HHS Public Access

Author manuscript

Sci Transl Med. Author manuscript; available in PMC 2023 July 04.

Published in final edited form as:

Sci Transl Med. 2023 January 04; 15(677): eadc9606. doi:10.1126/scitranslmed.adc9606.

Decreased Serotonin Transporter Activity in the Mitral Valve Contributes to Progression of Degenerative Mitral Regurgitation

Estibaliz Castillero[1,*], **Emmett Fitzpatrick**[2], **Samuel J. Keeney**[2], **Alex M. D'Angelo**[1], **Benjamin B. Pressly**[2], **Michael T. Simpson**[1], **Mangesh Kurade**[1], **W. Clinton Erwin**[1], **Vivian Moreno**[1], **Chiara Camillo**[1], **Halley J. Shukla**[2], **Vaishali V. Inamdar**[2], **Arbi Aghali**[2], **Juan B. Grau**[3], **Elisa Salvati**[2], **Itzhak Nissim**[4,5], **Lubica Rauova**[6], **Mark A. Oyama**[7], **Stanley J. Stachelek**[2], **Chase Brown**[8], **Abba M. Krieger**[9], **Robert J. Levy**[2], **Giovanni Ferrari**[1,10,*]

[1]Department of Surgery, Columbia University; New York, NY, 10032, USA.

[2]The Pediatric Heart Valve Center, and the Division of Cardiology, The Children's Hospital of Philadelphia; Philadelphia, PA, 19104, USA.

[3]Valley Hospital Heart Institute; Ridgewood, NJ, 07450, USA.

[4]Division of Human Genetics and Metabolic Disease, Children's Hospital of Philadelphia; Philadelphia, PA, 19104, USA.

[5]Department of Pediatrics, Biochemistry and Biophysics, Perelman School of Medicine at the University of Pennsylvania; Philadelphia, PA, 19104, USA.

[6]Division of Hematology, The Children's Hospital of Philadelphia; Philadelphia, PA, 19104, USA.

[7]Department of Clinical Sciences and Advanced Medicine, School of Veterinary Medicine, University of Pennsylvania; Philadelphia, PA, 19104, USA.

[8]Department of Surgery, University of Pennsylvania, Smilow Center for Translational Research; Philadelphia, PA 19104, USA.

[*]Corresponding author. gf2375@cumc.columbia.edu (G.F.), ec2929@cumc.columbia.edu (E.C.).

Author Contributions:

R.J.L. and G.F. acquired funding and conceptualized the original project. J.B.G. contributed to clinical conceptualization. E.C. and M.A.O contributed to mechanistic conceptualization. R.J.L, G.F and E.C. designed all experiments. R.J.L and G.F supervised all experiments. E.C. performed or directed all experiments unless otherwise noted. E.F., S.J.K. and H.J.S. performed the RT^2 profiler PCR assays. B.B.P., S.J.S, M.K and A.M.D. contributed to in vivo experiments. W.C.E. contributed to IHC experiments. W.C.E., M.T.S., V.M. and C.B. contributed to clinical data collection and analysis. V.M, C.C. and E.S. contributed to MVIC isolation and culture. I.N., L.R., M.A.O., B.B.P., S.J.S., V.V.I, A.A. and A.M.K. contributed to methodology. A.M.K. performed multivariate analysis. A.M.K. and E.C performed statistical analysis. E.C., E.F., S.J.K. and M.T.S. prepared the figures. E.C. wrote the original manuscript and post-submission material, E.F. and S.J.K. contributed to writing of the methods, R.J.L. and G.F. contributed to writing of background and discussion. R.J.L., G.F, E.C., M.A.O. and A.M.K. reviewed and edited the manuscript. All authors provided critical feedback and helped shape the research, analysis and manuscript.

Supplementary Materials

Materials and Methods.

Figs S1–S8.

Tables S1–S6.

MDAR Reproducibility Checklist.

Data file S1.

Competing interests:

M.A.O is a consultant for CEVA Sante Animale, Nestle Purina, ResQPharma, Inc. and Bantam Pharmaceutical, and this does not represent a conflict of interest concerning the present studies.

[9]Statistics Department, The Wharton School, University of Pennsylvania; Philadelphia, PA 19104, USA.

[10]Department of Biomedical Engineering, Columbia University; New York, NY, 10027, USA.

Abstract

Degenerative mitral valve (MV) regurgitation (MR) is a highly prevalent heart disease that requires surgery in severe cases. Here we show that a decrease in the activity of the serotonin transporter (SERT) accelerates MV remodeling and progression to MR. Through studies of a population of patients with MR, we show that selective serotonin reuptake inhibitor (SSRI) use and *SERT* promoter polymorphism 5-HTTLPR LL genotype were associated with MV surgery at younger age. Functional characterization of 122 human MV samples, in conjunction with in vivo studies in $SERT^{-/-}$ mice and wild type mice treated with the SSRI fluoxetine showed that diminished SERT activity in MV interstitial cells (MVICs) contributed to the pathophysiology of MR through enhanced serotonin receptor (HTR) signaling. SERT activity was decreased in LL MVICs partially due to diminished membrane localization of SERT. In mice, fluoxetine treatment or *SERT* knockdown resulted in thickened MV leaflets. Similarly, silencing of *SERT* in normal human MVICs led to upregulation of transforming growth factor (*TGF*)-beta-1 and collagen (*COL1A1*) in the presence of serotonin. In addition, treatment of MVICs with fluoxetine not only directly inhibited SERT activity, but also decreased *SERT* expression and increased *HTR2B* expression. Fluoxetine treatment and LL genotype were also associated with increased *COL1A1* expression in the presence of serotonin in MVICs, and these effects were attenuated by HTR2B inhibition. These results suggest that assessment of both 5-HTTLPR genotype and SERT-inhibiting treatments may be useful tools to risk-stratify patients with MV disease to estimate the likelihood of rapid disease progression.

One Sentence Summary:

Serotonin transporter genotypes and SSRI use influence the progression of degenerative mitral regurgitation.

INTRODUCTION

Degenerative mitral valve (MV) regurgitation (MR) is one of the most prevalent heart valve diseases (1). Currently, the only treatment for severe MR is surgery to repair or replace the degenerated MV leaflets. The molecular processes triggering abnormal extracellular matrix (ECM) remodeling in the MV leading to thickening and structural disorganization of the MV (myxomatous degeneration) are incompletely understood. Experimental and clinical evidence support a role for mechanisms related to serotonin (or 5-hydroxytryptamine, 5HT) in MV pathological remodeling (2–4). Signaling through serotonin G-protein-coupled receptors (HTRs) can upregulate transforming growth factor (TGF)-β1 leading to pro-fibrotic remodeling (5, 6). Indeed, conditions that result in increased circulating concentrations of serotonin have been shown to be associated with valvulopathies (7). Patients with neuroendocrine (carcinoid) tumors that secrete serotonin often present with ECM plaques in the right heart valve leaflets (2). Valve abnormalities were also observed in patients treated with the diet drug dexfenfluramine (3, 4) or the

anti-Parkinson's drug pergolide (8). Both dexfenfluramine and pergolide can function as agonists of HTR type 2B (HTR2B) (8, 9), which is one of the most abundant HTRs in the cardiovascular system. In addition to exerting HTR2B agonism, dexfenfluramine can increase serotonin signaling by inhibiting the serotonin transporter solute carrier family 6 member 4 (SLC6A4), also known as SERT (10). SERT reduces HTR-mediated signaling by transporting serotonin inside the cell. Inhibiting SERT is the mechanism of action of selective serotonin reuptake inhibitor (SSRI) antidepressants, which thereby increase serotonin signaling in the nervous system. Outside the nervous system, serotonin has multiple homeostatic functions ranging from facilitating platelet thrombus to gastrointestinal motility (11). Tissue-specific combinations of HTR types, serotonin transporters or local synthesis of serotonin may tightly regulate serotonin signaling at the tissue level.

We hypothesized that a decrease in the activity of SERT in the MV enhances the remodeling that leads to degenerative MR. To test this hypothesis, we focused on two clinically relevant scenarios: 1) decreased SERT activity caused by SSRI use, and 2) decreased SERT activity by specific *SERT*-promoter genotypes. *SERT* is subject to a polymorphism in the promoter (non-expressed) region of the gene (12); this polymorphism, abbreviated 5-HTTLPR, is a variable number tandem repeat (VNTR), 43 bases in length. Multiple studies have attempted to link 5-HTTLPR genotypes to serotonin-related disorders. To date, it is still not known to what extent 5-HTTLPR genotypes affect SERT activity in the MV. The aims of the study included testing the responsivity of MV interstitial cells (MVICs) from patients with MR from the three main 5-HTTLPR genotypes to serotonin stimulation. Our results confirmed that MV SERT activity is crucial in MV degenerative remodeling.

RESULTS

Gene expression of serotonin-associated proteins is altered in degenerative MR leaflets versus normal MV leaflets

First, we tested the hypothesis that MVs from patients with degenerative MR would have altered gene expression of serotonin-associated proteins in conjunction with changes in known markers of MV degeneration. The clinical and demographic data from the cohorts of patients with MR included in this study are detailed in table S1. The most common MR presentation among our cohort of patients was prolapse of the P2 segment of the posterior MV leaflet (Fig. 1A).

Immunohistochemistry (IHC) analysis of explanted MV of patients undergoing MV repair or replacement surgery for MR showed pathological remodeling of the MV. Movat's pentachrome staining (Fig. 1B) showed structural differences in the MR leaflets in comparison to anatomically normal leaflets. Well-defined layers in normal MVs contrast with MR MVs, which often have interstitial accumulation of elastic fibers and an increase in glycosaminoglycans intertwined with collagen. MVs from patients with MR had interstitial areas that showed accumulation of α-smooth muscle actin (αSMA)-positive cells, a marker of MVIC activation into a synthetic phenotype. The marker of proliferation Ki67 showed areas of MVIC proliferation in the interstitium of MR MVs that did not necessarily overlap with areas of MVIC activation. The matricellular protein osteopontin (OPN), involved in bone morphogenic protein (BMP)-mediated valve differentiation (13), was increased in MR

MVs. Employing RT^2 Profiler PCR arrays, we compared gene expression in degenerative MR leaflets (n=44) with normal MV leaflets (n=20). Table S2 includes the gene expression results of the complete gene panels. As expected, collagen gene expression (*COL1A1* and *COL1A2*) was increased in MR compared to normal MV (Fig. 1C). Among fibrotic remodeling markers, *TGFβ1*, *TGFβ2* and *BMP4* expression were increased in MR (Fig. 1C). These results illustrate the increased synthesis of ECM components in association with alterations in TGFβ/BMP signaling in degenerative MR. *SERT* expression was significantly decreased in MR vs. control MV ($P = 0.001$, Fig. 1D). Another serotonin transporter, vesicular monoamine transporter-2 (*VMAT2*), which sequesters intracellular serotonin into vesicles, was also decreased in MR. Expression of certain HTRs were also decreased in MR (Fig. 1D). *HTR2A* and *HTR2B* gene expression were not significantly changed (*HTR2A*, $P = 0.05$, *HTR2B*, $P = 0.3$). Fig. 1E shows the gene expression values of *SERT* from the RT^2 Profiler PCR array in each normal and MR sample. Immunofluorescence staining of MV tissue likewise indicated decreased SERT in MR MV versus normal (Fig. 1F and fig. S1). These results led us to hypothesize that serotonin might be able to exert increased HTR-dependent signaling in the MR MV due to reduced clearance of extracellular serotonin by reduced SERT and VMAT2.

