

# Hypersensitivity of Zebrafish *htr2b* Mutant Embryos to Sertraline Indicates a Role for Serotonin Signaling in Cardiac Development

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**Abstract:** Selective serotonin reuptake inhibitors (SSRIs) are antidepressants prescribed in 10% of pregnancies in the United States. Maternal use of SSRIs has been linked to an elevated rate of congenital heart defects, but the exact mechanism of pathogenesis is unknown. Previously, we have shown a decrease in cardiomyocyte proliferation, left ventricle size, and reduced cardiac expression of the serotonin receptor 5-HT<sub>2B</sub> in offspring of mice exposed to the SSRI sertraline during pregnancy, relative to offspring of untreated mice. These results suggest that disruption of serotonin signaling leads to heart defects. Supporting this conclusion, we show here that zebrafish embryos exposed to sertraline develop with a smaller ventricle, reduced cardiomyocyte number, and lower cardiac expression of *htr2b* relative to untreated embryos. Moreover, zebrafish embryos homozygous for a nonsense mutation of *htr2b* (*htr2b<sup>sa16649</sup>*) were sensitized to sertraline treatment relative to wild-type embryos. Specifically, the ventricle area was reduced in the homozygous *htr2b* mutants treated with sertraline compared with wild-type embryos treated with sertraline and homozygous *htr2b* mutants treated with vehicle control. Whereas long-term effects on left ventricle shortening fraction and stroke volume were observed by echocardiography in adult mice exposed to sertraline in utero, echocardiograms of adult zebrafish exposed to sertraline as embryos were normal. These results implicate the 5-HT<sub>2B</sub> receptor functions in heart development and suggest zebrafish are a relevant animal model that can be used to investigate the connection between maternal SSRI use and elevated risk of congenital heart defects.

**Key Words:** selective serotonin reuptake inhibitors, cardiac development, serotonin receptors, zebrafish, echocardiography

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## INTRODUCTION

Selective serotonin reuptake inhibitors (SSRIs) are the most commonly prescribed therapy for depression.<sup>1</sup> In the United States, depression affects 14%–20% of pregnant women, and approximately 10% of pregnant women take an SSRI.<sup>1–4</sup> Sertraline is one of the most commonly prescribed SSRIs.<sup>5</sup> A report published in 2005 showed that the infants of mothers taking SSRIs during pregnancy had an increased risk of congenital heart disease.<sup>6</sup> Multiple studies, including several meta-analyses, have subsequently described an association between SSRI exposure in pregnancy and congenital heart defects.<sup>7–12</sup> Despite this association, SSRI use during pregnancy has steadily been increasing over the past decade.

SSRIs inhibit the serotonin transporter, preventing reuptake of serotonin (5-HT).<sup>13</sup> As a result, this leads to an acute increase in extracellular 5-HT; however, concentrations of 5-HT become reduced with long-term SSRI exposure because of autoinhibitory feedback.<sup>13</sup> 5-HT is an important signaling molecule that regulates a variety of cellular processes that are implicated in heart development, including cell migration, proliferation, and differentiation.<sup>14</sup> 5-HT concentrations and SSRI exposure in vitro affect proliferation of embryonic cardiomyocytes.<sup>15,16</sup> Mice lacking tryptophan hydroxylase, a critical enzyme for 5-HT synthesis, develop cardiac insufficiency, as do mice with mutations in the serotonin transporter.<sup>17,18</sup> In addition, the serotonergic system is complex with several serotonin receptors important in ventricular development and cardiovascular regulation.<sup>19</sup> Notably, the 5-HT<sub>2B</sub> receptor, which is expressed on the cell surface of cardiomyocytes, is also critical for normal cardiac development.<sup>20</sup> Inactivating the 5-HT<sub>2B</sub> receptor in mice leads to increased embryonic and neonatal death. In surviving 5-HT<sub>2B</sub> receptor null mice, cardiomyocyte proliferation is reduced and dilated cardiomyopathy develops in adulthood.<sup>20</sup> By contrast, overexpression of 5-HT<sub>2B</sub> receptor in mice leads to ventricular hypertrophy because of an increase in both cardiomyocyte number and size.<sup>21</sup>

We and others have demonstrated adverse cardiac effects in animal models with disrupted serotonin signaling,

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from either SSRI or serotonin-norepinephrine reuptake inhibitor exposure during development.<sup>22–25</sup> Intrauterine fluoxetine exposure in mice leads to a reduction in left ventricular wall thickness and increased mortality.<sup>22</sup> We previously demonstrated that sertraline exposure in pregnant mice affects offspring cardiac development. Specifically, offspring exposed to sertraline in utero had reductions in neonatal cardiomyocyte cross-sectional area and proliferation, decreased expression of the 5-HT<sub>2B</sub> receptor, and reduced ventricular size in adulthood.<sup>23</sup>

