aggregation tests; H.R. and J.S. prepared all humans and mice samples for histological analysis; E.Q. prepared transfected cells for western blot controls; S.B. prepared two groups of transplanted mice; F.D. and N.F. provided advices for progenitors mobilization; B.G. trained E.A.D. and R.L. for histological analysis and provided advice; J.P.M. provided human resected valve samples; F.C. and O.H. designed, performed and analyzed study of BM progenitors in mice; E.A.D., R.L., L.Ma and L.Mo designed the study and wrote the manuscript.

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Disclosures:

The authors declare no competing financial interests.

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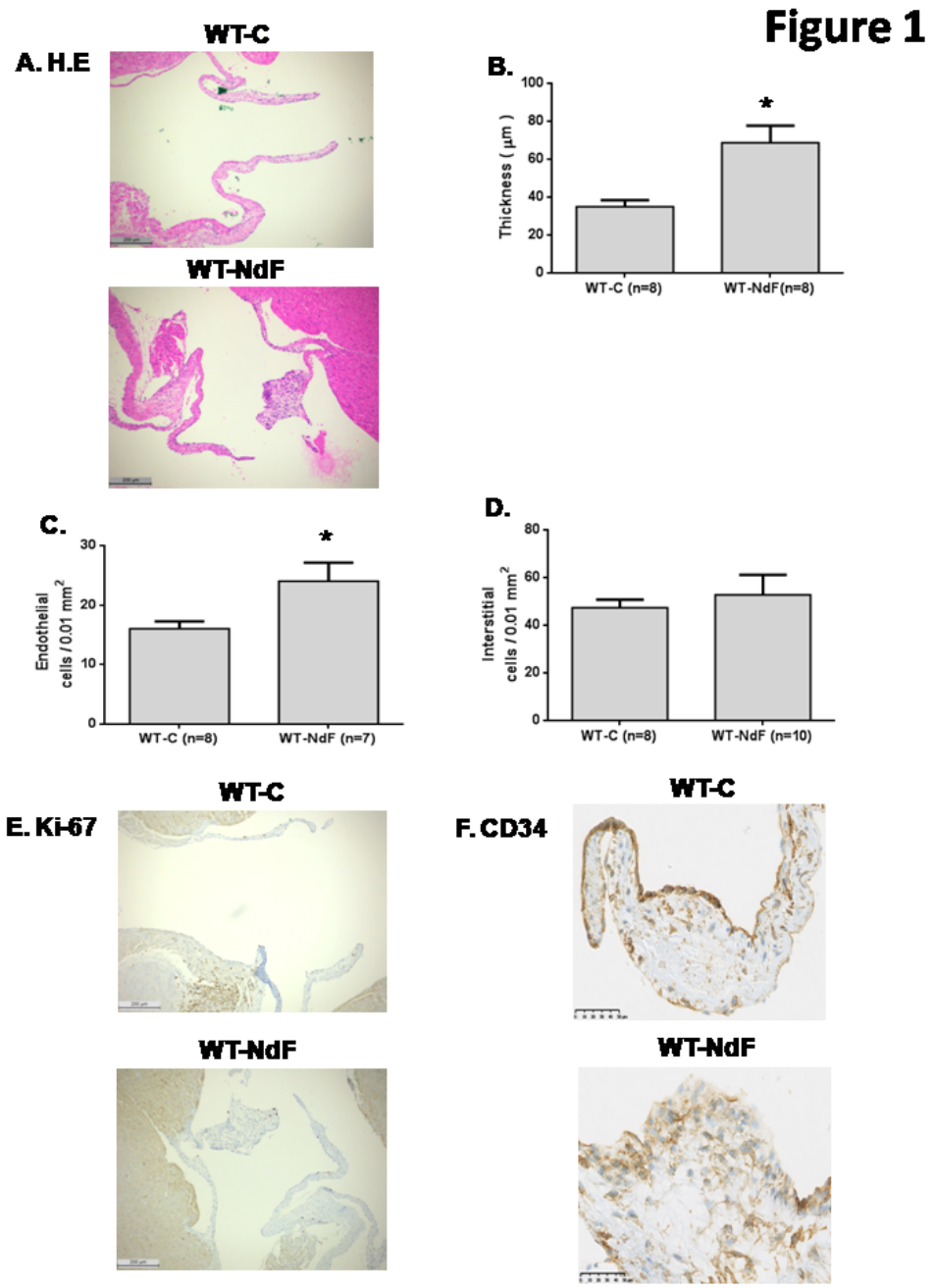