5-HTTLPR genotype LL is overrepresented in degenerative MR population

To study the possible implications of *SERT* promoter polymorphisms, which may be associated with changes in SERT activity, in degenerative MR, we performed clinical data analysis, including determination of the 5-HTTLPR genotype, of a subset of 225 patients with moderate to severe MR requiring MV cardiac surgery (Table S3). 41.3% of these patients were determined to be New York Heart Association (NYHA) Class III or IV. The age range at the time of surgery was 24 to 89 years. Most patients were men (71.1%). Genotypes are labeled according to the length of the 5-HTTLPR region as a result, “L” for long and “S” for short. The expected distribution of LS, LL and SS in the general population would be ≅50/25/25% according to the Hardy-Weinberg equilibrium. In the degenerative MR population studied, LS/LL/SS distribution was 49/33/18% (Fig. 2A), showing that the LL genotype is more abundant than the SS genotype in this cohort.

SSRI use and 5-HTTLPR genotype LL are associated with surgery at younger age in degenerative MR patients

Because our main hypothesis was that a decrease in the activity of SERT in the MV leads to degenerative MR due to relatively reduced serotonin deactivation and increased HTR signaling, we aimed to test whether SSRI use and 5-HTTLPR genotype LL were associated with accelerated MV degeneration and therefore, with the need for MV surgery at a younger age. We performed multivariate analyses according to age at the time of MV surgery (Fig. 2, B to D). Cox’s proportional hazards model was used, where the response variable was the age at MV surgery. In our cohort of patients with degenerative MR, SSRI use before MR surgery (20 of the 225 patients), was associated with increased hazard of having MR surgery at a younger age (Fig. 2B) after adjusting for co-variates in the Cox model. Sex, specifically male-over-female, was also significantly associated with increasing the hazard of having MR surgery at a younger age ($P = 0.004$). To confirm the result, we expanded the multivariate analysis to include 9441 patients from the Optum Database. This dataset included a number

of co-variates for analyses that were present in the primary 225 patient population as well as others that were not mutually available. In the Optum population (table S4), SSRI use (Fig. 2C) was also significantly associated with increasing the hazard of having MR surgery at a younger age ($P < 0.001$). Again, sex (male-over-female) was significantly associated with surgery at a younger age, along with alcohol dependence and obesity (all $P < 0.001$, Fig. 2C). Next, we tested interactions with 5-HTTLPR genotype LL in our local 225 subset cohort (Fig 2D). Although genotype LL was associated with surgery at older age (P=0.01), when considered together with sex (male-over female), there was a significant effect (P = 0.003) of LL-male-over-female of having surgery at younger age. Together, these results suggest that changes in SERT are associated with the need for MV surgery at younger age, especially in male patients, who make up the majority of the surgical MR population.

5-HTTLPR genotype LL and SSRI have a mild impact on MV-tissue level gene expression in degenerative MR

We next sought to confirm the impact of 5-HTTLPR genotype LL and SSRI in serotonin pathway genes and markers of pathological remodeling of the MV. Real time PCR with Taqman probes confirmed significant increases of *COL1A1* (P = 0.001) and *TGFβ1* (P = 0.007) and a decrease in *SERT* expression (P = 0.006) in the MV leaflets of patients with MR undergoing MV surgery compared to anatomically normal MV leaflets (Fig. 3A). *HTR2B* mRNA was significantly increased in this cohort of patients (P = 0.002), whereas *αSMA, HTR1B, HTR2A* and *HTR7* were not different from normal MVs. No correlation between age and *SERT* expression was observed (fig. S2A). We did not find an effect of sex in MV gene expression of *SERT* or *HTRs* (fig. S2B), although *αSMA* expression was higher in females than males (P = 0.03). When stratified by 5-HTTLPR genotype, *TGFβ1* expression was significantly higher in LL MV than in SS MVIC (P = 0.02) whereas *SERT, COL1A1*, *TGFβ1* expression trends were not significant ($P > 0.05$, fig. S3, A and B). The trends in gene expression were also similar to differences in immunofluorescent staining (fig. S3C). MR leaflets from patients with the SS genotype had the lowest content of SERT, HTR2B, the marker of TGFβ1-signaling phosphorylated (P-)SMAD2, and αSMA, among MR MVs. There was no significant difference in the expression of *SERT, COL1A1*, *HTRs*, *TGFβ1*, or *αSMA* in association with history of active SSRI use ($P > 0.05$, fig. S3, D and E). These results suggest that 5-HTTLPR genotype LL and SSRI result in small gene expression differences in the MVs of MR patients. LL genotype association with worsened MR may be mediated by a mechanism other than changes in *SERT* MV leaflet gene expression.

5-HTTLPR genotype LL and SSRI decrease the activity of SERT in MVICs

Next, we aimed to study the impact of 5-HTTLPR genotypes and SSRI in MVICs. Primary MVICs were isolated from human leaflets harvested from MR cases and normal leaflets (see table S5). *SERT* expression at baseline was not different in MR MVICs versus normal (Fig. 3B). Treatment with fluoxetine for one week resulted in significant downregulation of *SERT* expression in both normal and MR MVICs (Fig. 3C, $P < 0.001$, fig. S4A). There were no evident differences of *SERT* gene expression at baseline in MR MVICs among the three 5-HTTLPR genotypes. However, when MR MVICs were treated with fluoxetine for one week, the effect of fluoxetine decreasing *SERT* expression was strongest in the LL

genotype cells (Fig. 3D, $P = 0.006$). Despite literature suggesting that the LL variant in the promoter is associated with higher expression efficiency (12), the impact of the 5-HTTLPR genotypes in SERT activity in MVICs was not known. Hence, we analyzed SERT function in MVICs with a fluorescent SERT substrate (false fluorescence neurotransmitter, FFN246) (14). SERT activity at baseline was significantly lower in MR MVICs than in MVICs from normal MV (Fig. 3E, $P = 0.012$). Pretreatment with fluoxetine for 30 min decreased SERT activity (Fig. 3F, control and MR MVICs combined). SERT activity in LL genotype MR MVICs was markedly lower than in LS and SS (Fig. 3G, $P < 0.001$). The lowest uptake of FFN246 by LL MVICs was confirmed by confocal microscopy (Fig. 3H). The lower activity of SERT in LL MVICs was not associated with lower gene expression (fig. S4B). A potential mechanism for lower activity of SERT, despite similar gene expression among genotypes, could be decreased localization of SERT to the cellular membrane. Indeed, immunofluorescence analysis of unstimulated MR MVICs showed diffuse staining of SERT in LL MVICs (fig. S4C). To test this observation, we fixed MVIC after cold incubation with an anti-SERT antibody and performed immunofluorescence staining without membrane permeabilization, in order to stain for extracellular-facing membrane SERT. Our assay showed decreased membrane localization of SERT in LL MVIC (Fig. 3I). Testing the protocol in MVICs after *SERT* siRNA-mediated knockdown (Fig. 3I, right panel) confirmed the specific staining of SERT. This result was confirmed by a surface biotinylation assay (Fig. 3J), which likewise showed significantly decreased membrane SERT in MVIC from patients with MR carrying the LL genotype, which was significant ($P = 0.02$) in comparison with LS genotype. These results confirm that both clinical scenarios identified as either a predictor for younger MV repair surgery (SSRI use) or overrepresented in the MR surgical population (LL genotype) are characterized by lower SERT activity in MVICs. In the case of LL genotype, the lower activity of SERT is at least partially mediated by decreased SERT membrane localization in MVICs.

SERT downregulation alters HTR2B and ECM component gene expression in the MV

Because the clinical and gene expression data suggested that the conditions associated with downregulation of SERT may result in accelerated MR and gene expression changes in the MV, we sought to test whether downregulation of SERT was sufficient to induce anatomical or gene expression changes in the MV. For this purpose, we employed *SERT* knockout ($^{-/-}$) mice (fig. S5A). At 8 weeks of age, $SERT^{-/-}$ mice had thickened MV leaflets in comparison to wild type mice (Fig. 4A), and significantly increased expression of *COL1A1*, *αSMA* and *HTR2B* in the MV leaflets (Fig. 4B, all $P < 0.05$). These results showed that a decrease in the expression, and therefore activity, of SERT in the MV was sufficient to increase collagen synthesis in the MV and may induce changes in HTR. The observed changes in $SERT^{-/-}$ mice may be a consequence of a global absence of *SERT* during development. To test whether short-term downregulation of *SERT* in MVICs was sufficient to induce changes in *COL1A1* and markers of HTR signaling, we performed si RNA-mediated knockdown of *SERT* in MVICs from human normal MV specimens. Silencing of *SERT* (Fig. 4C, D) led to a 94% decrease in expression and was sufficient to increase the expression of *TGFβ1* (Fig. 4D). When MVICs were stimulated for 24 hours with serotonin after silencing, *COL1A1* expression was significantly higher than in MVIC without *SERT* silencing ($P = 0.04$). SERT silencing resulted in a decrease of the expression the serotonin-synthesis enzyme tryptophan

hydroxylase-1 (*TPH1*) whereas it did not impact the expression of HTRs or other genes relevant for MV biology, like OPN or filamin-A (fig. S5B). These results suggest that a short-term decrease of SERT activity is sufficient to induce changes in pro-fibrotic capacity of MVICs, although with a different gene expression profile than long-term loss of *SERT* in the MV. To test whether SERT inhibition by fluoxetine could also induce changes in the MV, we treated wild type mice with fluoxetine for 60 days (20 mg/kg/day). In comparison with untreated mice, the MV of mice treated with a high dose of fluoxetine were thickened (Fig. 4E), although the content of collagen in the MV leaflets did not appear increased (bottom row). The expression of *COL1A1* and *αSMA* in the MV leaflets was not changed by fluoxetine treatment (Fig. 4F). HTR2B was increased in the MVs of fluoxetine-treated mice (*HTR2B* mRNA $P = 0.045$, Fig. 4, F and G), including the thickened regions (Fig. 4G). Together, these results confirm that SERT inhibition can induce changes to anatomy, gene expression and protein abundance the MV.