Given the prevalence of SSRI use during pregnancy, it is important to better understand the effects of SSRIs on cardiac development. However, there are limitations to using rodent studies to determine whether the effects of SSRI exposure are direct or through a maternal or placental influence. Zebrafish provide an ideal model to address this issue because they develop externally. Zebrafish embryos develop in translucent eggs that can be easily visualized to monitor each stage of cardiac development after fertilization. Moreover, zebrafish embryos are easily amenable to pharmacologic administration and manipulation of gene expression.<sup>26</sup>

Here, we tested our hypothesis that sertraline exposure or 5-HT<sub>2B</sub> disruption in zebrafish embryos leads to reduced ventricular size in zebrafish. Using both the pharmacologic and genetic approaches, we were able to assess the role of serotonin signaling in heart development. Because the genetic networks that regulate vertebrate heart development are well conserved across species, our studies are expected to be highly relevant to human cardiac development.

## METHODS

### Zebrafish Strain, Maintenance, and Sertraline Exposure

Zebrafish were maintained as previously described.<sup>27</sup> All procedures were approved by the University of Iowa Animal Care and Use Committee. Embryos were obtained by natural mating and staged according to morphological criteria or hours postfertilization (hpf) at 28°C or 32°C, as previously described.<sup>28</sup> The following zebrafish lines were used: AB\*/Tuebingen, *Tg(cmlc2:GFP)*,<sup>29</sup> *Tg(nkx2.5:ZsYellow)*,<sup>30</sup> and *Tg(cmlc2:nls-EGFP)*.<sup>31</sup> Zebrafish embryos were exposed daily to either 1% dimethyl sulfoxide (DMSO) for vehicle control or the SSRI sertraline (5–20 μM in 1% DMSO) from 5.5 to 72 hpf or from 20 to 72 hpf.<sup>32,33</sup> The 5.5 hpf exposure window was used because at the blastula stage, myocardial precursors are undifferentiated and are specified bilaterally in the lateral marginal zone.<sup>26</sup> After migration toward the embryonic midline at approximately 15 hpf, the cardiac cells initiate differentiation and express cardiac myosins. A later exposure window was used from 20 to 72 hpf, reflecting a time point after myocardial precursor cell differentiation and migration to midline.

### Mutant Lines

Zebrafish embryos carrying the mutant allele *sa16649* of *htr2b* (*htr2b<sup>sa16649</sup>*) were purchased from the Zebrafish International Resource Center (ZIRC, Eugene, OR). This specific

nonsense mutation incorporates a C-to-A nucleotide transversion in exon 3, introducing a premature stop which results in a loss of 211 amino acids. To genotype the *sa16649* allele, DNA fragments were amplified using the following primer: forward, 5'-CCCTCGCAATCATGATGATCATTACTTGC-3'; reverse, 5'-GATATGACCATTTTCTCTGGTGAAGCAAAAGC-3'. polymerase chain reaction (PCR) amplicons were digested with Hind-III restriction enzyme (New England BioLabs Inc, Ipswich, MA). Bands of 154 base pairs for wild type and 121 + 33 base pairs for homozygous mutants were detected using electrophoresis.

### Live Images

To globally assess for toxicity, live embryos were mounted in 2.5% methylcellulose and bright-field images were taken on a Leica M165FC stereomicroscope with a Leica DFC290 color digital camera at 32, 48, and 72 hpf during the exposure window.

