RESEARCH ARTICLE

Genetic ablation of serotonin receptor 2B improves aortic valve hemodynamics of Notch1 heterozygous mice in a high-cholesterol diet model

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Abstract

Calcific aortic valve disease (CAVD) is a deadly disease that is rising in prevalence due to population aging. While the disease is complex and poorly understood, one well-documented driver of valvulopathy is serotonin agonism. Both serotonin overexpression, as seen with carcinoid tumors and drug-related agonism, such as with Fenfluramine use, are linked with various diseases of the valves. Thus, the objective of this study was to determine if genetic ablation or pharmacological antagonism of the 5-HT2B serotonin receptor (gene: Htr2b) could improve the hemodynamic and histological progression of calcific aortic valve disease. Htr2b mutant mice were crossed with Notch1+/− mice, an established small animal model of CAVD, to determine if genetic ablation affects CAVD progression. To assess the effect of pharmacological inhibition on CAVD progression, Notch1+/− mice were treated with 5-HT2B receptor antagonist SB204741. Mice were analyzed using echocardiography, histology, immunofluorescence, and real-time quantitative polymerase chain reaction. Htr2b mutant mice showed lower aortic valve peak velocity and mean pressure gradient—classic hemodynamic indicators of aortic valve stenosis—without concurrent left ventricle change. 5-HT2B receptor antagonism, however, did not affect hemodynamic progression. Leaflet thickness, collagen density, and CAVD-associated transcriptional markers were not significantly different in any group. This study reveals that genetic ablation of Htr2b attenuates hemodynamic development of CAVD in the Notch1+/− mice, but pharmacological antagonism may require high doses or long-term treatment to slow progression.

Introduction

The aortic valve (AV) controls unidirectional oxygenated blood flow from the left ventricle into the systemic circulation. Comprised of a compliant tri-leaflet structure, this valve permits unimpeded blood flow during systole yet forms a strong coaptation to prevent regurgitation during diastole. Calcific aortic valve disease (CAVD) is characterized by the accumulation of
stiff fibrotic and/or calcific deposits on the leaflet cusps which can impede blood flow, causing aortic stenosis (AS) and leading to heart failure and death if left untreated [1]. AS is one of the deadliest cardiovascular diseases and is becoming an increasing public health concern in aging populations [1]. The disease is estimated to be present in 1.7% of patients over the age of 65 and 3.4% over the age of 75 [2, 3]. Severe AS is particularly deadly, carrying a two-year survival rate as low as 22% in those with the most highly impeded blood flow [4]. Estimates from 2017 indicate AS accounts for approximately 17,000 deaths in North America per year [5].

The molecular pathophysiology of CAVD is poorly understood. Originally thought to be a passive, degenerative process, research in recent decades has revealed a complex, actively regulated biological process leading to disease [6]. Roles have been defined for pathological activation of resident cells including valvular endothelial cells (VECs) and valvular interstitial cells (VICs). VICs broadly contribute to CAVD through two mechanisms: dystrophic and osteogenic calcification. Dystrophic calcification is characterized by a shifting of VICs from a quiescent phenotype to a synthetic and contractile phenotype with upregulation of alpha smooth muscle actin (αSMA) and cadherin-11 [7]. In osteogenic calcification, VICs can also transition to an osteoblast-like phenotype characterized by upregulation of Runt-related transcription factor 2 (Runx2), alkaline phosphatase, and ectopic bone formation [7, 8].

There are currently no pharmaceutical options for slowing or treating CAVD. Surgical and transcatheter AV replacements can restore functionality; however, those suffering from late stage cardiovascular disease also often carry higher risk for complications from surgical interventions and many are left untreated [9]. Therefore, a pharmaceutical alternative would be of great benefit.

One of the earliest and best-documented mechanisms driving diseases of the heart valves is the serotonin signaling pathway. Endocrine diseases leading to overexpression of serotonin, such as carcinoid tumors, have been linked with multiple valvulopathies, and serotonin receptor agonists such as Fenfluramine (an anti-obesity drug marketed in the 1990s) have been clearly shown to exacerbate valve disease [10, 11]. Fenfluramine was found to specifically activate 5-HT$_{2B}$, the serotonin receptor 2B subtype [12, 13]. Signaling through 5-HT$_{2B}$ has been implicated in several small animal models of cardiovascular disease. Prior work has shown that activating the 5-HT$_{2B}$ receptor via selective agonism can lead to valve pathology in a rat model [14]. Additionally, 5-HT$_{2B}$ ablation in mice causes decreased ventricular mass and diminished systolic function [15]. 5-HT$_{2B}$ signaling has also been shown to be involved in isoproterenol and angiotensin II induced cardiac hypertrophy, primarily affecting myocardial fibroblasts [16, 17]. Studies in dogs show increased 5-HT$_{2B}$ gene expression in symptomatic myxomatous mitral valve disease and protein co-localizes with αSMA positive myofibroblasts [18]. Recent studies indicate 5-HT$_{2B}$ activation induces early hallmarks of pathological mitral valve remodeling which is attenuated by genetic knockout and receptor antagonism. Interestingly the phenomenon may be mediated by bone-marrow derived endothelial progenitor cells [19]. Our laboratory has previously shown that antagonism of 5-HT$_{2B}$ can reduce AV myofibroblast activation by repressing non-canonical TGF-β signaling in vitro [20], indicating that 5-HT$_{2B}$ may be a therapeutic target for CAVD.

These clinical and basic research findings led us to hypothesize that 5-HT$_{2B}$ may be a novel target for pharmacological treatment of CAVD. Therefore, we evaluated the impact of both genetic and pharmacological ablation of 5-HT$_{2B}$ in the well-established Notch1$^{+/−}$ mouse model of CAVD, based on the heritable form in patients with NOTCH1 mutations [21–25]. Briefly, we found that genetic ablation mitigated CAVD phenotypes, while pharmacological targeting was not efficacious.