SERT downregulation increases *COL1A1* in a HTR2B-mediated mechanism

We sought to investigate whether low SERT activity in MR MVIC has a direct local impact in *COL1A1* expression. One-week treatment of fluoxetine did not increase *COL1A1* expression in MR MVIC (Fig. 5A), or in normal MVIC (fig. S6A), although both normal and MR MVIC showed a short-term response to upregulate *COL1A1* mRNA in response to fluoxetine (fig. S6A). Next, we tested *COL1A1* expression after stimulation of MVICs with serotonin or TGFβ1 for 24 hours. The effect of serotonin on upregulation of *COL1A1* expression in MVICs was dose dependent (fig. S6B). Treatment of MVICs with 10μM serotonin significantly upregulated *COL1A1* expression to a similar degree as TGFβ1, but was only significantly in MR MVIC ($P < 0.001$, Fig. 5B). Among MR MVICs, cells with the SS genotype (which have the highest SERT activity at baseline, Fig. 3G) were less responsive to serotonin stimulation of *COL1A1* expression (Fig. 5C). Higher SERT activity can result in increased clearance of extracellular serotonin, and therefore could reduce the amount of serotonin available to exert signaling through HTR receptors. We found that in response to brief treatment with serotonin (1μM, 10 minutes), phosphorylation of extracellular-signal regulated kinase 1/2 (ERK), which can occur downstream of HTR2B, was less pronounced in SS MVIC in comparison with LL and LS MVIC (Fig. 5, D and E, fig. S7A). We therefore investigated whether the induction of *COL1A1* expression after SERT downregulation could be mediated by HTR2B signaling. Treatment with the HTR2B inhibitor LY272015 (LY) prevented the serotonin-induced increase in *COL1A1* expression (Fig. 5F) in MR MVICs, whereas a HTR2A inhibitor (ketanserin) did not prevent serotonin-induced *COL1A1* mRNA upregulation. The LY effect preventing serotonin-induced upregulation was more pronounced in LL MVICs (Fig. 5G). Moreover, LY co-treatment was able to prevent serotonin-induced *COL1A1* mRNA upregulation in normal MVICs with siRNA-mediated knock-down of *SERT* (fig. S7B). Basal *HTR2B* expression in MR MVIC was higher than in normal MVIC (Fig. 5H). In agreement with the results found in mice, treatment with fluoxetine increased *HTR2B* mRNA in both normal and MR MVICs (Fig. 5I). However, a short-term response to upregulate *HTR2B* mRNA in response to fluoxetine was present in MR MVICs but not normal MVICs (fig. S7, C and D). Increase of *HTR2B* expression in MR MVICs by fluoxetine treatment was highest in LL MVICs (Fig. 5J). LL MR MVICs treated with fluoxetine for one week

and subsequently stimulated with serotonin for 24h further upregulated *COL1A1* mRNA (Fig. 5K). LY attenuated the increase in *COL1A1* mRNA in response to fluoxetine and serotonin treatments. These results suggest that treatment of MR patients with fluoxetine may exacerbate increased ECM remodeling. This effect may be exacerbated in patients carrying the LL genotype due to further decreased SERT activity and further upregulation of *HTR2B* in response to fluoxetine. Together, these results show that downregulation of SERT activity in MR, genotype L, and fluoxetine, are each associated with increased *COL1A1* expression due to enhanced HTR2B signaling.

DISCUSSION

In this study, we showed that decreased SERT activity, by 5-HTTLPR genotype or by SSRI use, could induce a pathological phenotype in the MV leaflets of patients with primary MR. Serotonin can induce several cellular processes with implications for cardiovascular homeostasis and pathophysiology including mitogenesis, hypertrophy, fibrosis, oxidative damage, inotropy vasopression and coagulation (15, 16, 17). Mitogenic and profibrotic actions of serotonin are particularly relevant in the context of valvular disease. Serotonin can induce *TGFβ1* expression (18) in VIC and contribute to ECM secretion. Stimulation of mitogenesis and upregulation of TGFβ1 by serotonin are at least partially mediated by HTR2 and ERK mechanisms (19). Downregulation of SERT results in more extracellular serotonin available to exert signaling through HTR receptors, and this is indeed the mechanism of action of SSRI and the rationale for their use to treat depression. Downregulation of SERT not only increases HTR2B signaling, but also *HTR2B* expression in certain physiological contexts, including primary MR. Upregulation of *HTR2B* in the fibrotic myocardium of $SERT^{-/-}$ mice had previously been reported (20). Here, we showed that $SERT^{-/-}$ mice also have increased *HTR2B* expression in the MV. Our results suggest that the effect of SERT downregulation increasing *COL1A1* expression can be attenuated by HTR2B antagonism. It is well-established that HTR signaling, particularly through HTR type-2 receptors, has important roles in cardiovascular pathophysiology (21). We previously showed that HTR2B antagonism, but not HTR2A antagonism, could prevent MV thickening induced by chronic angiotensin-II infusion in mice (22). In the current study, HTR2B antagonism attenuated the pro-fibrotic effect of SERT downregulation at the MVIC level. The role of HTR2 receptors in valve pathophysiology may extend beyond serotonin mechanisms: HTR2B exacerbated the influence of inflammation in aortic valve calcification (23), exemplifying the connections between serotonin and other molecular stimuli of valve remodeling.

The lower activity of SERT in LL MVIC we report contrasts with findings and interpretations in other systems, in which LL genotype increased *SERT* expression and possibly activity (12, 24). The association between LL genotype and lower SERT activity in MVIC appears to be consequence of post-transcriptional mechanisms that ultimately result in lower SERT content at the MVIC membrane surface. A decrease in the amount of membrane SERT in LL MVICs may be a consequence of either a decrease of trafficking to the membrane upon synthesis or recycling, an increase in SERT endocytosis, or both. Lower activity of SERT in LL MR MVICs may result in a compensatory increase in *SERT* expression, which could contribute to 5-HTTLPR-dependent changes in SERT expression

beyond promoter-length-mediated effects. Associations between LL genotype and certain cardiovascular conditions have been reported, including myocardial infarction risk (25) or pulmonary hypertension risk (26). The large body of literature on 5-HTTLPR functional effects is not without controversy (27). Associations between 5-HTTLPR genotypes and disease may be dependent of the ethnicity of the cohort (28), which may affect 5-HTTLPR genotype distribution (29). Other variations in the SERT gene may also impact transcriptional efficiency and activity in MVIC and MV disease progression (30).

Gene and drug independent mechanisms likely mediate in part the decrease in SERT in MV disease. MR may be associated with chronic platelet activation (31), which would result in increased release of serotonin from platelets; this mechanism may mediate the across-the-board downregulation of SERT in the MV of patients with MR. Our mechanistic studies show that, irrespectively of the factor resulting in SERT downregulation (MR, 5-HTTLPR genotype, fluoxetine or gene knockdown), lower SERT activity is enough to increase *COL1A1* expression in the MV. Our results show a spectrum in which LL corresponds to the least favorable end (overrepresentation in surgical population, surgery at younger age in males, increased *COL1A1* expression in response to serotonin) and SS to the most favorable (mildest changes in gene expression in MV, lowest P-ERK in response to serotonin stimulation and lowest increase in *COL1A1* expression in response to serotonin). Importantly, our multivariate analysis indicated that the influence of LL genotype and SSRI use in the risk of needing MV surgery at young age may be compounded. Our in vitro studies showed that the acute effect of fluoxetine downregulating SERT activity was similar among genotypes, however, the effect of chronic fluoxetine treatment in MR MVIC upregulating *HTR2B* was strongest in LL MVIC.

We interpret the role of SERT in primary MR not as a risk factor for causing MV disease, but as a risk factor for enhancing progression of the disease. SERT downregulation had been previously reported in the MV of pigs after surgical ligation (32) and in dogs with late-stage degenerative MR, but not in early canine MV degeneration (33). It is possible that during early remodeling in MV diseases, compensatory mechanisms overcome the negative influence of SERT downregulation (by SSRI or LL) in MV physiology, while in valves with ongoing degeneration the ultimate downregulation of SERT in tissue, enhanced by LL genotype and compounded by SSRI use, may enhance structural degeneration leading to the need for surgery.

The use of SSRIs has been associated with both negative and protective actions in the cardiovascular system (34). In some cases, SSRI use appear to slow down progression of atherosclerosis and reduce inflammatory markers. Potentially negative changes in right ventricular dimensions were found associated with SSRI use, but without changes in ejection fraction (34). Cardiac arrythmias have been associated with SSRI use, but they are often well tolerated and don’t often warrant SSRI discontinuation (34). A 2013 multicenter study did not find an association between the use of the SSRI benfluorex and valvulopathies (35). However, a meta-analysis by our group supports the view that serotonergic drugs are associated with valvular heart disease (36). Our present results indicate that the use of SSRI may have different impact depending on 5-HTTLPR genotypes. Even though our multivariate analysis showed an association between male sex to have surgery at younger

age, as well as an interaction of LL genotype and male sex, these effects should be interpreted with caution. Criteria for referral for MV surgery are largely based on metric measurements, and women may meet criteria later in the course of the disease due to having smaller cardiac volumes (37). Therefore, differences in age at time of MV surgery between male and female do not necessarily reflect different rates of progression of MV disease. Our study supports the notion of using HTR2B blockade as a pharmacological strategy to prevent the negative role of SERT downregulation in degenerative MR progression. Several HTR2 antagonist are FDA-approved and used as antidepressants (mirtazapine), antipsychotics (amisulpride, cariprazine) or antihistamines (cyproheptadine). Inhibiting HTR2B may be cardioprotective beyond the MV: HTR2B in cardiac fibroblasts contributes to scar formation and impaired cardiac function after myocardial infarction (38). The HTR2A/B antagonist sarprogelate has shown beneficial effects in heart failure preventing cardiac hypertrophy (39).