Figure 1: Chronic nordexfenfluramine administration induces mitral valve remodelling in mice

The mitral valve architecture has been assessed in wild-type control mice or treated for 28 days with NdF (1 mg/kg/d). A) H.E = hematoxylin eosin stained slides were used to quantify B) Thickness, C) Endothelial cellularity and D) Interstitial cellularity in WT-C (wild-type control) and WT-NdF (wild-type nordexfenfluramine) (using the same grid-line reticle) (scale bar = 200 µm). Immunohistochemical staining (scale bar = 50 µm) for Ki-67 (E) and CD34 (F); arrows indicate positive nuclei for Ki-67. Values are means ± SEM (n=number per group). Statistical significant difference by unpaired t-test versus control is indicated by *$p<0.05$.

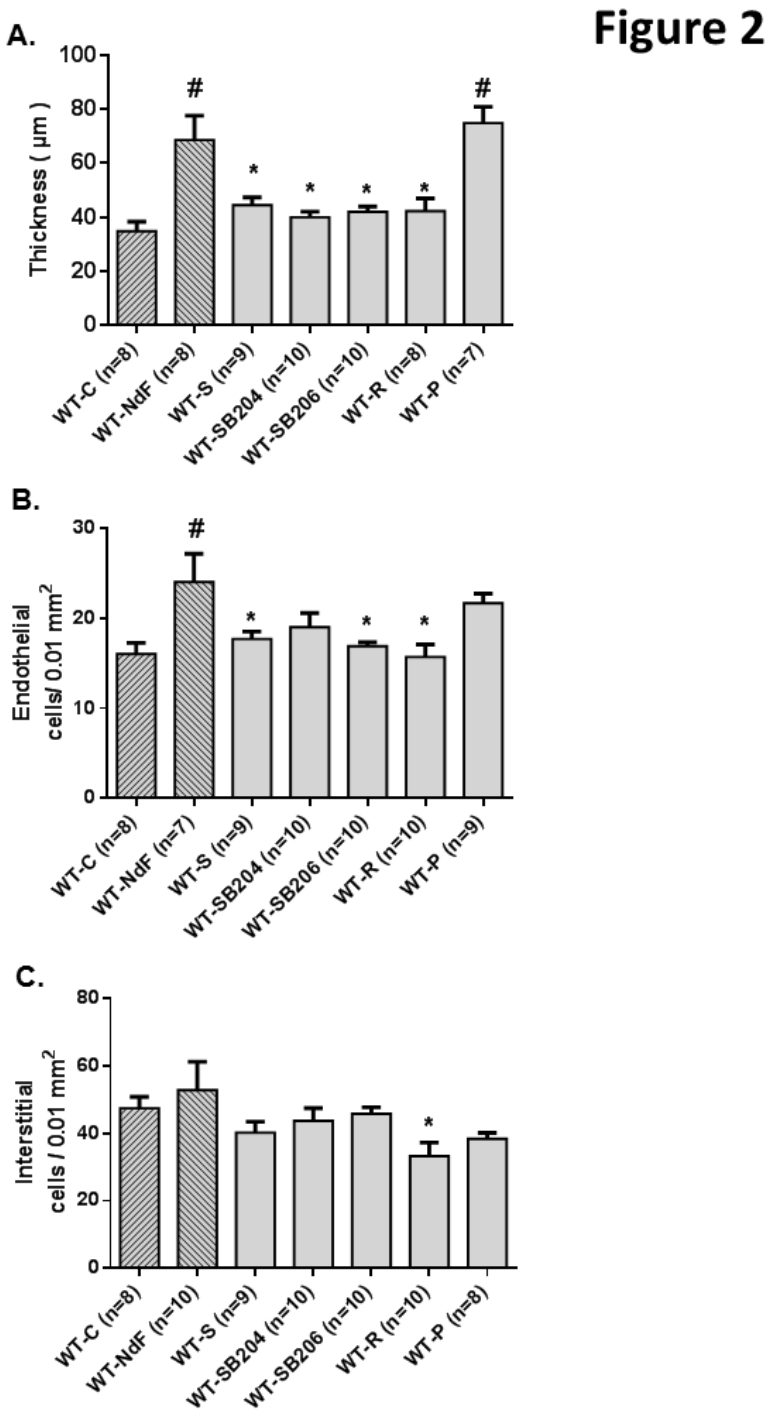