Competing interests: The authors have declared that no competing interests exist.
Materials and methods

Mouse models

All animal procedures were approved and carried out in accordance with relevant guidelines and regulations established by the Institutional Animal Care and Use Committee at Vanderbilt University. Briefly, Notch1\(^{+/−}\) and Htr2b\(^{-/-}\) mice were crossed to create Notch1; Htr2b double mutant animals [26, 27]. In a separate cohort, Alzet minipumps (model 1004) delivering either the high-affinity 5-HT\(_{2B}\) antagonist SB204741 (Tocris Biosciences; 1 mg kg\(^{-1}\)d\(^{-1}\)) or vehicle (50% dimethyl sulfoxide [Sigma-Aldrich] and polyethylene glycol-400 [Fisher Chemical]) were implanted subcutaneously in Notch1\(^{-/-}\) mice at 4 months of age (Fig 1). SB204741 is a well-validated and specific 5-HT\(_{2B}\) receptor antagonist commonly used in pharmacological studies targeting this ligand [28, 29]. SB204741 dosing varies widely across the literature, but we use 1 mg kg\(^{-1}\)d\(^{-1}\) to allow for longer time course administration with good results [30].

All mice were fed a 1% cholesterol Western diet (TestDiet 5TJT) for a total of 3.5 months beginning at 2.5 months of age. Food and water were provided ad libitum. Mice were sacrificed at 6 months of age using carbon dioxide inhalation followed by cervical dislocation before harvesting biological samples for processing and analysis.

Echocardiography

*In vivo* transthoracic echocardiography was performed by the Vanderbilt Cardiovascular Physiology Core using a Vevo 2100 small animal imaging system. Mice were lightly anesthetized (average heart rate of 465 bpm) using isoflurane inhalation and placed supine on a heated stage in order to obtain a clear image of the aortic root. AV jet velocity profiles were estimated by aortic pulsed-wave Doppler imaging. A custom MATLAB script was implemented to determine the maximum systolic velocity and mean pressure gradient [31]. Three images were analyzed per mouse yielding a total of ~100 cardiac cycles that were quantified and averaged to reliably quantify AV hemodynamic characteristics.

Concurrently, parasternal short-axis M-mode imaging was performed to study the effects on cardiac function and left ventricular performance. Systolic and diastolic measurements were performed to obtain estimates of ejection fraction and ventricular mass. For each mouse, ~9 cardiac cycles were averaged to obtain representative cardiac function metrics. Electrocardiographic measurements were acquired while imaging to allow for cardiac cycle-based gating of measurements using R wave peaks.

Histological and immunofluorescence staining

Mice were euthanized at 6 months of age and aortic roots were immediately dissected and either flash frozen or embedded in optimal cutting temperature (OCT) compound. Embedded samples were stored at -80˚ C until they were cut at 10 μm serial sections in a -20˚ C cryostat. For all tissue analysis, 3–5 sections were sampled across separate regions of the valve to produce a representative summary of valve characteristics.

To assess microstructural differences between AVs from different groups Masson’s Trichrome staining (Sigma-Aldrich) was used according to manufacturer instructions. Briefly, nuclei (black) were highlighted with Weigert’s iron hematoxylin, muscle and cytoplasm (red) with Beibrich scarlet-acid fuchsin, and collagen (blue) with aniline blue post-phosphotungstic and phosphomolybdic acid wash; slides were treated with Bouin’s solution to improve color intensity. Slides were rinsed in acetic acid, dehydrated in progressively concentrated ethyl alcohol baths, cleared with xylene, and mounted in organic mounting media. Brightfield images were taken using the Nikon Eclipse E800 microscope under a 4X magnification objective.
To assess osteogenic calcification, Alizarin Red S staining was performed (Sigma-Aldrich). Fresh frozen sections were washed with 1X phosphate buffered saline and deionized water. Sections were then incubated for one hour in 14 mM Alizarin Red S solution. Sections were rinsed in water, then dipped 20 times each in sequential baths of acetone, 1:1 acetone/xylene solution, and xylene. Slides were then mounted in organic mounting media. Brightfield images were taken using a Nikon Eclipse E800 microscope under a 4x magnification objective.

Fig 1. The effects of genetic ablation and pharmacological inhibition of 5-HT\textsubscript{2B} on CAVD development were studied using a high-cholesterol diet Notch\textsuperscript{1+/-} mouse model. A. Mutant mice were aged to six months on high-cholesterol diet and doppler and M-mode echocardiography was utilized to determine hemodynamic phenotype in the valve and left ventricle. B. Notch\textsuperscript{1+/-} mice were aged to six months with SB204741 infusion between 4 and 6 months. Doppler and M-mode echocardiography were performed and 4 and 6 months. All mice were sacrificed at 6 months. Black bar = 1 month.

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To assess the level of myofibroblast activation or osteoblast-like phenotype induction between groups, sections were stained for αSMA and Runx2. The downstream effector of 5-HT2B activation, phospho-p44/42 mitogen-activated protein kinase (phospho-ERK1/2) was also stained. Sections were fixed and permeabilized in 4% paraformaldehyde/0.1% Triton-X solution. Non-specific binding was reduced by incubating in 10% bovine serum albumin in 1X phosphate buffered saline for one hour. Sections were then incubated in rabbit polyclonal anti-αSMA, rabbit monoclonal anti-Runx2, or rabbit monoclonal anti-phospho-ERK1/2 at 4˚C overnight (Abcam ab5694, 1:100; Cell Signaling Technology 12556, 1:100; Cell Signaling Technology 4370, 1:200). Slides were thoroughly washed the next day then incubated in goat anti-rabbit IgG Alexa Fluor 647 secondary antibody for 1 hour at room temperature (Thermo-Fisher A-21245, 1:1000). Slides were thoroughly washed then mounted with ProLong Gold Antifade with DAPI (Cell Signaling Technology 8961), sealed, and stored at -20˚ C. Fluorescent images were taken using an Olympus BX53 microscope equipped with a high resolution Qimaging Retiga 3000 camera under a 20X magnification objective.

Quantitative image analysis

Custom MATLAB scripts for quantitative image analysis were implemented to identify changes in leaflet composition and microstructure. Images from different samples were acquired with the same set of exposures and background levels in order to allow for quantification and comparison of image intensities.