Our study has several limitations. The Optum data set does not distinguish between degenerative MR and ischemic MR. Patients requiring concomitant coronary bypass surgery were excluded to hypothetically exclude patients with ischemic MR, but some of these patients likely remain in the cohort. Methodological concerns prevented us from obtaining reliable measurements of serotonin in blood of MR patients in comparison to controls, therefore a variation in the amount of blood serotonin in primary MR cannot be ruled out as a factor. We focused in MVICs for in vitro studies, but MV endothelial cells, and bone marrow derived-cells can also contribute to MV remodeling (21) and may respond differently to SSRI and 5-HTTLPR than MVIC. The results of our in vitro experiments were not sub-analyzed by sex due to insufficient power. Even though we did not find differences by sex in human or mouse MV tissue, we cannot rule out possible differences at the MVIC level associated with sex. Fluoxetine was the only SSRI tested in vivo and in vitro, other SSRI may differently impact the MV. The in vivo dose of fluoxetine (40) aimed to achieve functional SERT occupancy at the high end of clinically relevant serum concentrations. Therefore, the remodeling effects observed in the MV of mice treated with fluoxetine corresponded to higher concentrations than average in patients taking SSRI.

We conclude that diminished SERT activity contributes to the pathophysiology of MR through enhanced HTR signaling in MVICs in MR leaflets. Although further study is needed, we suggest that assessment of 5-HTTLPR genotype and consideration of SERT-inhibiting drug regimens might be useful tools to risk-stratify patients with MV disease in estimating the likelihood of rapid disease progression.

MATERIALS AND METHODS

Study design

The objective of this study were to determine the impact of SERT downregulation on MV remodeling in degenerative MR. Clinical data, MV specimens from patients with MR and normal MV samples were collected and analyzed. The specific specimens used for each assay is detailed in table S5. Identification of 5-HTTLPR genotype was performed in DNA isolated from patient samples. All mouse studies were performed with IACUC approval. Mice lacking a functional serotonin transporter ($SERT^{-/-}$, B6.129(Cg)-Slc6a4tm1Kpl/J) and

C57BL/6J wild type mice treated with or without fluoxetine were employed to investigate the effect of SERT knockdown or inhibition in MV remodeling. Power analyses were conducted to determine minimum sample sizes, with an alpha (p value) of 0.5 to achieve of power of 0.8, unless specified in the figure legend. Replicates refer to biological replicates from cells from different subjects, unless it is specifically stated in the figure legend that the group includes biological replicates from cell subpopulations from the same subject. Randomization, blinding and replication practices are specified in each applicable section.

Human specimens

All human subjects' research in this study, including the use of human tissues, conformed to the principles outlined in the Declaration of Helsinki. All patient information was de-identified. Exclusion criteria for this study included Marfan's syndrome, congenital MV abnormalities, endocarditis, rheumatic heart disease, ischemic MR, a history of cancer, autoimmune diseases, previous mitral surgery, and any history of cardiac trauma. To the best of our knowledge no patients with the X-linked Filamin-A mutation associated with MR were included.

Patient enrollment: MR patients

Patients with MR referred for first time surgery at the participating hospitals from 2009–2022 were enrolled in this study. Informed consent per IRB approval was obtained at either The Hospital of the University of Pennsylvania (IRB Protocol #809349), The Valley Hospital (IRB Protocol#11.0009), or Columbia University Irving Medical Center (IRB Protocol #AAAR6796) upon admission prior to surgery.

Patient enrollment: Normal tissue

Normal MV tissue was comprised by two groups: i) MV tissue from patients undergoing cardiac transplant with no MV disease, and ii) MV tissue isolated from healthy hearts from cardiac donors that were allocated for cardiac transplant but ultimately not transplanted for logistic reasons. Group i: Normal MV leaflets, together with de-identified clinical data, were obtained from the heart transplant service of the Hospital of the University of Pennsylvania with IRB approval (IRB Protocol #802781). Normal leaflet retrievals excluded valve leaflets from patients with MV disease. Group ii: Subjects with no known cardiopulmonary disease whose organs were listed but were unable to be placed at the time of organ recovery for heart transplantation and who consented to donate tissue for research purposes by Live On New York (previously New York Organ Donor Network) were included in this study.

SERT$^{-/-}$ mice studies

With IACUC approval, mice lacking a functional serotonin transporter (*SERT*$^{-/-}$, B6.129(Cg)-Slc6a4tm1Kpl/J) were obtained from Jackson Laboratories between ages 4–6 weeks. WT mice of C57BL/6J background were used as control. Groups were comprised of equal numbers of male and female mice. Mice were sacrificed at 8 weeks of age and hearts were perfused with phosphate-buffered saline (PBS) and harvested. A subset of hearts (n≥5/group) were stored in formalin for subsequent histological analysis. A separate subset of hearts (n≥5/group) were micro-dissected and MV leaflets were processed for RNA isolation.

Fluoxetine mouse studies

C57BL/6 mice (equal numbers of males and females) were purchased from Jackson Laboratories at 60 days of age. With IACUC approval, two groups of mice were studied (n=10 each), a fluoxetine group, treated with 20mg/kg/day provided in drinking water with monitoring, and an untreated group. Mice were randomized into control or treatment groups. The dose was aimed to achieve functional SERT occupancy and produce drug serum concentrations on the high end of clinically relevant serum concentrations (40). Mice were sacrificed after 8 weeks and hearts were perfused with PBS and harvested as described above.

MVIC isolation and culture

Human MV leaflets were minced and digested with 1 mg/ml collagenase type 2 (Worthington Biochemical Corporation) and 100 IU/mL hyaluronidase (Worthington) in complete growth medium for 16h-24h at 37°C. After digestion, the cell suspension was pelleted in a centrifuge (5 min, 220 g). Cells were cultivated in complete growth medium (Advanced DMEM containing 4.5g/ml glucose, supplemented with 10% fetal bovine serum (FBS), 4 mM L-glutamine, 1% penicillin/streptomycin). Phenotypic validation of MR and normal MVICs was performed by assessing expression of MVIC markers using PCR (αSMA Hs00426835_g1, and desmin, Hs00157258_m1, both FAM-based Taqman probes from Thermo) and confirming the absence of expression of the endothelial cell marker CD31 (Hs01065279_m1, Thermo). Multiple vials of MVIC subpopulations from passages 0–2 were cryopreserved from each subject. Cryorecovered MVICs at passages 2–6 were used for all experiments. MVIC from each 5-HTTLPR genotype were randomized into different in vitro experiments as detailed in table S5.

Statistical analysis

Clinical data, both from the local patient population and the Optum dataset, were assessed using Cox's proportional hazards model where the response variable was the age at MV surgery. Thus, the multivariate analysis approach used needs clarification since cross sectional data were used to determine age at surgery rather than the ideal endpoint of risk of MR. However, since MR surgery is only performed for end-stage disease, this approach was empirically used with the caveat just stated. The rationale for this is that cardiac surgery for MR is the only treatment option, and because of the substantial risks involved, it is deferred as long as possible. MR surgery is never elective. Younger age at surgery indicates more rapidly progressive disease. The covariates were then pared down to a subset that were of statistical significance. RT^2 profiler PCR array results were analyzed by Student's t tests for significance per the manufacturer's analytical software (Qiagen). The data were tested for normality: when it failed, nonparametric tests were used in lieu of parametric tests. The parametric tests that provided the p-values were t-test, two-way or one-way analysis of variance (ANOVA), depending on the number of covariates in the analysis. Values between the covariates were compared using Bonferroni test to control for multiplicity. When the data were sufficiently skewed suggesting the use of a nonparametric test, Mann Whitney U test, paired Kolmogorov-Smirnov test, or Kruskal Wallis were employed with Dunn's test for multiple comparisons. Pearson's correlation coefficient was

used to determine correlations. Analyses were performed in Prism software (GraphPad Software). For all analyses, p values were 2 sided, and $P < 0.05$ was considered significant. Data were expressed as means ± standard error, unless noted. All data and statistic tests employed, including conditions for assumption of normality, are included in the figure legends, supplementary materials and Data File S1.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements:

The authors thank R.A. Norris for his helpful advice on microdissection of mitral valve leaflets from mice and subsequent RNA isolation. The authors also thank P.W. Groeneveld, University of Pennsylvania, for his helpful advice about planning the national database studies. The authors are grateful to K. Margulies and K. Bedi, The University of Pennsylvania, for their assistance in obtaining anatomically normal mitral valve leaflets as permitted by IRB Protocol #802781. The authors thank G. Anderson, Yale University, for providing expertise in serum serotonin measurements. The authors thank P.A. Kurlansy and M. Argenziano, Columbia University, for their revision of the manuscript prior to submission. These studies used the resources of the Herbert Irving Comprehensive Cancer Center Confocal and Specialized Microscopy Shared Resource and the Molecular Pathology core funded in part through Center Grant P30CA013696.

Funding:

This work was supported, in part, by a National Heart, Lung and Blood Institute of the National Institutes of Health (R01-HL131872) (to G.F. and R.J.L.), the Kibel Fund for Aortic Valve Research (to G.F. and R.J.L.), the Valley Hospital Foundation ‘Marjorie C. Bunnel’ charitable fund (to G.F.), the Andrew Sabin Family Foundation Cardiovascular Research Laboratory (to G.F.), the Erin Beatty Fund (to R.J.L.), and the William J. Rashkind Endowment of the Children’s Hospital of Philadelphia (to R.J.L.).

Data and materials availability:

All data and methods associated with this study are present in the paper or the Supplementary Materials. The $SERT^{-/-}$ mice are available from the corresponding author under a material transfer agreement with Columbia University.