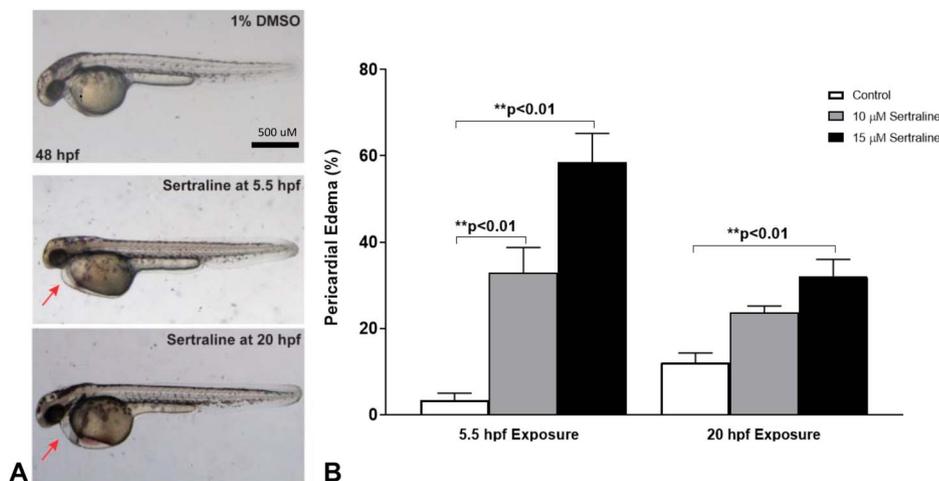
### In situ Hybridization

To evaluate cardiac development at specific stages, in situ hybridization was performed as previously described.<sup>34</sup> The following cardiac specific markers were used: *ventricular myosin heavy chain* (*vmhc*; 32, 48 hpf),<sup>35</sup> *nkx 2.5* (32 hpf),<sup>35</sup> and *cardiac myosin light chain 2* (*cmlc2*; 48 hpf).<sup>36</sup> Whole embryos were mounted in 100% glycerol and photographed using a Leica M165FC stereomicroscope with a Leica DFC290 color digital camera. To evaluate whether expression of cardiac markers was altered in the embryo treatment groups, each embryo per experiment was imaged and ventricle size was measured using ImageJ software.

### Heart Dissection, Confocal Imaging, and Cardiomyocyte Counts

Using the *Tg(cmlc2:nls-EGFP)*<sup>31</sup> zebrafish line, embryos were treated with either 1% DMSO or sertraline from 5.5 to 72 hpf. Hearts were dissected at 48 hpf according to the methods described by Lombardo et al.<sup>37</sup> In brief, embryos were transferred into centrifugation tubes. Embryos were anesthetized with tricaine according to our animal protocols. Tricaine solution was removed, and embryos were washed with Leibovitz's L-15 medium (L-15) and 10% fetal bovine serum (FBS) and maintained on ice. Additional FBS was added, and the solution was pipetted up and down until the yolk was completely disrupted. After deyolking, embryos were pipetted up and released dropwise onto the surface of the L-15/10% FBS solution, causing the heart to release. Embryos were then run through different sized filters (Falcon Cell Strainer, Corning, NY) to eliminate debris. The last filter used was turned upside down, and the hearts were flushed into a well. GFP-positive hearts were manually separated under the fluorescence stereomicroscope. Confocal images were taken using a Zeiss inverted LSM 700 laser scanning confocal microscope with either an EC Plan-Neo 40X/NA 1.3 oil or an LD C-Apo 40X/NA 1.1 water objective. Z-stacks were acquired at 0.5 μM intervals using the following settings: 1024 × 1024 pixels, 8 speed, 4

**FIGURE 1.** Sertraline exposure leads to pericardial edema. A, Live images of zebrafish embryos at 48 hours post fertilization (hpf) treated with 1% dimethyl sulfoxide (DMSO) or sertraline. Time sertraline exposure began is indicated in top right corner. Pericardial edema, a marker of disrupted development, is shown by the red arrow. B, The percentage of embryos with pericardial edema at 72 hpf after being treated with 1% DMSO or sertraline from 5.5 to 72 hpf or 20 to 72 hpf. Data shown as mean  $\pm$  SEM.  $^{***}P < 0.01$  compared to control.



averaging. To quantify the number of cardiomyocytes in each image, ImageJ software was used and GFP-labeled cardiomyocytes were counted by 3 blinded investigators.

### Proliferation Studies

Using the *Tg(cmlc2:nls-EGFP)*<sup>31</sup> zebrafish line, embryos were treated with either 1% DMSO or sertraline from 5.5 to 72 hpf. Hearts were dissected at 72 hpf according to the methods described as above.<sup>37</sup> BrdU was used to assess cardiomyocyte proliferation. BrdU treatment with 5 mg/mL, 1% DMSO in E3 medium for 16 hours occurred at 28°C. Fixed embryos were rinsed and permeabilized as described.<sup>31</sup> Immunofluorescent staining was with anti-GFP (clone B-2, Santa Cruz Biotechnology; 1:200) and rhodamine secondary (ThermoFischer Scientific, Waltham, MA). Images were taken on Confocal Zeiss 700 as described above.