For Masson’s Trichrome stained images, colorimetric segmentation in an HSL color space was performed in order to identify the area fractions of collagen (blue; H = 150˚-250˚, S = 0.1–1.0, L = 0.1–0.93) and cytoplasm/myocardium (red; H = 250˚-25˚, S = 0.1–1.0, L = 0.1–0.93) in each field of view [32]. Area fractions for each constituent were computed as the ratio of positive pixels to non-background pixels within the specified AV leaflet area.

For immunofluorescence images, co-staining of DAPI and either αSMA or Runx2 was imaged in separate fluorescent channels at the same location. Total valve leaflet area was estimated from DAPI images based on a manually defined leaflet boundary. Segmentation of individual nuclei was also performed [33]. Briefly, DAPI positive pixels were separated from the image background based on a fuzzy c-means segmentation, and individual nuclear boundaries were estimated using a modified watershed transform. Contacting nuclei were identified and split based on a concave object separation algorithm [34]. αSMA area fractions were then computed as the ratio of positive pixels to total leaflet pixels based on the defined leaflet boundary. Runx2 expression was defined based on the ratio of positive pixels to total pixels within each identified nuclear boundary where Runx2 positive nuclei were defined as having greater than 50% Runx2 stain coverage. Positive nuclei counts were normalized to the number of total nuclei. αSMA and Runx2 positive pixels were defined as those with intensity values greater than the median stain intensity value within the leaflet boundary.

Real-time quantitative polymerase chain reaction

Flash frozen aortic roots containing intact AVs were suspended in Trizol Reagent (Thermo-Fisher 15596026) and homogenized using a bead homogenizer (BioSpec Products). RNA was isolated using phenol-chloroform extraction as described based on manufacturer protocols. RNA purity and concentration were measured using the NanoDrop UV Spectrophotometer (ThermoFisher ND-ONE-W). cDNA was generated with the SuperScript IV First-Strand Synthesis System (ThermoFisher 18091150) based on manufacturer protocols. SYBR Green PCR Master Mix (ThermoFisher 4309155) was used to facilitate detection during target amplification. The CFX96 Real-Time PCR Detection System (Bio-Rad) was used to monitor fluorescent
intensity during amplification. cDNA was initially denatured at 95˚ C for four minutes, then
cycled 40 times through the following cycle: 10 sec 95˚ C, 10 sec at 55˚ C anneal, plate read. A
melt curve was generated after cycling by increasing temperature from 65 to 95˚ C at 0.5˚ C
for 5 sec and performing a plate read at each increment. Bio-Rad CFX Manager 3.1 software
was used to automatically calculate the cycle quantification value at which samples amplified
at a high enough value to be detected. Relative changes in transcript expression were deter-
mined by normalizing to the geometric mean of two housekeeping genes: Gapdh and Actb.
Statistics were performed on untransformed ΔCt values. All primers are included below
(Table 1).

Primers were designed using the Integrated DNA Technologies PrimerQuest tool. Primers
were designed to span one exon and with a 3’ bias. Product sizes of less than 300 were selected.
Sequences were verified using NCBI Primer-BLAST to fully match the target transcript and
have no significant off target interactions.

Statistical analysis
Data are presented as mean +/- standard error of the mean (SEM), unless otherwise noted. All
data sets were tested for normality using the D’Agostino-Pearson omnibus normality test.
Data sets passing the normality test were analyzed using parametric tests: Student’s t-test for 2
group comparison and one-way analysis of variance (ANOVA) for 3 or more groups. Small
groups (sample size less than 7) or groups with non-normal distribution were analyzed using
non-parametric tests: Mann-Whitney U test for 2 group comparison and Kruskal-Wallis one-
way analysis of variance for 3 or more groups. For analyzing absolute change from four to six
months in the drug study, the one-sample t test or Wilcoxon signed-rank test was used for
parametric and non-parametric data, respectively. To account for multiple comparisons, sta-
tistical significance was corrected in post-hoc tests using Tukey or Dunn’s correction for
parametric and non-parametric tests, respectively. For all tests, a p-value of 0.05 was consid-
ered statistically significant. Data storage and statistical analysis was performed using Micro-
soft Excel, MATLAB r2019a, and GraphPad Prism 8.4.1.

Results
Genetic ablation of Htr2b rescues hemodynamic metrics of aortic valve
disease
At 6 months, peak velocity is 15–20% higher than the average peak velocity found in the
LDLr–/–/ApoB100/100/IGF-II+/– mutant model of CAVD [35]. Using a previously reported
threshold of 1320 mm/s [35], incidence of severe CAVD decreased with ablation of Htr2b gene
(S1 Fig). Ablation of Htr2b from Notch1+/– mice decreases the average peak velocity during the
cardiac cycle with a corresponding decrease in mean pressure gradient across the AV, relative
to Notch1+/–;Htr2b+/+ (Fig 2A and 2B). Left ventricular mass was not changed with Htr2b
Additionally, the function of the left ventricle was not altered based on ejection fraction measurements (Fig 2D). Together, this suggests the observed changes in hemodynamics are intrinsic to the AV and not due to altered cardiac function.

5-HT<sub>2B</sub> antagonism does not affect hemodynamic metrics of aortic valve disease

Based on results from the mutant study, we aimed to determine if the beneficial effects of Htr2b ablation on CAVD progression could be recapitulated using a pharmacological inhibitor specific to the 5-HT<sub>2B</sub> receptor. Following the initiation of SB204741 treatment (after 6 weeks of high cholesterol diet), the absolute change in peak velocity, mean gradient, left ventricular mass, and ejection fraction was calculated from 4 to 6 months of age. There was no significant change in valve metrics of peak velocity or mean pressure gradient (Fig 3A and 3B). Further,
there was a slight increase in left ventricle mass but no significant change in ejection fraction (Fig 3C and 3D). Despite AV improvement following genetic ablation, 5-HT\textsubscript{2B} antagonism by SB204741 does not reduce CAVD progression in Notch1\textsuperscript{+/−} mice. Both the Htr2b\textsuperscript{-/−} and SB204741 models were assayed for compensatory expression of Htr2a as a potential mechanism of disease. Htr2a expression was non-significantly decreased with administration of SB204741 (S3 Fig).