References

1. Nkomo VT, Gardin JM, Skelton TN, Gottdiener JS, Scott CG, Enriquez-Sarano M, Burden of valvular heart diseases: a population-based study. Lancet. 368, 1005–1011 (2006). [PubMed: 16980116]
2. Moller JE, Pellikka PA, Bernheim AM, Schaff HV, Rubin J, Connolly HM, Prognosis of carcinoid heart disease: analysis of 200 cases over two decades. Circulation. 112, 3320–3327 (2005). [PubMed: 16286584]
3. Connolly HM, Crary JL, McGoon MD, Hensrud DD, Edwards BS, Edwards WD, Schaff HV, Valvular heart disease associated with fenfluramine-phentermine. N. Engl. J. Med 337, 581–588 (1997). [PubMed: 9271479]
4. Volmar KE, Hutchins GM. Aortic and mitral fenfluramine-phentermine valvulopathy in 64 patients treated with anorectic agents. Arch. Pathol. Lab. Med 125, 1555–1561 (2001). [PubMed: 11735689]
5. Disatian S, Orton EC. Autocrine serotonin and transforming growth factor beta 1 signaling mediates spontaneous myxomatous mitral valve disease. J. Heart Valve Dis 18, 44–51 (2009). [PubMed: 19301552]
6. Hutcheson JD, Ryzhova LM, Setola V, Merryman WD, 5-HT(2B) antagonism arrests non-canonical TGF-beta1-induced valvular myofibroblast differentiation. J. Mol. Cell. Cardiol 53, 707–714 (2012). [PubMed: 22940605]

7. Møller JE, Connolly HM, Rubin J, Seward JB, Modesto K, Pellikka PA. Factors associated with progression of carcinoid heart disease. N. Engl. J. Med 348, 1005–1015 (2003). [PubMed: 12637610]
8. Zanettini R, Antonini A, Gatto G, Gentile R, Tesei S, Pezzoli G, Valvular heart disease and the use of dopamine agonists for Parkinson's disease. N. Engl. J. Med 356, 39–46 (2007). [PubMed: 17202454]
9. Rothman RB, Baumann MH, Savage JE, Rauser L, McBride A, Hufeisen SJ, Roth BL, Evidence for possible involvement of 5-HT(2B) receptors in the cardiac valvulopathy associated with fenfluramine and other serotonergic medications. Circulation. 102, 2836–2841 (2000). [PubMed: 11104741]
10. Rothman RB, Ayestas MA, Dersch CM, Baumann MH, Aminorex, fenfluramine, and chlorphentermine are serotonin transporter substrates. Implications for primary pulmonary hypertension. Circulation. 100, 869–875 (1990).
11. Berger M, Gray JA, Roth BL, The expanded biology of serotonin. Annu. Rev. Med 60, 355–366 (2009). [PubMed: 19630576]
12. Lesch KP, Bengel D, Heils A, Sabol SZ, Greenberg BD, Petri S, Benjamin J, Müller CR, Hamer DH, Murphy DL, Association of anxiety-related traits with a polymorphism in the serotonin transporter gene regulatory region. Science. 274, 1527–1531 (1996). [PubMed: 8929413]
13. Castillero E, Howsmon DP, Rego BV, Keeney SJ, Driesbaugh KH, Kawashima T, Xue Y, Camillo C, George I, Gorman RC, Gorman JH, Sacks MS, Levy RJ, Ferrari G, Altered Responsiveness to TGFβ and BMP and Increased CD45+ Cell Presence in Mitral Valves Are Unique Features of Ischemic Mitral Regurgitation. Arterioscler Thromb Vasc Biol. 41, 2049–2062 (2021). [PubMed: 33827255]
14. Henke A, Kovalyova Y, Dunn M, Dreier D, Gubernator NG, Dincheva I, Hwu C, Šebej P, Ansorge MS, Sulzer D, Sames D, Toward Serotonin Fluorescent False Neurotransmitters: Development of Fluorescent Dual Serotonin and Vesicular Monoamine Transporter Substrates for Visualizing Serotonin Neurons. ACS Chem. Neurosci 16, 925–934 (2018).
15. Oyama MA, Elliott C, Loughran KA, Kossar AP, Castillero E, Levy RJ, Ferrari G, Comparative pathology of human and canine myxomatous mitral valve degeneration: 5HT and TGF-β mechanisms. Cardiovasc. Pathol 46, 107196 (2020). [PubMed: 32006823]
16. Ayme-Dietrich E, Lawson R, Da-Silva S, Mazzucotelli JP, Monassier L. Serotonin contribution to cardiac valve degeneration: new insights for novel therapies? Pharmacol Res. 140, 33–42 (2019). [PubMed: 30208338]
17. Brattelid T, Qvigstad E, Birkeland JA, Swift F, Bekkevold SV, Krobert KA, Sejersted OM, Skomedal T, Osnes JB, Levy FO, Sjaastad I, Serotonin responsiveness through 5-HT2A and 5-HT4 receptors is differentially regulated in hypertrophic and failing rat cardiac ventricle. J. Mol. Cell. Cardiol 43, 767–779 (2007) [PubMed: 17936780]
18. Jian B, Connolly J, Savani RC, Narula N, Liang B, Levy R. Serotonin mechanisms in heart valve disease I: Serotonin-induced up-regulation of transforming growth factor-beta1 via G-protein signal transduction in aortic valve interstitial cells. Am. J. Pathol 161, 2111–2121 (2002). [PubMed: 12466127]
19. Xu J, Jian B, Chu R, Lu Z, Li Q, Dunlop J, Rosenzweig-Lipson S, McGonigle P, Levy RJ, Liang B, Serotonin mechanisms in heart valve disease II: the 5-HT2 receptor and its signaling pathway in aortic valve interstitial cells. Am. J. Pathol 161, 2209–2218. (2002). [PubMed: 12466135]
20. Mekontso-Dessapa A, Brouri F, Pascal O, Lechat P, Hanoun N, Lanfumey L, Seif I, Benhaiem-Sigaux N, Kirsch M, Hamon M, Adnot S, Eddahibi S, Deficiency of the 5-hydroxytryptamine transporter gene leads to cardiac fibrosis and valvulopathy in mice. Circulation, 113, 81–89 (2006). [PubMed: 16380550]
21. Ayme-Dietrich E, Aubertin-Kirch G, Maroteaux L, Monassier L. Cardiovascular remodeling and the peripheral serotonergic system. Arch. Cardiovasc. Dis 110, 51–59 (2017). [PubMed: 28017279]
22. Driesbaugh KH, Branchetti E, Grau JB, Keeney SJ, Glass K, Oyama MA, Rioux N, Ayoub S, Sacks MS, Quackenbush J, Levy RJ, Ferrari G. Serotonin receptor 2B signaling with interstitial cell activation and leaflet remodeling in degenerative mitral regurgitation. J. Mol. Cell. Cardiol 115, 94–103 (2018). [PubMed: 29291394]

23. Fong F, Xian J, Demer LL, Tintut Y, Serotonin receptor type 2B activation augments TNF-α-induced matrix mineralization in murine valvular interstitial cells. J. Cell. Biochem 122, 249–258 (2021). [PubMed: 32901992]
24. Heils A, Teufel A, Petri S, Stober G, Riederer P, Bengel D, Lesch KP, Allelic variation of human serotonin transporter gene expression. J. Neurochem 66, 2621–2624 (1996). [PubMed: 8632190]
25. Fumeron F, Betoulle D, Nicaud V, Evans A, Kee F, Ruidavets JB, Arveiler D, Luc G, Cambien F, Serotonin transporter gene polymorphism and myocardial infarction: Etude Cas-Temoins de l'Infarctus du Myocarde (ECTIM). Circulation. 25, 2943–2945 (2002).
26. Jiao YR, Wang W, Lei P, Jia H, Dong J, Gou Y, Chen C, Cao J, Wang Y, Zhu Y, 5-HTT, BMPR2, EDN1, ENG, KCNA5 gene polymorphisms and susceptibility to pulmonary arterial hypertension: A meta-analysis. Gene. 680, 34–42 (2019). [PubMed: 30218748]
27. Culverhouse RC, Saccone NL, Horton AC, Ma Y, Anstey KJ, Banaschewski T, Burmeister M, Cohen-Woods S, Etain B, Fisher HL, Goldman N, Guillaume S, Horwood J, Juhasz G, Lester KJ, Mandelli L, Middeldorp CM, Olié E, Villafuerte S, Air TM, Araya R, Bowes L, Burns R, Byrne EM, Coffey C, Coventry WL, Gawronski KAB, Glei D, Hatzimanolis A, Hottenga JJ, Jaussent I, Jawahar C, Jennen-Steinmetz C, Kramer JR, Lajnef M, Little K, Zu Schwabedissen HM, Nauck M, Nederhof E, Petschner P, Peyrot WJ, Schwahn C, Sinnamon G, Stacey D, Tian Y, Toben C, Van der Auwera S, Wainwright N, Wang JC, Willemsen G, Anderson IM, Arolt V, Åslund C, Bagdy G, Baune BT, Bellivier F, Boomsma DI, Courtet P, Dannlowski U, de Geus EJC, Deakin JFW, Easteal S, Eley T, Fergusson DM, Goate AM, Gonda X, Grabe HJ, Holzman C, Johnson EO, Kennedy M, Laucht M, Martin NG, Munafò MR, Nilsson KW, Oldehinkel AJ, Olsson CA, Ormel J, Otte C, Patton GC, Penninx BWJH, Ritchie K, Sarchiapone M, Scheid JM., Serretti A, Smit JH, Stefanis NC, Surtees PG, Völzke H, Weinstein M, Whooley M, Nurnberger JI, Breslau N, Bierut LJ, Collaborative meta-analysis finds no evidence of a strong interaction between stress and 5-HTTLPR genotype contributing to the development of depression. Mol. Psychiatry 23,133–142 (2018). [PubMed: 28373689]
28. Areeshi MY, Haque S, Panda AK, Mandal RK. A serotonin transporter gene (SLC6A4) polymorphism is associated with reduced risk of irritable bowel syndrome in American and Asian population: a meta-analysis. PLoS One. 8, e75567 (2013) [PubMed: 24069428]
29. Murdoch JD, C Speed W, Pakstis AJ, Heffelfinger CE, Kidd KK, Worldwide population variation and haplotype analysis at the serotonin transporter gene SLC6A4 and implications for association studies. Biol. Psychiatry 74, 879–89 (2013). [PubMed: 23510579]
30. Murphy DL, Moya PR, Human serotonin transporter gene (SLC6A4) variants: their contributions to understanding pharmacogenomic and other functional G×G and G×E differences in health and disease. Curr. Opin. Pharmacol 11, 3–10 (2011). [PubMed: 21439906]
31. Martini F, Zuppiroli A, Gori A, Chiarantini E, Fedi S, Prisco D, Cellai A, Boddi V, Abbate R, Dolara A, Gensini G, Platelet and blood clotting activation in patients with mitral valve prolapse. Thromb. Res 83, 299–306 (1996). [PubMed: 8870174]
32. Cremer SE, Zois NE, Moesgaard SG, Ravn N, Cirera S, Honge JL, Smerup MH, Hasenkam JM, Sloth E, Leifsson PS, Falk T, Oyama MA, Orton C, Martinussen T, Olsen LH, Serotonin markers show altered transcription levels in an experimental pig model of mitral regurgitation. Vet. J 203,192–198 (2015). [PubMed: 25599900]
33. Scruggs SM, Disatian S, Orton EC. Serotonin transmembrane transporter is down-regulated in late-stage canine degenerative mitral valve disease. J. Vet. Cardiol 12, 163–169 (2010). [PubMed: 21036114]
34. Nezafati MH, Eshraghi A, Vojdanparast M, Abtahi S, Nezafati P. Selective serotonin reuptake inhibitors and cardiovascular events: A systematic review. J. Res. Med. Sci 21, 66 (2016). [PubMed: 27904611]
35. Maréchaux S, Jeu A, Jobic Y, Ederhy S, Donal E, Réant P, Abouth S, Arnasteen E, Boulanger J, Ennezat PV, Garban T, Szymanski C, Tribouilloy C, Impact of selective serotonin reuptake inhibitor therapy on heart valves in patients exposed to benfluorex: a multicentre study. Arch. Cardiovasc. Dis 106, 349–356 (2013). [PubMed: 23876809]
36. Fortier JH, Pizzarotti B, Shaw RE, Levy RJ, Ferrari G, Grau J, Drug-associated valvular heart diseases and serotonin-related pathways: a meta-analysis. Heart. 105, 1140–1148 (2019). [PubMed: 31129607]