Figure 2: Effect of serotonergic system blockade in wild-type mitral valves
The mitral valve architecture has been determined in NdF-treated mice in the presence of various blockers. A) Thickness, B) Endothelial cells and C) Interstitial cells counting in wild-type treated for 28 days with NdF (1 mg/kg/d) (WT-NdF), or NdF with sarpogrelate (2 mg/kg/d) (WT-S), or NdF with SB204741 (1 mg/kg/d) (WT-SB204), or NdF with SB206553 (1 mg/kg/d) (WT-SB206), or NdF with ritanserin (2 mg/kg/d) (WT-R), and NdF with para-cholorophenylalanine (100 mg/kg/d) (WT-P); (# $p < 0.05$ vs. WT-C; *$p<0.05$vs. WT-NdF) statistical significance (ANOVA, Dunnett's post hoc tests), n=number of animals per group.

Figure 3

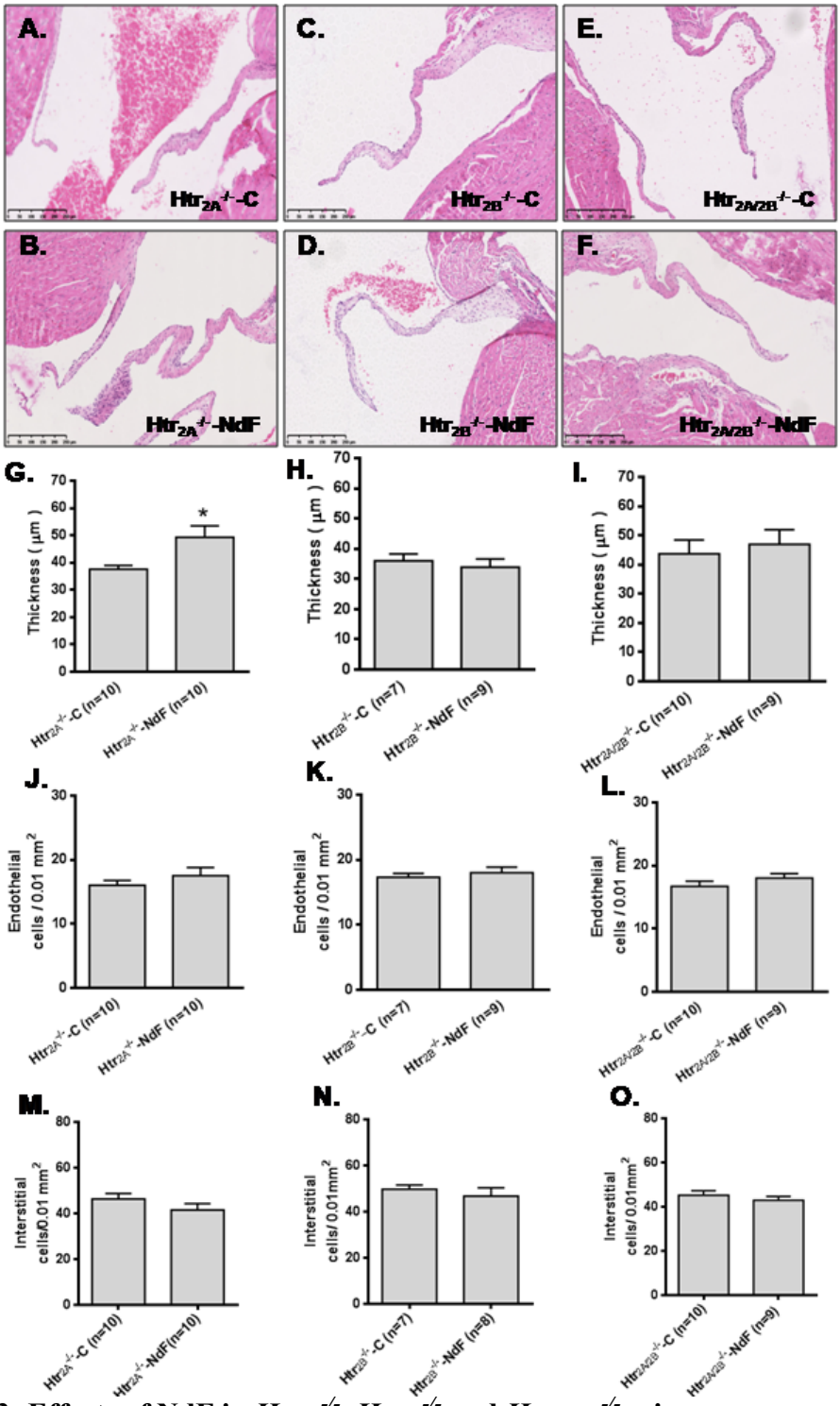

Figure 3: Effects of NdF in *$Htr_{2A}^{-/-}$*, *$Htr_{2B}^{-/-}$* and *$Htr_{2A/2B}^{-/-}$* mice