5-HT\textsubscript{2B} genetic ablation or pharmacological inhibition does not significantly change leaflet microstructure

Masson’s trichrome staining was used to analyze collagen abundance and thickness of AV leaflet cross sections (Fig 4A and 4D). There was wide variation, particularly across wild-type and DMSO samples, and despite trends toward decreased thickness and increased collagen density, we observed no statistically significant changes (Fig 4B and 4C, S2 Fig). Similarly, there were no detected differences in leaflet thickness or collagen density in drug treatment groups despite the same trends as observed in the genetic ablation study (Fig 4E and 4F).
5-HT<sub>2B</sub> genetic ablation or pharmacological inhibition does not significantly change myofibroblast activation or osteoblast-like phenotype induction

To determine if myofibroblast activation played a role in the difference observed in mutant AV hemodynamic phenotypes, immunofluorescent labeling of the αSMA protein was performed (Fig 5A and 5B). Similarly, Runx2 was stained to determine if there was evidence of osteoblast-like phenotype shift in valve cells and Alizarin Red S staining was used to identify osteogenic calcification (Fig 6A and 6B). There was no significant difference in αSMA and high sample-to-sample variance was observed, particularly in the Notch1<sup>+/−</sup>; Htr2b<sup>+/+</sup> group (Fig 5C). Analysis of Runx2 staining revealed a 50–60% positivity rate of all valve cells independent of genotype and treatment, but there was little to no evidence of widespread calcification based on the Alizarin Red S stain. No significant differences in Runx2 expression were observed between groups (Fig 6C). phospho-ERK1/2 staining showed high variability which may indicate a multimodal distribution, similar to αSMA (S4 Fig). The only samples showing definitively positive phospho-ERK1/2 staining were in Notch1<sup>+/−</sup>;Htr2b<sup>+/+</sup> and DMSO groups.

5-HT<sub>2B</sub> genetic ablation or pharmacological inhibition does not significantly change fibrotic CAVD transcripts

To further investigate the molecular mechanisms leading to differing hemodynamic phenotypes in mutant mice, RT-qPCR was performed for several genes of interest. Levels of these transcripts were not significantly changed, further pointing to a myofibroblast independent mechanism (Fig 7A). Ccn2, the gene for the fibrotic marker connective tissue growth factor...
(CTGF) showed an overall group difference ($p < 0.05$; Kruskal-Wallis 1-way ANOVA) but no differences when accounting for multiple comparisons ($p = 0.0655$ between $\text{Notch}^+/\text{Htr}^{+/+}$ and $\text{Notch}^+/\text{Htr}^{+/--}$; $p = 0.0826$ between $\text{Notch}^+/\text{Htr}^{+/+}$ and $\text{Notch}^+/\text{Htr}^{+/--}$).

RT-qPCR was performed in order to potentially detect any early markers of disease that may be present before symptoms can be detected by imaging. No significant changes were detected in the typical fibrotic CAVD markers of $\text{Acta}2$, $\text{Cdh}11$, $\text{Col}1a1$, or $\text{Ccn}2$ (Fig 7B).

**Discussion**

CAVD is a significant health concern in aging populations of developed countries, which are shifting toward more sedentary lifestyles. While the pathophysiology of the disease is poorly understood, serotonin signaling is a relatively well characterized driver of valve disease. The aim of this study was to assess the possibility of slowing or treating CAVD by genetically deleting or pharmacologically inhibiting $5\text{-HT}_{2B}$ in a high-cholesterol diet $\text{Notch}^+/\text{Htr}^{+/+}$ mouse model.

We found that genetic mutation of the $5\text{-HT}_{2B}$ receptor—via $\text{Htr}^{+/+}$ deletion—mitigates hemodynamic progression of aortic stenosis.

We began by testing echocardiographic outcomes of genetic and pharmacological blockade of $5\text{-HT}_{2B}$. Genetic knockdown of $\text{Htr}^{+/+}$ improved echocardiographic metrics of aortic stenosis (Fig 2), but receptor blockade with SB204741 had no effect (Fig 3). This may indicate that pharmacological inhibition of $5\text{-HT}_{2B}$ using SB204741 is insufficient in blocking receptor

![Figure 5](https://doi.org/10.1371/journal.pone.0238407.g005)
action compared to complete genetic deletion. This is supported by the fact that Notch1\(^{+/+}\); Htr2b\(^{+/+}\) mice saw only slight, non-significant improvement over Notch1\(^{+/+}\); Htr2b\(^{+/-}\) mice. In other words, only complete Htr2b knockout mice had significant changes. A stronger hemodynamic phenotype tends to develop between 6 and 9 months of age when using HFD (unpublished observations). It is possible that the mice in our study were not aged long enough to see appreciable differences in CAVD phenotypes. The age-range in this study is comparable to

Fig 6. Neither 5-HT\(_2B\) genetic ablation nor pharmacological inhibition show a difference in osteoblast-like phenotype or osteogenic calcification. A. Fluorescent (left column) and Alizarin Red S (right column) brightfield imaging was carried out on mutant mice. B. Fluorescent (left column) and Alizarin Red S (right column) brightfield imaging was carried out on vehicle and drug treated mice as described. C. Nuclei greater than 50% positive for Runx2 stain were counted and normalized by total nuclei. Mean+/−SEM, \(^{\star}p<0.05\) following Kruskal-Wallis 1-way ANOVA with Dunn’s post-test. White/black bar = 200 \(\mu\)m.

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Fig 7. Htr2b mutant and SB204741-treated mice do not have altered transcription of genes associated with fibrotic/myofibroblast driven CAVD. A. RT-qPCR was used to assess the levels of mRNA transcripts of Acta2, Cdh11, Col1a1, and Ccn2. No significant differences were detected. B. Similar targets were interrogated in drug treatment groups. Similarly, there were no differences. Mean+/−SE, \(^{\star}p<0.05\) following Kruskal-Wallis 1-way ANOVA with Dunn’s post-test.