37. Kandula V, Kislitsina ON, Rigolin VH, Thomas JD, C Malaisrie S, Andrei AC, Ramesh A, Kruse J, Cox JL, McCarthy PM Does gender bias affect outcomes in mitral valve surgery for degenerative mitral regurgitation? Interact Cardiovasc Thorac Surg. 33, 325–332 (2021). [PubMed: 33893493]

38. Snider JC, Riley LA, Mallory NT, Bersi MR, Umbarkar P, Gautam R, Zhang Q, Mahadevan-Jansen A, Hatzopoulos AK, Maroteaux L, Lal H, Merryman WD, Targeting 5-HT2B Receptor Signaling Prevents Border Zone Expansion and Improves Microstructural Remodeling After Myocardial Infarction. Circulation. 143, 1317–1330 (2021). [PubMed: 33474971]

39. Shimizu K, Sunagawa Y, Funamoto M, Honda H, Katanasaka Y, Murai N, Kawase Y, Hirako Y, Katagiri T, Yabe H, Shimizu S, Sari N, Wada H, Hasegawa K, Morimoto T, The Selective Serotonin 2A Receptor Antagonist Sarpogrelate Prevents Cardiac Hypertrophy and Systolic Dysfunction via Inhibition of the ERK1/2-GATA4 Signaling Pathway. Pharmaceuticals (Basel). 14, 1268 (2021) [PubMed: 34959669]

40. Nackenoff AG, Moussa-Tooks AB, McMeekin AM, Veenstra-VanderWeele J, Blakely RD. Essential Contributions of Serotonin Transporter Inhibition to the Acute and Chronic Actions of Fluoxetine and Citalopram in the SERT Met172 Mouse. Neuropsychopharmacology. 41, 1733–1741 (2016). [PubMed: 26514584]

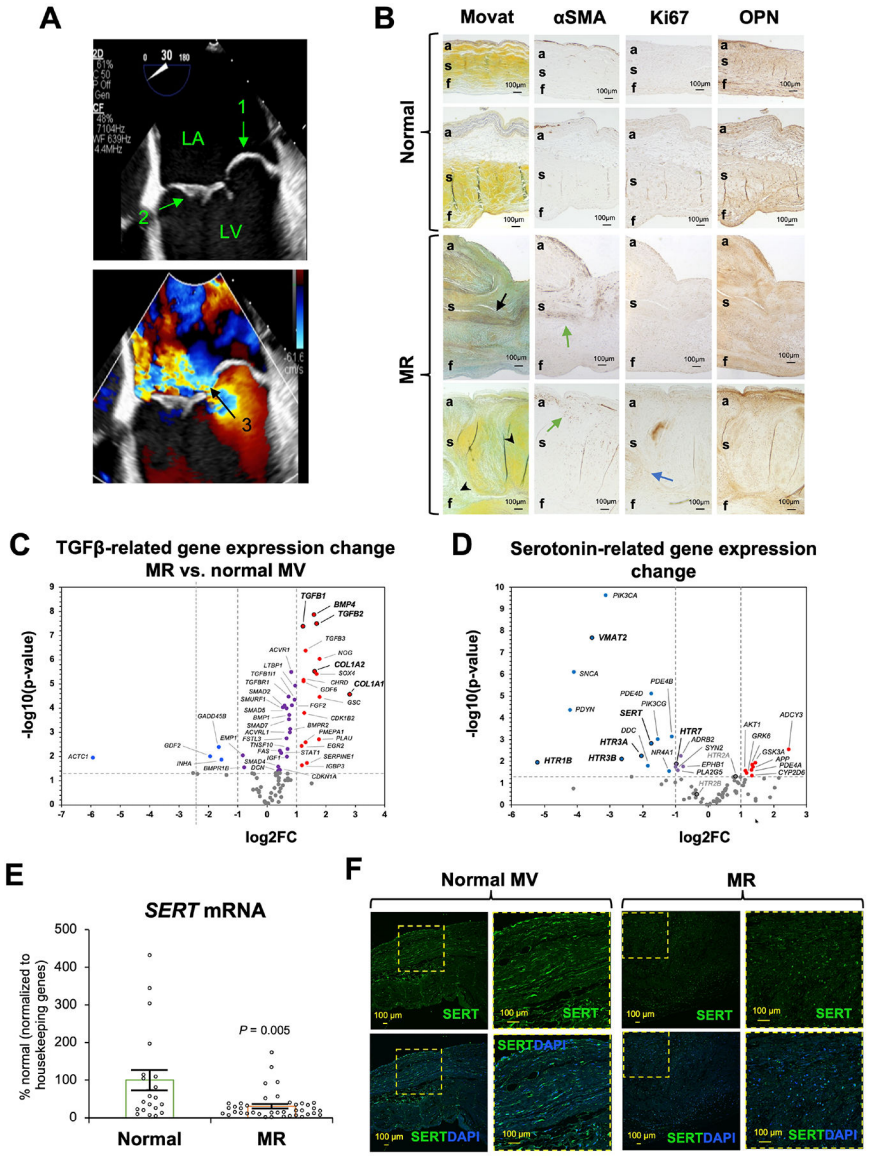

Figure 1: Phenotype of degenerative MR MV leaflets includes *SERT* expression downregulation.

(A) Representative echocardiography images of a patient with MR prior to MV surgery; LA, left atrium; LV, left ventricle. Arrow 1 (top panel, green) indicates the thickened and prolapsed posterior mitral leaflet. Arrow 2 (top panel, green) indicates the thickened anterior mitral leaflet with shallowed coaptation depth. Arrow 3 (bottom panel, black) indicates torrential anterior directed MR jet. **(B)** Representative IHC staining of normal MV samples from heart donors (top two rows) and MR MV samples recessed during surgery (bottom two rows); a, zona atrialis; f, zona fibrosa; s, zona spongiosa. Movat's stain shows accumulation of elastic fibers (stained in black, black arrow) and an increase in glycosaminoglycans (stained in blue, black arrowheads) intertwined with collagen (stained in yellow). IHC of αSMA shows areas with increased MV interstitial cell activation in MR MV (green arrows). IHC of Ki67 shows occasional areas of cell proliferation in MR MV (blue arrow). Right column shows IHC for the matricellular protein osteopontin (OPN). Volcano plots

of gene expression results by RT^2 profiler PCR panels of (**C)** TGFβ-related genes and **(D)** serotonin-related genes in MR tissue (n=44) in comparison with normal MV tissue (n=20). Horizontal dotted lines denote the genes with −log10 > 1.3 (corresponding to a p value = 0.05 by Student t test, purple dots), vertical dotted lines define genes with a log2 fold change > 1 (red dots) or < −1 (blue dots), corresponding to fold change = 2 and 0.5 versus normal, respectively. **(E)** Scatter dot plot of *SERT* expression from RT^2 profiler PCR in normal (n=20) and MR MV tissue (n=44). Re-analysis of data included in panel D. Error bars indicate standard error. P value by Mann Whitney U test. **(F)** Representative immunofluorescence staining of SERT in normal and MR MV tissue (green). Within each sample, yellow dotted box area is shown with higher agnification to the right of the main image. Nuclei are visualized by DAPI stain (blue). All IHC/immunofluorescence were n≥4/group. All gene expression results were calculated by the 2−ΔΔCT method and normalized to housekeeping genes.

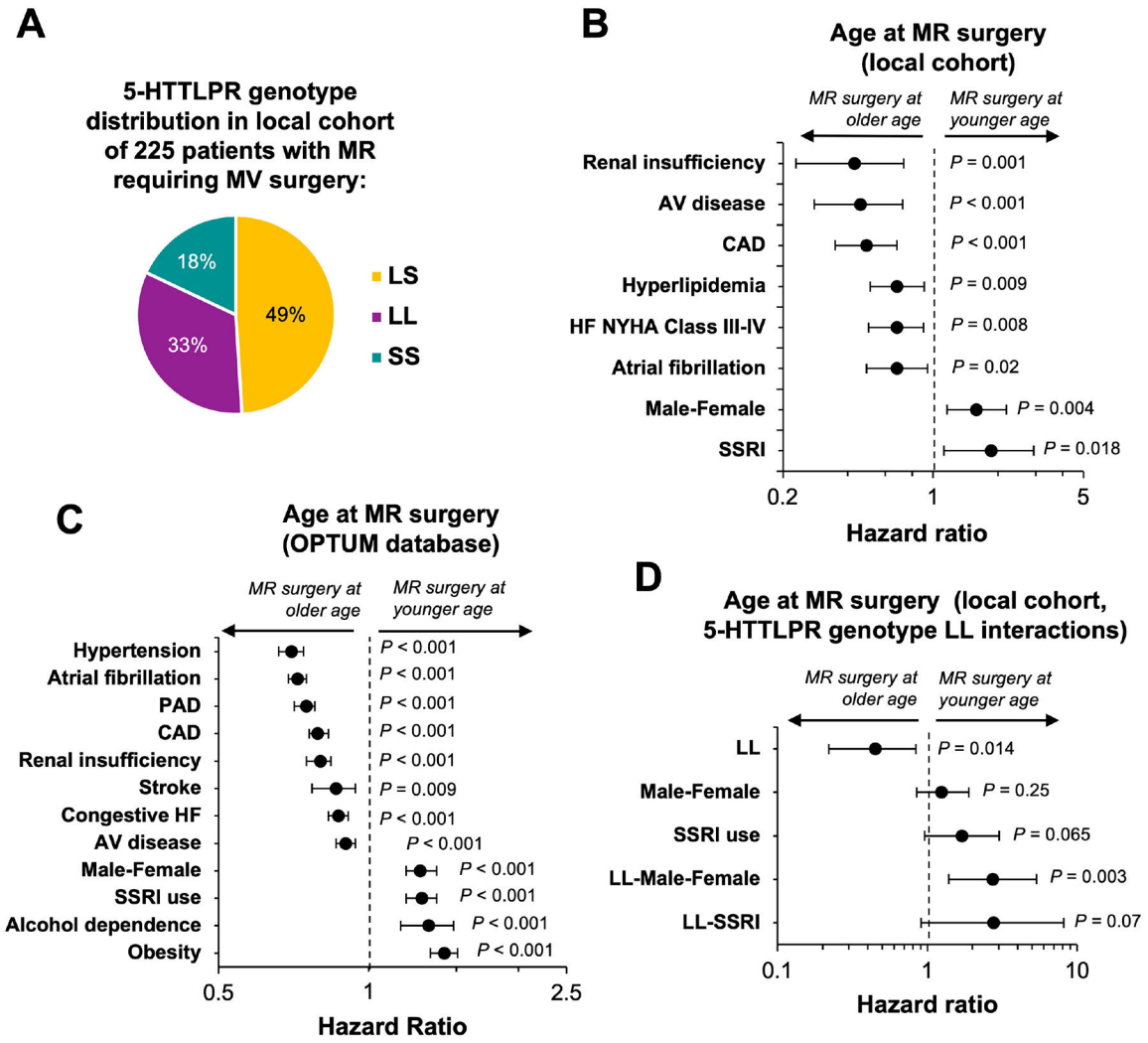

Figure 2: SSRI use and 5-HTTLPR genotype LL impact the timing and need for MV surgery in degenerative MR patients.