A. to F. correspond to typical histological aspects of the different *Htr_2* knockout mice lines (*$Htr_{2A}^{-/-}$, $Htr_{2B}^{-/-}$, $Htr_{2A/2B}^{-/-}$*) (controls and treated for 28 days with NdF (1 mg/kg/d)) stained with hematoxylin eosin (scale bar = 250 µm); G., H. and I. represent measured thickness; J., K. and L. represent endothelial cells count and M., N. and O. represent counted interstitial cells count. Values are means ± SEM (n=number of animals per group). Statistical significant difference by unpaired t-test versus knockout control is indicated by * $P<0.05$.

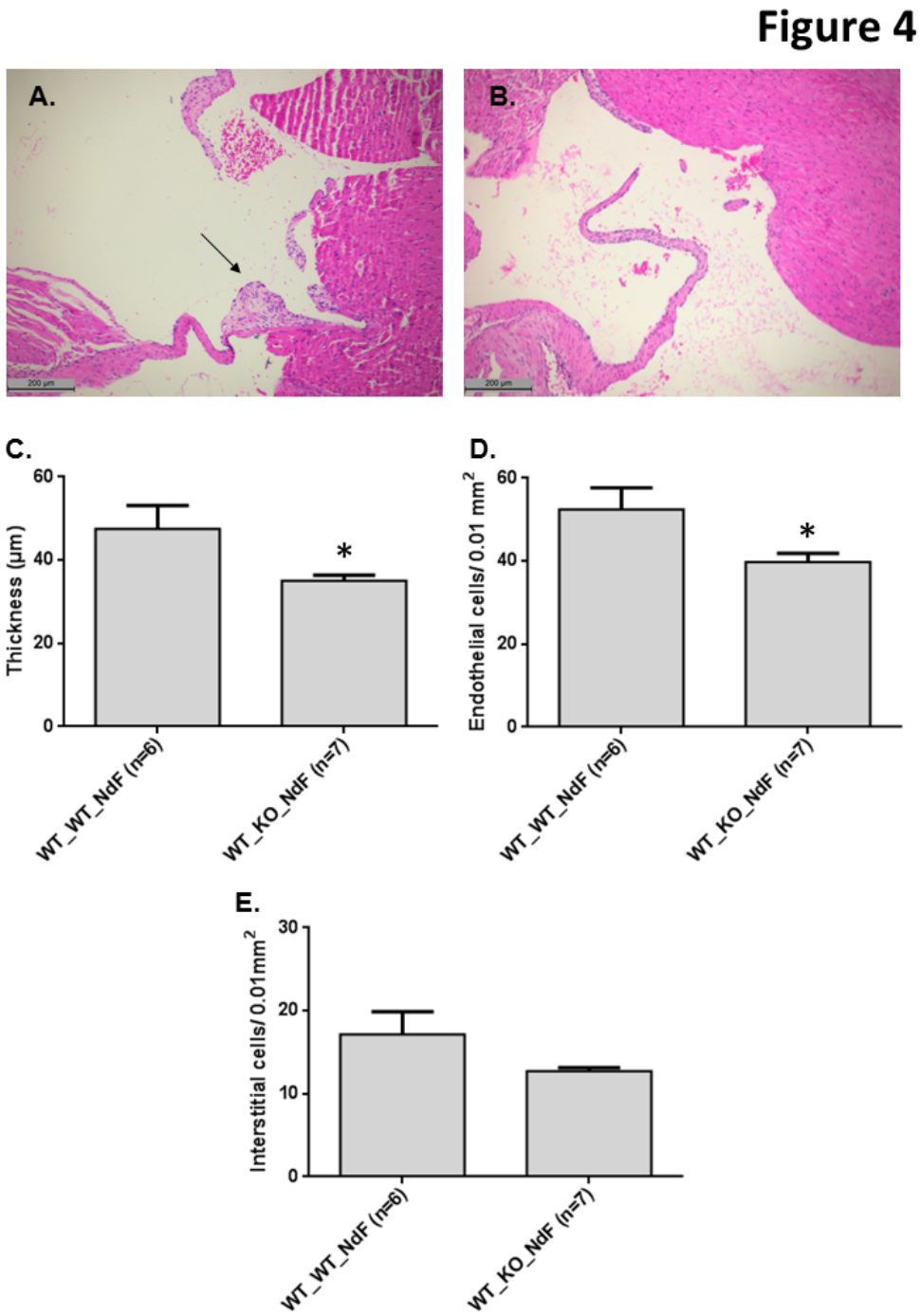

Figure 4: Mice with restricted ablation of 5-HT_{2B} receptors to BM are resistant to NdF-induced valvulopathy