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early adulthood in humans whereas CAVD generally does not become prevalent until advanced age.

On assaying leaflet morphology and collagen structure, we saw no significant changes in any group (Fig 4). This was expected in drug treatment studies as we saw no significant differences in hemodynamic profile; therefore, we expected the structure was likely unchanged. Trends in mutant mice may indicate reduced leaflet hypertrophy in mutants which could lead to improvements in the AV hemodynamic profile. However, variability in the studied groups—particularly Notch1+/−;Htr2b+/+—precludes a clear conclusion from being drawn. The lack of change in collagen density may indicate an alternate structural cause for differences in the systolic AV flow profile in mutant mice, such as accumulation of glycosaminoglycans, undetected by using the trichrome staining method [6, 8, 36].

We also assessed myofibroblast activation by αSMA staining. Serotonin signaling through the 2B receptor has shown to play a role in the activation of quiescent VICs into activated myofibroblasts. These cells play a role in the fibrosis and calcification that lead to impaired blood flow found in CAVD. One of the integral parts of myofibroblast activation is the adoption of a contractile phenotype that is characterized by abundant stress fibers primarily composed of the protein αSMA [7]. Yet again, we saw no significant changes (Fig 5). It is possible that the myofibroblast activation phenotype may be a bimodal distribution and if a longer time course was studied, more mice would develop the elevated αSMA phenotype seen in one Notch1+/−;Htr2b+/+ mouse. However, when combined with collagen deposition based on trichrome staining, it appears the myofibroblast-driven fibrotic phenotype is likely not the cause of the differing phenotype which may point to a difference in glycosaminoglycan structure, osteogenic calcification, or cellular inflammation [6, 36, 37]. ERK1/2, a signaling molecule downstream of 5-HT2B activation showed similar activation variability to αSMA and may also be a multimodal distribution. Notch1 is known to downregulate Runx2 mediated osteoblast-like phenotype in CAVD [21, 38]. Indeed, immunostaining in this study shows Runx2 expression is present in Notch1+/− mice with around 50% of resident cells positive for the protein. Our data show Htr2b ablation nor receptor inhibition alter Runx2 expression or osteogenic calcification, suggesting the hemodynamic phenotype observed in mutant mice is not based on canonical osteogenic signaling pathways (Fig 6). Immune cell recruitment plays a key role in other 5-HT2B-mediated fibrotic disease and has been shown to contribute to the Notch1+/− model of CAVD, suggesting it may be involved here [25, 30, 39]. This may also indicate a phenotype arising from cells other than myofibroblasts. For instance, valvular endothelial cells are involved in CAVD progression via nitric oxide signaling and endothelial-mesenchymal transition [40, 41]. These alternative cellular mechanisms have not been rigorously interrogated in the context of 5-HT2B associated CAVD and may be a fruitful avenue of investigation.

RT-qPCR studies (Fig 7) also reveal little about the causative mechanisms of the changes found in the hemodynamics of the mutants. Changes in myofibroblast markers Acta2 and Cdh11 were not changed in mutants or drug groups. Colla1 was also not altered in any group, as would be expected based on histology analysis. Finally, Ccn2, a marker of fibrotic remodeling, was different at a group level in mutants but after correcting for multiple comparisons we observed no differences. This was also true between drug treatment groups. In addition, we did not find compensatory expression of 5-HT2B receptor (S3 Fig). 5-HT2C receptor subtype has been shown to be undetectable in the cardiovascular system and was not studied [28].

Overall, our results indicate that double mutant mice exhibit an improved hemodynamic phenotype at six months of age compared to Notch1+/−;Htr2b+/+ mice. However, pharmacologic inhibition of 5-HT2B by SB204741 did not affect the development of CAVD between four and six months of age. This may be due to antagonism of the receptor not being a sufficient intervention to slow progression of the disease. SB204741 is known to be a selective antagonist
of the 2B receptor subtype but has relatively low potency [28]. While we have had success using a 1 mg kg$^{-1}$ d$^{-1}$ in previous studies in the cardiovascular system, studies using SB204741 in other diseases like chronic liver disease or pancreatic cancer require higher dosages of up to 10 mg kg$^{-1}$ d$^{-1}$ [30, 42, 43]. Additionally, our window of intervention may have been too early as six months of age is still relatively early for the disease to present.

Separately, results from the genetic study suggest prophylactic 5-HT$_{2B}$ inhibition may be sufficient for attenuating CAVD initiation, but therapeutic inhibition is insufficient for slowing the progression of existing disease. Histological analysis revealed mutant and drug treated mice did not significantly vary in level of leaflet hypertrophy or collagen density. Rough measurements in trichrome stains of aortic valve annulus diameter show a slight increase in Htr2b mutant mice which may result in a larger outflow area leading to lower flow and pressure across the valve but we were unable to confirm this using in vivo echocardiography (inadequate resolution for reliable measurements). Due to the potential variability introduced in the fixation, sectioning, and staining process in valve morphology we can only speculate in this potential explanation but caution that further investigation is needed to confirm this phenotype. There was also no difference in a subset of markers associated with myofibroblast activation and fibrosis that are generally detected in CAVD. Indeed, at the transcript level, there was little difference in general markers of CAVD.

Findings from the current study highlight the involvement of 5-HT$_{2B}$ signaling on CAVD initiation and progression based on improved AV hemodynamics following Htr2b deletion. Future studies focused on the therapeutic potential of targeted 5-HT$_{2B}$ receptor blockers should include a longer time course (12–18 months) to study the impact on the later stages of CAVD where the hemodynamic, tissue, and molecular phenotypes should be stronger and interrogate the effect of 5-HT$_{2B}$ inhibition before and after disease onset. Additionally, unbiased transcriptomic and proteomic approaches, such as mRNA sequencing, can be used to identify potential pathways of interrogation that may have been missed using a targeted approach.