(A) Pie chart illustrating the distribution of 5-HTTLPR genotypes in a local cohort of MR patients (n=225). **(B-D)** Multivariate Cox model analysis results of patients with degenerative MR undergoing cardiac surgery. Data were assessed employing Cox's proportional hazards model where the response variable was the age at MV surgery. Results corresponding to **(B)** our local cohort (n=225) and **(C)** Optum database population (n=9441). **(D)** Sub-analysis of the local cohort (n=225) showing interactions with 5-HTTLPR genotype LL. Values are shown as hazard ratio ± lower and upper 95th percentile confidence limits, reflecting the hazard of requiring surgery at younger ages (hazard ratio>1.0) compared to older age at surgery (hazard ratio<1.0). AV, aortic valve; CAD, coronary artery disease; HF, heart failure, NYHA, New York Heart association; SSRI, serotonin reuptake inhibitor; PAD, peripheral artery disease; male-female refers to "male over female" variable in the multivariate model.

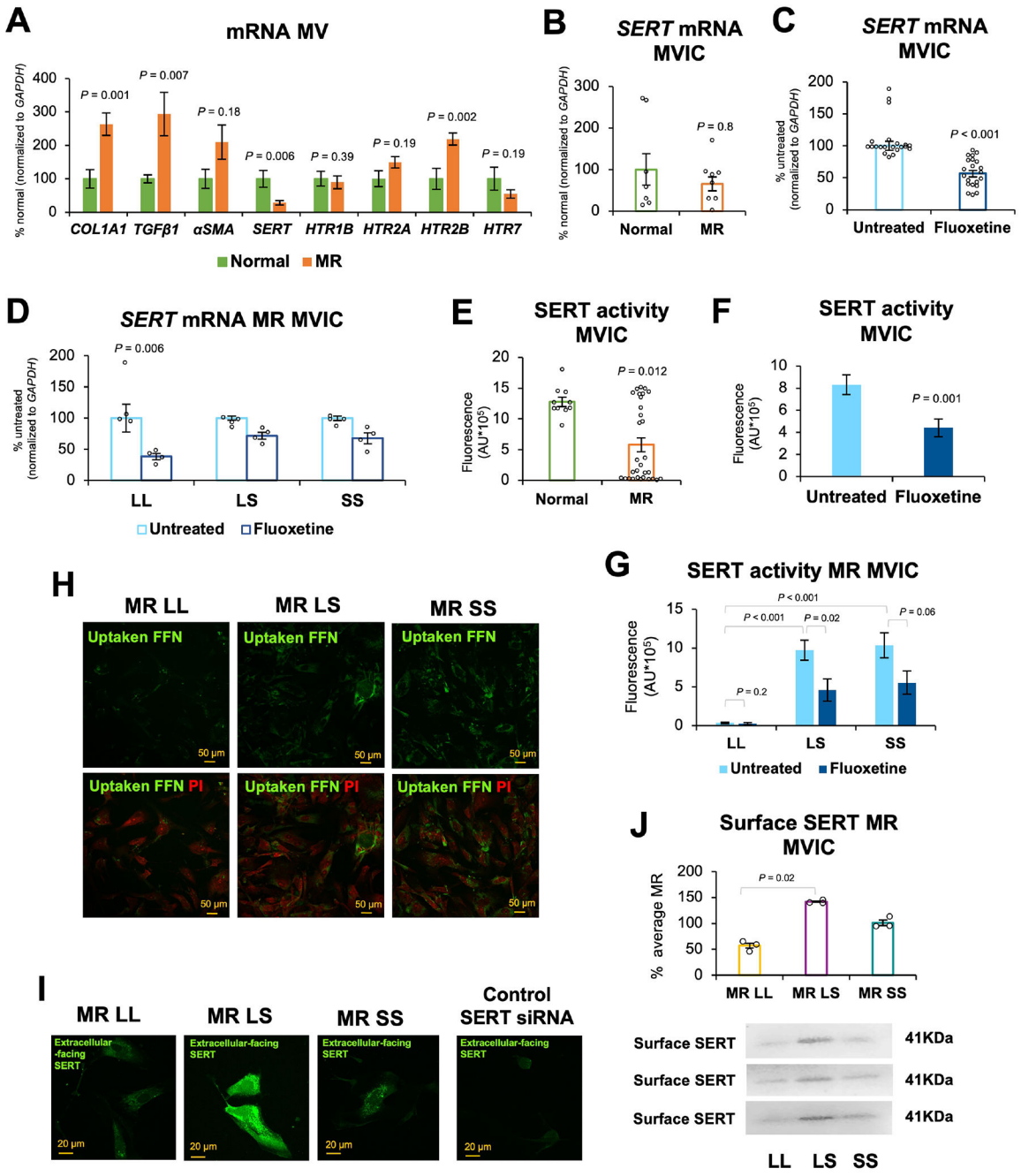

Figure 3: 5-HTTLPR genotype LL and the SSRI fluoxetine decrease the activity of SERT in MVICs.

(A) Gene expression in a sub-cohort of MV tissue from human normal MV tissue (n=9) and MV from patients with degenerative MR undergoing mitral valve surgery (n=32). **(B)** Gene expression of *SERT* at baseline in MVIC isolated from normal and MR MV (n≥8/group). **(C)** Gene expression of *SERT* in MVIC (normal and MR combined) after seven-day treatment with 1μM fluoxetine (n≥21/group, biological replicates corresponding to MVIC subpopulations from 13 MR patients). **(D)** Gene expression of *SERT* in MR MVIC stratified by 5-HTTLPR genotype after seven-day treatment with 1μM fluoxetine

(n≥4/group). Re-analysis of data included in panel C. All gene expression results calculated by the 2−ΔΔCT method and presented as % of the mean of corresponding control (normal or untreated) normalized to *GAPDH*. **(E)** SERT activity by FFN246 uptake assay at baseline in MVIC isolated from normal and MR MV (n≥10/group, biological replicates corresponding to MVIC subpopulations from 3 normal subjects and 13 MR patients). **(F)** SERT activity in MVIC (normal and MR combined) with or without 30-minute pre-treatment with 1μM fluoxetine (n=65/group, biological replicates corresponding to MVIC subpopulations from 13 MR patients). **(G)** SERT activity in MR MVIC stratified by 5-HTTLPR genotype with or without 30-minute pre-treatment with 1μM fluoxetine (n≥24 biological replicates corresponding to MVIC subpopulations from ≥ 4 MR patients/group. Re-analysis of data included in panel F). **(H)** Representative images of visualization of FFN246 uptake (green) in MVIC by confocal microscopy. Propidium iodide (PI) stain was performed to aid in cell localization (red). **(I)** Representative confocal microscopy of extracellular SERT in cultured MR MVIC (green), performed with antibody incubation before fixation and without cell permeabilization. Staining performed in control MVIC with silencing RNA-mediated knockdown of *SERT* is shown to indicate non-specific signal (right panel). All immunofluorescence, n≥4/group. **(J)** Quantification of Western blot of SERT in the extracellular cytosolic membrane fraction in MR MVIC following surface biotinylation pull-down assay, and representative images from blots with different samples in each (n=3/group, estimated chance or type-I error = 0.027). All data are shown as % of the mean of all samples in each blot. Error bars indicate standard error. In vitro assays were run a minimum of three times. P values by Mann Whitney U test when two groups were compared (panels A, B, C, E, F and G), two-way ANOVA with Bonferroni (panel D), or Kruskal Wallis with Dunn’s multiple comparison test (panels G and J), when appropriate.