Reduced mitral valve thickness and endothelial cellularity after chronic NdF administration (1mg/kg/day, during 28 days, subcutaneously) in lethally irradiated mice transplanted with BM cells from $Htr_{2B}^{-/-}$ (B: WT_KO_NdF) compared to mice transplanted with wild-type BM (A: WT_WT_NdF) (scale bar = 200 µm). Mitral valve thickness (C), endothelial (D) and interstitial cellularity (E) of transplanted mice exposed to NdF. Values are means ± SEM (n=number of animals per group). Statistical significant difference by unpaired t-test versus mice transplanted with wild-type BM is indicated by * $P<0.05$.

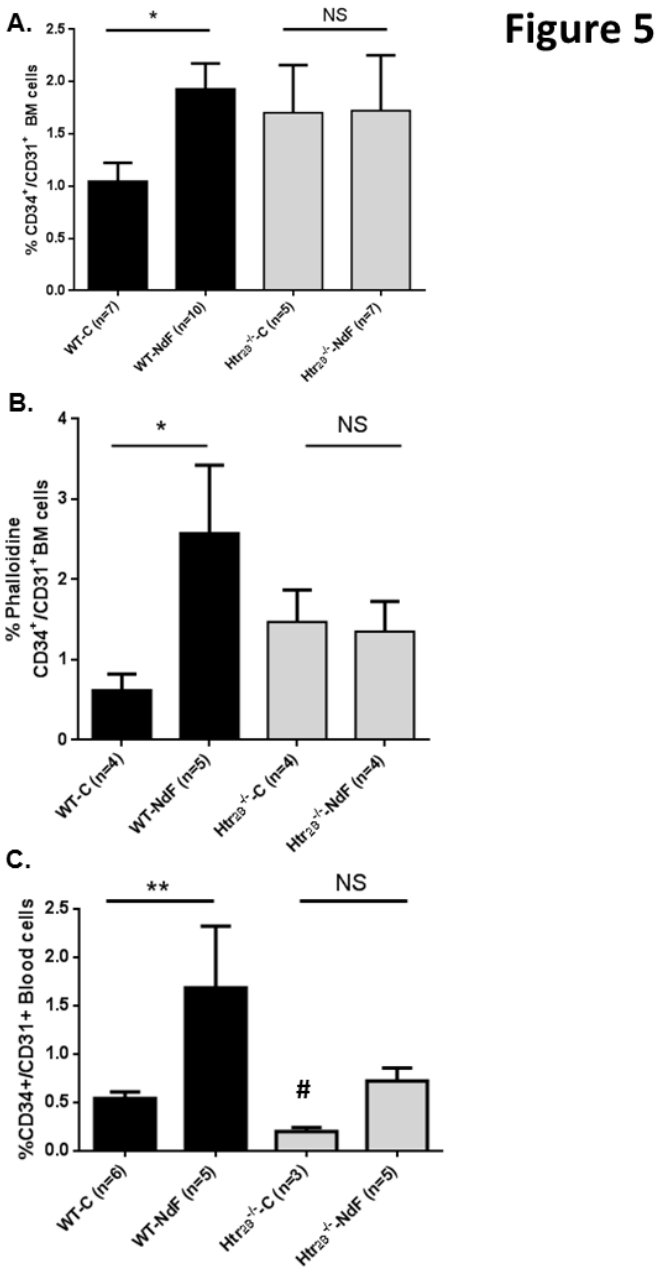

Figure 5: 5-HT$_{2B}$ receptor stimulation triggers the mobilization of CD34$^+$/CD31$^+$ cells from BM into the blood