**Limitations**

Due to prohibitive cost and logistical concerns of housing mice and providing specialized diet ad libitum we were only able to age mice to 6 months of age, limiting the development of CAVD and AS phenotypes which correlate strongly with advanced age. A relatively low dosage of SB204741 was used due to the long time course of treatment and previous studies from our lab having success with this dosage. Annulus diameter was unable to be verified on parasternal long-axis echoes due to insufficient resolution making measurement unreliable. Aortic valve anatomy is variable across coronal/frontal plane studied. While efforts to sample several regions were developed, this is a potentially confounding aspect of the analysis. Molecular interrogation of causative pathways was limited due to the small amount of high-quality mRNA and tissue sections that can be extracted from single mouse aortic roots.

**Conclusions**

The results from this study provide evidence that Htr2b mutant mice may have more compliant leaflets allowing for unimpeded blood flow in the Notch1$^{+/−}$ model of CAVD. However, the molecular changes underlying this tissue-level difference are unclear. We also found that despite significant improvement following genetic deletion, 5-HT$_{2B}$ antagonism using SB204741 was unable to cause a significant hemodynamic or histological difference between four and six months of age. Future studies are needed to fully evaluate the therapeutic potential of targeting 5-HT$_{2B}$ signaling for the treatment and management of CAVD.
Supporting information

S1 Fig. Portion of mice with hemodynamic CAVD based on criteria from [33]. 1320 mm/s is the 95th percentile of peak velocity found in ref. [33]. Using this criteria, 33%, 25%, and 12.5% of wild-type, knockout heterozygous, and knockout null mice developed hemodynamic CAVD, respectively.

S2 Fig. High morphological variability in Notch1+/−;Htr2b+/− and DMSO groups. Statistical comparison is difficult due to wide variability in tissue characteristics in A. wild-type and B. DMSO treatment groups.

S3 Fig. Genetic ablation or pharmacological inhibition did not result in compensation of other 5-HT receptor subtypes. Randomly selected samples from wild-type, knockout, DMSO, and SB204741 groups were selected and RT-qPCR was performed on the Htr2a gene. Expression was very low and there was no significant change. Mean±SE, *P<0.05, Mann-Whitney U-test.

S4 Fig. High phospho-ERK1/2 stain variability in Notch1+/−;Htr2b+/− and DMSO groups. Statistical comparison is difficult due to wide variability in tissue characteristics in A. wild-type and B. DMSO treatment groups.

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References

1. Lindman BR, Clavel M-A, Mathieu P, Iung B, Lancellotti P, Otto CM, et al. Calcific aortic stenosis. Nat Rev Dis Prim [Internet]. 2016 Mar 3; 2:16006. Available from: https://doi.org/10.1038/nrdp.2016.6 PMID: 27188578

2. Nkomo VT, Gardin JM, Skelton TN, Gottlieber JS, Scott CG, Enriquez-Sarano M. Burden of valvular heart diseases: a population-based study. Lancet (London, England). 2006 Sep; 368(9540):1005–11. https://doi.org/10.1016/S0140-6736(06)69209-8 PMID: 16980116
3. Osnabrugge RLJ, Mylotte D, Head SJ, Van Miegem NM, Nkomo VT, LeReun CM, et al. Aortic Stenosis in the Elderly: Disease Prevalence and Number of Candidates for Transcatheter Aortic Valve Replacement: A Meta-Analysis and Modeling Study. J Am Coll Cardiol [Internet]. 2013; 62(11):1002–12. Available from: http://www.sciencedirect.com/science/article/pii/S0735109713020792 https://doi.org/10.1016/j.jacc.2013.05.015 PMID: 23727214

4. Otto CM, Burwash IG, Legget ME, Munt BI, Fujikoa M, Healy NL, et al. Prospective study of asymptomatic valvular aortic stenosis: Clinical, echocardiographic, and exercise predictors of outcome. Circulation. 1997

5. VS S., Alvaro A, BE J., BM S., CC W., CA P., et al. Heart Disease and Stroke Statistics—2020 Update: A Report From the American Heart Association. Circulation [Internet]. 2020 Mar 3; 141(9):e139–596. Available from: https://doi.org/10.1161/CIR.0000000000000757 PMID: 31992061

6. Rajamannan NM, Evans FJ, Akaiwa E, Grande-Allen KJ, Demer LS, Heistad DD, et al. Calcific Aortic Valve Disease: Not Simply a Degenerative Process. Circulation [Internet]. 2011 Oct 17; 124(16):1783 LP–1791. Available from: http://circ.ahajournals.org/content/124/16/1783.abstract https://doi.org/10.1161/CIRCULATIONAHA.110.066767 PMID: 22007101

7. Bowler MA, Merryman WD. In vitro models of aortic valve calcification: Solidifying a system. Vol. 24. aspetjournals.org/content/57/1/75.abstract

8. Rajamannan NM, Subramaniam M, Rickard D, Stock SR, Donovan J, Springer M, et al. Human aortic valve calcification is associated with an osteoblast phenotype. Circulation. 2003; 107(17):2181–4. https://doi.org/10.1161/01.CIR.0000097059.21548.E9 PMID: 12719280

9. Iung B, Cacheri A, Baron G, Messika-Zeitoun D, Delahaye F, Tomos P, et al. Decision-making in elderly patients with severe aortic stenosis: why are so many denied surgery? Eur Heart J [Internet]. 2005 Sep 26; (24):2714–20. Available from: https://doi.org/10.1093/eurheartj/ehi471 PMID: 16141261

10. Druce M, Rockall A, Grossman AB. Fibrosis and carcinoid syndrome: from causation to future therapy. Nat Rev Endocrinol [Internet]. 2009; 5(5):276–83. Available from: https://doi.org/10.1038/nrendo.2009.51 PMID: 1944261

11. Connolly HM, Crary JL, McGoon MD, Hensrud DD, Edwards BS, Edwards WD, et al. Valvular Heart Disease Associated with Fenfluramine–Phentermine. N Engl J Med [Internet]. 1997 Aug 28; 337 (9):581–8. Available from: https://doi.org/10.1056/NEJM199708283370901 PMID: 9271479