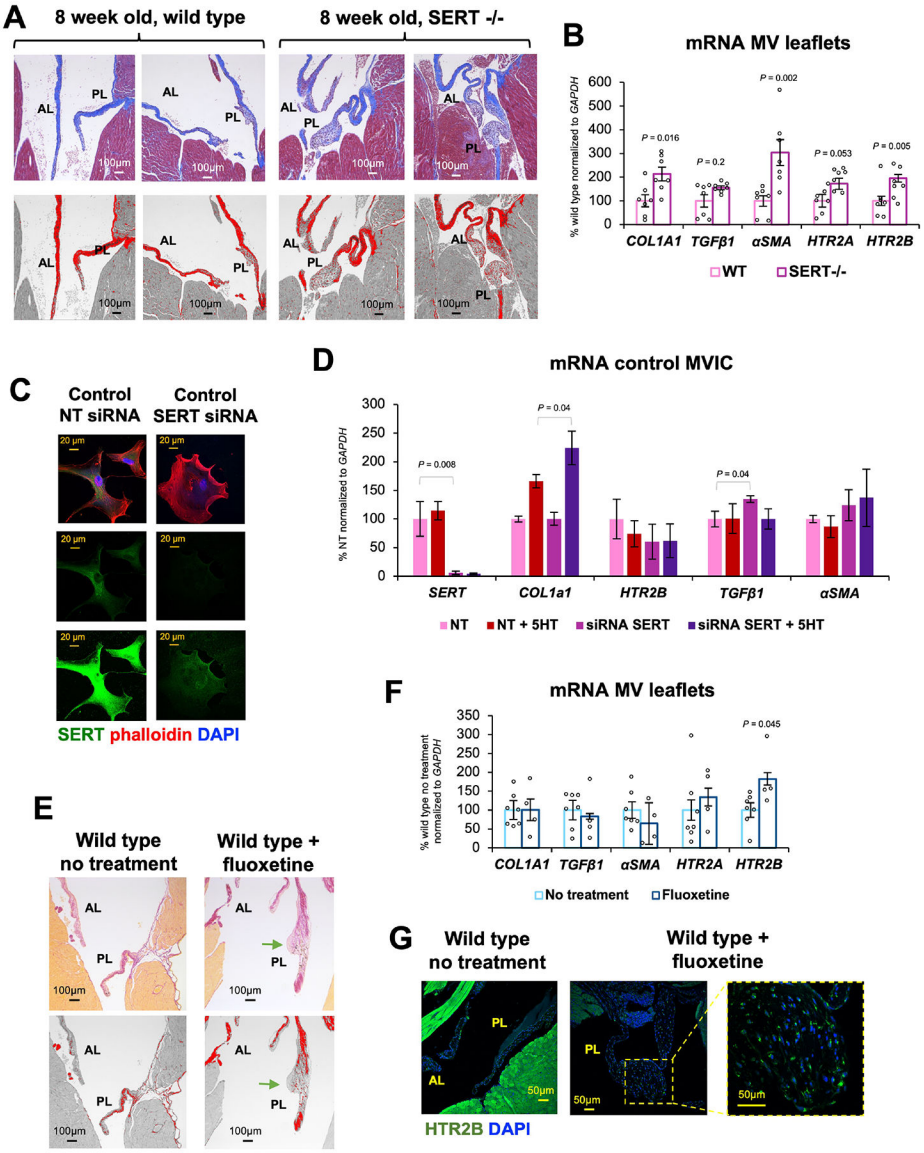

Figure 4: SERT downregulation alters *HTR2B* expression and increases ECM component gene expression in the MV.

(A) Representative trichrome staining of heart sections of 8-week-old wild type and *SERT*$^{+/-}$ mice, showing the MV leaflets; AL, anterior leaflet; PL, posterior leaflet. Bottom row shows colorimetric thresholding to highlight collagen in red. **(B)** Gene expression of *COL1A1*, *TGFβ1*, *αSMA*, *HTR2A* and *HTR2B* and in MV leaflets from 8-week-old wild type (WT) and *SERT*$^{+/-}$ mice (n =7/group). **(C)** Representative confocal microscopy of SERT (green) in human control MVIC with silencing RNA (siRNA)-mediated knockdown of *SERT* or non-targeting (NT) RNA, performed with cell permeabilization. Phalloidin (red) was used to visualize cell structure and nuclei are visualized by DAPI stain (blue). Bottom row images have been overexposed to show SERT signal in knocked-down MVIC. **(D)** Gene expression of *SERT*, *COL1A1*, *HTR2B*, *TGFβ1* and *αSMA* in human control MVIC with *SERT* or NT siRNA, treated for 24 hours with or without 10μM of serotonin (5HT, n=5/group). **(E)** Representative Picrosirius red staining of heart sections of 120-day-old

wild type mice treated with or without 20 mg/kg/day fluoxetine for 60 days, showing the thickened MV leaflets (green arrows). Bottom row shows colorimetric thresholding to highlight collagen in red. **(F)** Gene expression of *COL1A1*, *TGFβ1*, *αSMA*, *HTR2A* and *HTR2B* in MV leaflets from wild type mice treated with or without fluoxetine (n≥4/group). **(G)** Representative immunofluorescence staining of HTR2B (green) in MV leaflets from wild type mice treated with or without fluoxetine. DAPI used to denote nuclei (blue). Green signal in the myocardium (top left and bottom right corners of left panel) includes unspecific tissue autofluorescence. All IHC, n≥4/group. All gene expression results calculated by the 2−ΔΔCT method and presented as % of the mean of corresponding control (WT or non-treated) normalized to *GAPDH*. Assays were run a minimum of three times. Error bars indicate standard error. P values by Mann Whitney U test (panel B) or Student's T-test (panel F) when two groups were compared, two-way ANOVA with Bonferroni multiple comparison test (panel D), when appropriate.

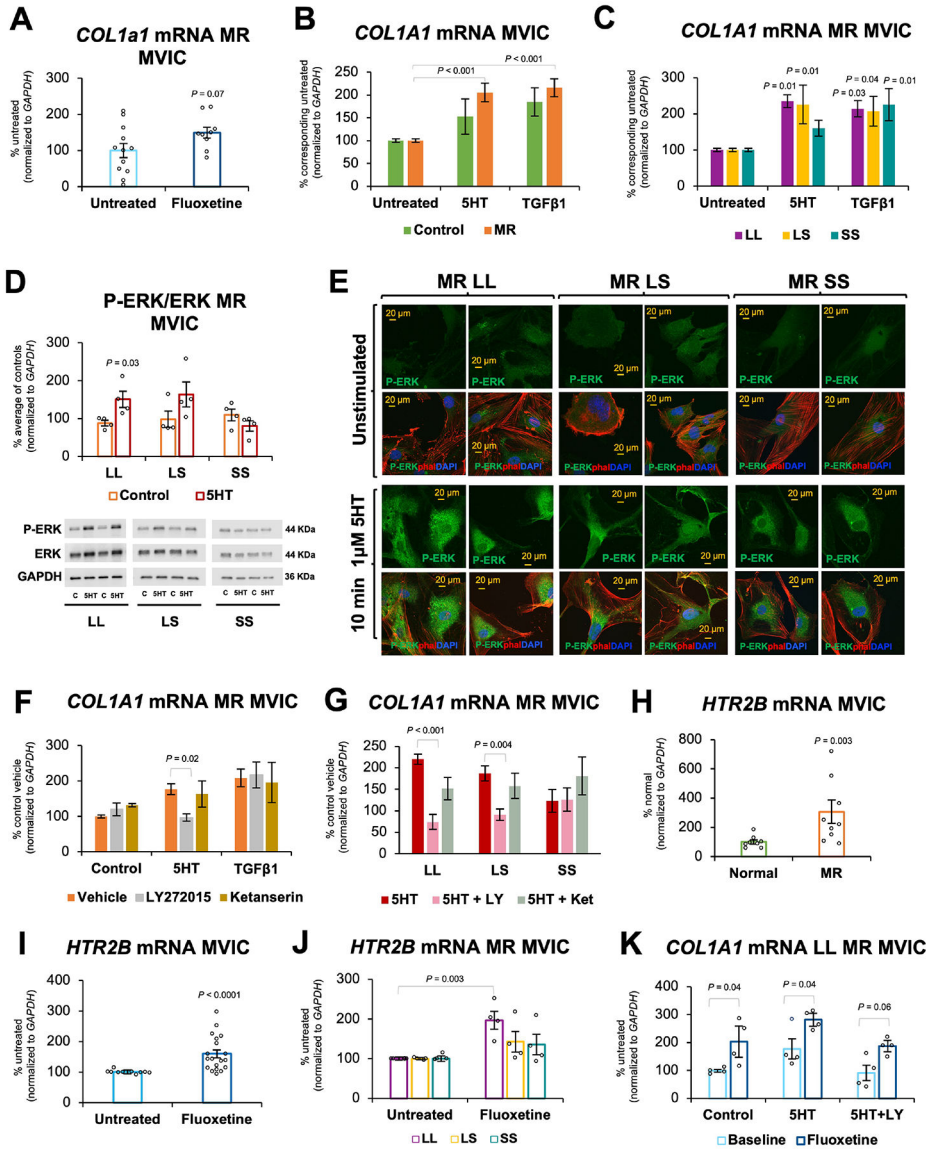

Figure 5: SERT downregulation increases *COL1A1* expression through a HTR2B-mediated mechanism.

(A) Gene expression of *COL1A1* in human MVIC (normal and MR combined) after seven-day treatment with 1μM fluoxetine (n≥8/group, biological replicates corresponding to MVIC from 8 normal subjects and 10 MR patients). **(B)** *COL1A1* expression in control and MR MVIC after 24-hour treatment of serotonin (5HT, 10μM), TGFβ1 (10ng/ml) or vehicle (n≥5/group, biological replicates corresponding to MVIC from 5 normal subjects and 17 MR patients). **(C)** Gene expression of *COL1A1* in MR MVIC after 24-hour treatment of serotonin (10μM), TGFβ1 (10ng/ml) or vehicle, stratified by 5-HTTLPR genotype (n=5/group. Re-analysis of data included in panel B). *P* values correspond to comparisons versus corresponding untreated. **(D)** Phosphorylated (P-) ERK (normalized to total ERK and GAPDH) in MR MVIC treated for 10 minutes with 1μM serotonin or vehicle. Quantification above and representative western blots below (n=4/group). **(E)** Representative confocal microscopy of P-ERK (green) in MR MVIC treated for 10 minutes with 1μM serotonin

or vehicle. Phal indicates phalloidin (red), nuclei are visualized by DAPI stain (blue). All IHC, n≥4/group. **(F)** *COL1A1* expression in MR MVIC after 24-hour treatment of serotonin (10μM), TGFβ1 (10ng/ml) with or without HTR2B-inhibitor LY272015 (100nM), HTR2A-inhibitor ketanserin (10μM), or vehicle (n≥6/group). **(G)** *COL1A1* expression in MR MVIC after 24-hour treatment of serotonin with or without inhibitor LY272015 (LY) or ketanserin (ket), stratified by 5-HTTLPR genotype (n≥4/group. Re-analysis of data included in panel F). **(H)** *HTR2B* expression at baseline in MVIC isolated from normal and MR MV(n≥8/group). **(I)** *HTR2B* expression in human MVIC (normal and MR combined) after seven-day treatment with 1μM fluoxetine (n≥10/group, biological replicates corresponding to MVIC subpopulations from 4 normal subjects and 12 MR patients). **(J)** *HTR2B* expression in MR MVIC after seven-day treatment with fluoxetine, stratified by 5-HTTLPR genotype (n≥4/group. Re-analysis of data included in panel I). **(K)** *COL1A1* expression in LL MR MVIC after seven-day treatment with 1μM fluoxetine, with or without subsequent 24-hour treatment of serotonin (10μM) and LY272015 (LY, 100nM) (n≥4/group). All gene expression results calculated by the $2^{-\Delta\Delta CT}$ method and presented as % of the mean of corresponding control normalized to *GAPDH*. Assays were run a minimum of three times, with a minimum of two cell-lines per assay. Error bars indicate standard error. P values by Student's T-test (panel A) or Mann Whitney U test (panels D, H and I) when two groups were compared, two-way ANOVA with Bonferroni multiple comparison test (panels B, C, F, G, J, and K), when appropriate.