After a single subcutaneous NdF injection (3 mg/kg), the number of free CD34$^+$/CD31$^+$ cells (corresponding to endothelial progenitors, flushed from the femur and tibia, and not sequestered in osteoblastic niches), increased significantly after 15 minutes in the BM of WT mice, but not in the BM of *Htr$_{2B}$*$^{-/-}$ mice, as shown by the quantification in percent of total BM cells (A). In the BM of WT mice, following the NdF injection an increase in actin polymerization was observed in CD34$^+$/CD31$^+$ cells, whereas no change was noticed in cells of *Htr$_{2B}$*$^{-/-}$ mice (B). Simultaneously, in the WT mice, the blood single NdF injection induced a significant increase in endothelial progenitors, whereas the amount of these progenitors in the blood of *Htr$_{2B}$*$^{-/-}$ mice did not change (C). These data suggest that endothelial progenitors mobilization by NdF is a 5-HT$_{2B}$ receptor dependent mechanism and originates from BM; values are means ± SEM (n=number per group), statistical difference tested by non parametric Mann Withney test is shown by * P <0.05 and ** p<0.01 **(#: p<0.05 vs WT-C)**.

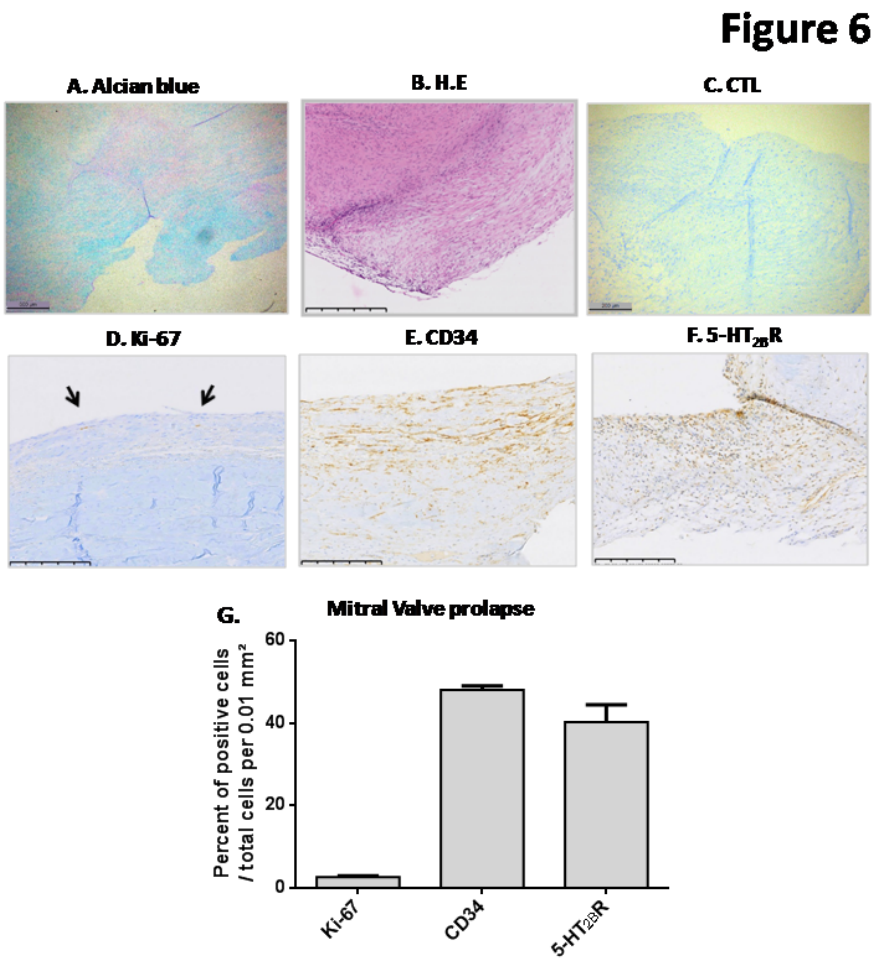

Figure 6: Human mitral valve lesions contain numerous 5-HT_{2B} positive cells and show a low proliferation rate

(A) Alcian blue staining shows severe deposition of glycosaminoglycans (blue) in the spongiosa and disrupted, disorganized collagen (pink) (scale bar = 500 µm). (B) Hematoxylin and eosin (HE) staining shows cell rich areas (scale bar = 500 µm). In valve lesions, immunostaining for Ki-67 reveals a low rate of proliferation (D); arrows indicate positive nuclei for Ki-67 (scale bar = 250 µm). Immunostaining for CD34 (E) and 5-HT_{2B} receptors (F) revealed cells located in the surface and under the surface of valve leaflets (F) (scale bar = 250 µm). (G) Immunostaining for Ki-67, CD34 and 5-HT_{2B} receptors were quantified (using the same grid-line reticle, focusing valvular lesions) and shown as the percentage of cells located in valvular lesions; Values are means ± SEM, obtained in 3 distinct sites from 4 human mitral valves. Labeling control (C) represents same mitral valve tissue processed with same method but without primary antibodies.