12. Fitzgerald LW, Burn TC, Brown BS, Patterson JP, Corjay MH, Valentine PA, et al. Possible Role of Valvular Serotonin 5-HT<sub>2B</sub> Receptors in the Cardiopathy Associated with Fenfluramine. Mol Pharmacol [Internet]. 2000 Jan 1; 57(1):75 LP–179. Available from: http://molpharm.aspetjournals.org/content/57/1/75.abstract

13. RR B., BM H., SJ E., Laura R, Ace M, HS J., et al. Evidence for Possible Involvement of 5-HT2B Receptors in the Cardiac Valvulopathy Associated With Fenfluramine and Other Serotonergic Medications. Circulation [Internet]. 2000 Dec 5; 102(23):2836–41. Available from: https://doi.org/10.1161/01.cir.102.23.2836 PMID: 11104741

14. Elangbam CS, Job LE, Zadrozny LM, Barton JC, Yoon LW, Gates LD, et al. 5-Hydroxytryptamine (5HT)-induced valvulopathy: Compositional valvular alterations are associated with 5HT2B receptor and 5HT transporter transcript changes in Sprague-Dawley rats. Exp Toxicol Pathol [Internet]. 2008; 60 (4):253–62. Available from: http://www.sciencedirect.com/science/article/pii/S0940299308000523 https://doi.org/10.1016/j.etp.2008.03.005 PMID: 18511249

15. NC G., Pierre H, Nadia M, Jean-Luc V, DM P., Laurent M, et al. Abilation of Serotonin 5-HT2B Receptors in Mice Leads to Abnormal Cardiac Structure and Function. Circulation [Internet]. 2001 Jun 19; 103 (24):2973–9. Available from: https://doi.org/10.1161/01.heart.103.24.2973 PMID: 11413089

16. Fabricce J, Jacques C, Alexandre S, Nelly E, NC G., L Jean-Marie, et al. Involvement of the Serotonin 5-HT2B Receptor in Cardiac Hypertrophy Linked to Sympathetic Stimulation. Circulation [Internet]. 2004 Aug 24; 110(8):969–74. Available from: https://doi.org/10.1161/01.CIR.0000139856.20505.57 PMID: 15302781

17. Laurent M, Marc-André L, Fabricce J, Pascal B, Luc M, Jacques de C. Serotonin 5-HT2B Receptor Blockade Prevents Reactive Oxygen Species–Induced Cardiac Hypertrophy in Mice. Hypertension [Internet]. 2008 Aug 1; 52(2):301–7. Available from: https://doi.org/10.1161/HYPERTENSIONAHA.107.105551 PMID: 18591460

18. Cremer SE, Moesgaard SG, Rasmussen CE, Zois NE, Falk T, Reimann MJ, et al. Alpha-smooth muscle actin and serotonin receptors 2A and 2B in dogs with myxomatous mitral valve disease. Res Vet Sci [Internet]. 2015; 100:197–206. Available from: http://www.sciencedirect.com/science/article/pii/S0034528815000946 https://doi.org/10.1016/j.rvsc.2015.03.020 PMID: 25843893
19. Ayre-Dietrich E, Lawson R, Côté F, de Tapia C, Da Silva S, Ebel C, et al. The role of 5-HT2B receptors in mitral valvulopathy: bone marrow mobilization of endothelial progenitors. Br J Pharmacol [Internet]. 2017 Nov 1; 174(22):4123–39. Available from: https://doi.org/10.1111/bph.13981 PMID: 28806488

20. Hutcheson JD, Ryzhova LM, Setolia V, Merryman WD. 5-HT2B antagonism arrests non-canonical TGF-β1-induced valvular myofibroblast differentiation. J Mol Cell Cardiol [Internet]. 2012; 53(5):707–14. Available from: http://www.sciencedirect.com/science/article/pii/S0022317312003010X https://doi.org/10.1016/j.yjmcc.2012.08.012 PMID: 22940605

21. Garg V, Muth AN, Ransom JF, Schluterman MK, Barnes R, King IN, et al. Mutations in NOTCH1 cause aortic valve disease. Nature. 2005; 437(7056):270–4. https://doi.org/10.1038/nature03940 PMID: 16025100

22. Meritxell N, Donal M, Beatriz M-P, Yolanda B, CJ C., Francisco F-A, et al. Diet-Induced Aortic Valve Disease in Mice Haploinsufficient for the Notch Pathway Effector RBPJK/CSSL. Arterioscler Thromb Vasc Biol [Internet]. 2011 Jul 1; 31(7):1580–8. Available from: https://doi.org/10.1161/ATVBAHA.111.227561 PMID: 21493891

23. Chen J, Ryzhova LM, Sewell-Loftin MK, Brown CB, Huppert SS, Baldwin HS, et al. Notch1 mutation leads to valvular calcification through enhanced myofibroblast mechanotransduction. Arterioscler Thromb Vasc Biol. 2015; 35(7):1587–605. https://doi.org/10.1161/ATVBAHA.114.305095 PMID: 26023079

24. CC R., BM A., Caleb SJ, David MW. Targeting Cadherin-11 Prevents Notch1-Mediated Calcific Aortic Valve Disease. Circulation [Internet]. 2017 Jun 13; 135(24):2449–50. Available from: https://doi.org/10.1161/CIRCULATIONAHA.117.027771 PMID: 28668953

25. Raddatz MA, Huffstater TM, Bersi MR, Reinfeld BI, Madden MZ, Booton SE, et al. Macrophages Promote Aortic Valve Cell Calcification and Alter STAT3 (Signal Transducer and Activator of Transcription 3) Splicing. Arterioscler Thromb Vasc Biol. 2020 Apr;2020.01.24.919001.

26. Conlon RA, Reaume AG, Rossant J. Notch1 is required for the coordinate segmentation of somites. Development [Internet]. 1995 May 1; 121(5):1533 LP–1545. Available from: http://dev.biologists.org/content/121/5/1533.abstract PMID: 7788282

27. Nebigil CG, Choi D-S, Dierich A, Hickel P, Le Meur M, Messaddeq N, et al. Serotonin 2B receptor is required for heart development. Proc Natl Acad Sci [Internet]. 2000 Aug 15; 97(17):9508 LP–9513. Available from: http://www.pnas.org/content/97/17/9508.abstract https://doi.org/10.1073/pnas.97.17.9508 PMID: 10944220

28. Maroteaux L, Ayre-Dietrich E, Aubertin-Kirsch G, Banas S, Quentin E, Lawson R, et al. New therapeutic opportunities for 5-HT2 receptor ligands. Pharmacol Ther [Internet]. 2017; 170:14–36. Available from: http://www.sciencedirect.com/science/article/pii/S0163725816301875 https://doi.org/10.1016/j.pharmthera.2016.10.008 PMID: 27771435

29. Forbes IT, Jones GE, Murphy OE, Holland V, Baxter GS. N-[1-Methyl-5-indolyl]-N’-(3-methyl-5-isothiazolyl)urea: A Novel, High-Affinity 5-HT2B Receptor Antagonist. J Med Chem. 1995; 38(6):855–7. https://doi.org/10.1021/jm00006a001 PMID: 7699699

30. Bloodworth NC. Bone-Marrow Derived Proangiogenic Cells Mediate Pulmonary Arteriole Stiffening via Serotonin 2B Receptor Dependent Mechanism. Circ Res. 2017; 73:1–45.

31. Ferruzzi J, Di Achille P, Tellides G, Humphrey JD. Combining in vivo and in vitro biomechanical data reveals key roles of perivascular tethering in central artery function. Proc Natl Acad Sci [Internet]. 2018 Sep 7; 13(9):e0201379. Available from: https://doi.org/10.1073/pjnl.2018.09.013 PMID: 30192758

32. Schroer AK, Bersi MR, Clark CR, Zhang Q, Sanders LH, Hatzopoulos AK, et al. Cadherin-11 blockade reduces inflammation-driven fibrotic remodeling and improves outcomes after myocardial infarction. JCI Insight [Internet]. 2019 Sep 19; 4(18). Available from: https://doi.org/10.1172/jci.insight.131545

33. Bersi MR, Khosravi R, Wujciak AJ, Harrison DG, Humphrey JD. Differential cell-matrix mechanoadaptations and inflammation drive regional propensities to aortic fibrosis, aneurysm or dissection in hypertension. J R Soc Interface [Internet]. 2017 Nov 30; 14(136):20170327. Available from: https://doi.org/10.1098/rsif.2017.0327 PMID: 29118111

34. Wienert S, Heim D, Saeger K, Stenzinger A, Beil M, Hufnagl P, et al. Detection and Segmentation of Cell Nuclei in Virtual Microscopy Images: A Minimum-Model Approach. Sci Rep [Internet]. 2012; 2(1):503. Available from: https://doi.org/10.1038/srep00503 PMID: 22787560

35. Annabi M-S, Clisson M, Fleury M-A, Voisine M, Hervault M, Shen M, et al. Sex-differences in echocardiographic assessment of aortic valve in young adult LDLr−/−/ApoB100/100/GF-II−/− mice. Exp Gerontol [Internet]. 2020; 140:111075. Available from: http://www.sciencedirect.com/science/article/pii/S053155652030423X https://doi.org/10.1016/j.exger.2020.11.01075 PMID: 32861845

36. Grande-Allen KJ, Osman N, Ballinger ML, Madani H, Marasco S, Lough PJ. Glycosaminoglycan synthesis and structure as targets for the prevention of calcific aortic valve disease. Cardiovasc Res [Internet].
37. Raddatz MA, Madhur MS, Merryman WD. Adaptive immune cells in calcific aortic valve disease. Am J Physiol Circ Physiol. 2019 Jul; 317(1):H141–55. https://doi.org/10.1152/ajpheart.00100.2019 PMID: 31050556

38. Nigam V, Srivastava D. Notch1 represses osteogenic pathways in aortic valve cells. J Mol Cell Cardiol [Internet]. 2009; 47(6):828–34. Available from: http://www.sciencedirect.com/science/article/pii/S0022282809003290 https://doi.org/10.1016/j.yjmcc.2009.08.008 PMID: 19695258

39. Launay J-M, Hervé P, Callebert J, Mallat Z, Collet C, Doly S, et al. Serotonin 5-HT2B receptors are required for bone-marrow contribution to pulmonary arterial hypertension. Blood. 2012; 119(7):1772–80. https://doi.org/10.1182/blood-2011-06-358374 PMID: 22186990

40. Bosse K, Hans CP, Zhao N, Koenig SN, Huang N, Guggilam A, et al. Endothelial nitric oxide signaling regulates Notch1 in aortic valve disease. J Mol Cell Cardiol [Internet]. 2013; 60:27–35. Available from: http://www.sciencedirect.com/science/article/pii/S0022282813001272 https://doi.org/10.1016/j.yjmcc.2013.04.001 PMID: 23583836

41. Paranya G, Vineberg S, Dvorin S, Kaushal S, Roth SJ, Rabkin E, et al. Aortic valve endothelial cells undergo transforming growth factor-β-mediated and non-transforming growth factor-β-mediated trans-differentiation in Vitro. Am J Pathol. 2001 https://doi.org/10.1016/s0002-9440(10)62520-5 PMID: 11583961

42. Ebrahimkhani MR, Oakley F, Murphy LB, Mann J, Moles A, Perugorria MJ, et al. Stimulating healthy tissue regeneration by targeting the 5-HT2B receptor in chronic liver disease. Nat Med [Internet]. 2011; 17 (12):1668–73. Available from: https://doi.org/10.1038/nm.2490 PMID: 22120177

43. Jiang S-H, Li J, Dong F-Y, Yang J-Y, Liu D-J, Yang X-M, et al. Increased Serotonin Signaling Contributes to the Warburg Effect in Pancreatic Tumor Cells Under Metabolic Stress and Promotes Growth of Pancreatic Tumors in Mice. Gastroenterology [Internet]. 2017; 153(1):277–291.e19. Available from: http://www.sciencedirect.com/science/article/pii/S0016508517302718 https://doi.org/10.1053/j.gastro.2017.03.006 PMID: 28315323