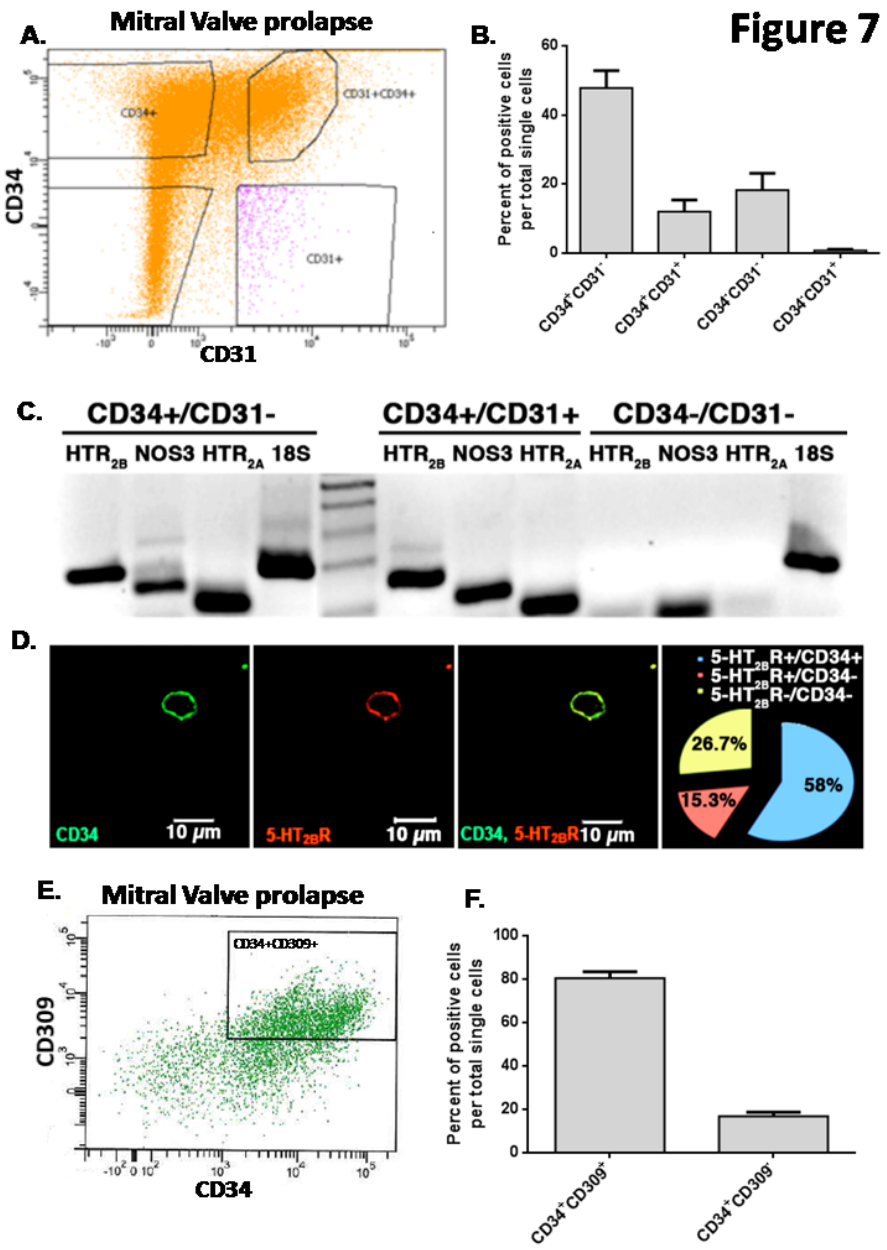

Figure 7: 5-HT$_{2B}$ positive cells extracted from human mitral valve lesions are endothelial progenitors

In mitral valve prolapse (n=8), after collagenase treatment, 60% of single cells sorted by FACS are CD34^{+}(B). Values are means +/- SEM and representative data from one experiment is displayed (A). (C) Expression of *HTR$_{2A}$*, *HTR$_{2B}$*, *NOS3* and *18S* RNAs isolated from three major valvular cell populations were analyzed by RT-qPCR, showing the expression of *HTR$_{2A}$* and *HTR$_{2B}$* mRNA in CD34^{+} cells. (D) Double staining of mitral valvular cells (n=3) with antibodies directed against CD34 (green) and 5-HT$_{2B}$ receptors (5-HT$_{2B}$R-red). The merged pictures show co-localization of CD34 and 5-HT$_{2B}$ receptors proteins and quantification shows about 60% of co-staining. Size bar = 10µm. Flow cytometric analysis of CD34 and CD309 expression after magnetic sorting of CD34^{+} cells isolated from mitralvalve prolapse (n=3), after collagenase treatment. The CD34 and CD309 double positive cells were counted, after MACS isolation (F). Values are means +/- SEM and representative data from one experiment is displayed (E).

Table 1: Effects of nordexfenfluramine on hemodynamic and morphometric parameters in wild-type mice.

	Day 1			**Day 28**		
Parameters	**WT-C (n=10)**	**WT-NdF (n=10)**	p value	**WT-C (n=10)**	**WT-NdF (n=10)**	p value
BW, mg	30±0.6	28±0.6	ns	30±0.9	29±0.9	ns
HW/BW, mg/g	-	-	-	5.38±0.07	5.42±0.09	ns
SAP, mmHg	96±2.5	101±2	ns	123±1.5	126±2	ns
HR, bpm	480±20	441±10	ns	575±17	570±13	ns
PWT, mm	0.76±0.02	0.77±0.02	ns	0.73±0.02	0.65±0.04	ns
SWT, mm	0.79±0.02	0.78±0.02	ns	0.78±0.02	0.67±0.03	ns
LV mass, mg	103±8	97±4	ns	103±8	103±9	ns
LV mass index, mg/g	3.43±0.20	3.56±0.23	ns	3.33±0.29	3.76±0.13	ns
EDLVD, mm	3.77±0.19	3.68±0.14	ns	4.10±0.09	4.28±0.08	ns
ESLVD, mm	2.22±0.16	2.55±0.11	ns	2.23±0.11	2.5±0.11	ns
SF, %	38±2.5	40±2	ns	38±1.5	42±1.5	ns
CO, ml/min	50±5.5	46±5.5	ns	55±3.5	53±2.5	ns

Values are means ± SEM,- represents undefined parameters, **p** = statistical significance (unpaired t-test), **ns** = not significant, **n** = number per group, **NdF** = nordexfenfluramine, **WT-C** = wild-type control group, **WT-NdF** = wild-type nordexfenfluramine treated group, **BW** = body weight, **HW** = heart weight, **SAP** = systolic arterial pressure, **PWT** = posterior wall thickness, **SWT** = septum wall thickness, **HR** = heart rate, **LV** = left ventricle, **EDLVD** = end diastolic left-ventricular diameter, **ESLVD** = end systolic left-ventricular diameter, **SF** = shortening fraction, **CO** = cardiac output.

Table 2: Blood 5-HT and urine 5-HIAA in wild-type mice.

	WT-C (n=9)	**WT-NdF (n=10)**	**WT-S (n=9)**	**WT-SB204 (n=10)**	**WT-SB206 (n=10)**	**WT-R (n=10)**	**WT-P (n=9)**
Blood 5-HT (µg/L)	1930±162	1801±81	1646±163	2160±93	2213±89	1877±76	596±48#*
Urine 5-HIAA (µmol/L)	36±7	64±5#	39±4*	46±6	75±10#	44±5	16±3*

Values are means ± SEM, ($p<0.05$) statistical significance (ANOVA, post hoc Dunnett's multiple comparison test; # vs. WT-C or * vs. WT-NdF).
Seven groups of WT mice: Wild-type mice infused with vehicle (WT-C), or treated for 28 days with NdF (WT-NdF), or NdF with sarpogrelate (WT-S), or NdF with SB204741 (WT-SB204), or NdF with SB206553 (WT-SB206), or NdF with ritanserin (WT-R), and NdF with para-cholorophenylalanine (WT-P);

Table 3: Blood 5-HT and urine 5-HIAA in transgenic mice.

	***Htr*$_{2A}^{-/-}$**		***Htr*$_{2B}^{-/-}$**		***Htr*$_{2A/2B}^{-/-}$**	
	C (n=9)	NdF (n=11)	C (n=7)	NdF (n=9)	C (n=10)	NdF (n=9)
Blood 5-HT (µg/L)	1750±69	1774±102	1656±109	1834±79	2144±53	1795±75*
Urine 5-HIAA (µmol/L)	52±4	51±7	74±7	57±6	43±4	85±5*

Values are means ± SEM, *($p<0.05$) statistical significance (unpaired t-test); C = control (untreated mice).