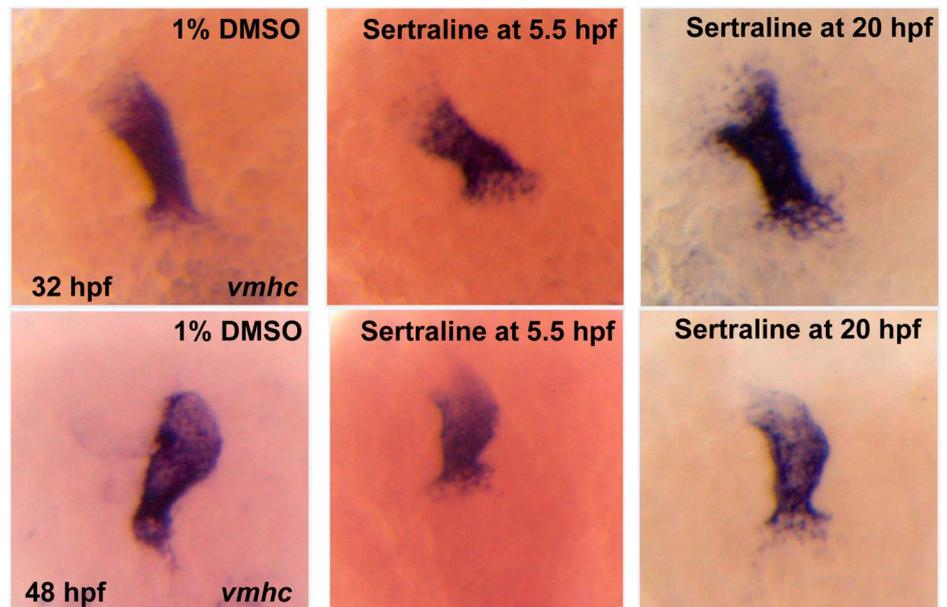
### RNA Isolation and Quantitative Real-Time PCR

To determine the cardiac expression of *htr2b*, heart was dissected as described above from sertraline and 1% DMSO-treated embryos at 48 hpf and transferred into Trizol (Life Technologies, Grand Island, NY). To obtain adequate cardiac mRNA, approximately 100 embryos were used per group as suggested by Lombardo et al.<sup>37</sup> Samples were then processed according to the mRNA isolation protocol from Trizol. In brief, samples were homogenized using a QIAshredder spin column (QIAGEN, Hilden, Germany). Chloroform was added to the solution and incubated at room temperature for 3 minutes. The samples were then centrifuged at 12,000 $\times$ g for 15 minutes at 4°C and the aqueous phase transferred to a new tube. An equal volume of 100% EtOH was added to each sample's aqueous phase, and samples were transferred to the Zymo RNA Clean and Concentrator-25 purification kit (Zymo Research, Irvine, CA). The manufacturer's protocol was followed, with RNA eluted in RNase-free water. RNA concentration and purity were assessed by absorbance measurement. RNA integrity was assessed by electrophoresis on a 1% agarose gel. cDNAs were then synthesized using the iScript cDNA Synthesis kit (Bio-Rad Laboratories,

Hercules, CA). Quantitative real-time PCR was performed with the iQ SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA). Primers (forward: 5'-TGCTGGTAGGACTGCTGGTG-3', reverse: 5'-TGATGGACGCAGTGGAGAAC-3') were used to detect *htr2b*, and primers (forward: 5'-GAGAAGTTCGAGAAGGAAGC-3', reverse: 5'-CGTAGTATTTGCTGGTCTCG-3') were used to detect the housekeeping gene *eef1a1a* as a reference.

### Echocardiography

To assess long-term cardiovascular effects from embryonic exposure, baseline echocardiograms were performed beginning at 4 months of age on zebrafish that received either 1% DMSO or sertraline as embryos. Zebrafish embryos that received 15 µM sertraline from 5.5 to 72 hpf did not survive beyond 1 week of life, so subsequent sertraline doses were decreased to 5, 7.5, and 10 µM. Echocardiograms were performed as described<sup>38</sup> using a Vevo 2100 imager fitted with a 50-Mhz linear array probe (VisualSonics Inc, Toronto, ON, Canada) by an investigator blinded to group assignment. Zebrafish were anesthetized with tricaine. Zebrafish were placed abdominal side facing upwards on a small sponge. The sponge allowed fish to be in stable position while keeping them moist with the water system. The zebrafish body was covered with a thin plastic sheet to protect the gills from ultrasound gel. The probe was applied gently to the chest, and ventricular long-axis and short-axis images were acquired and stored off-line for analysis blinded to treatment condition. Spongiform architecture of zebrafish myocardium precludes ultrasonographic delineation of the blood-endocardium interface. Ventriculographic measurements were therefore based on visual identification of epicardial borders only. Thus, for example, stroke volume is reported as the change in total ventricular volume, including myocardium, from end-diastole to end-systole. Fractional area of change was determined using ventricular long-axis images. Fractional shortening was determined using ventricular short-axis images. End-diastolic volume, stroke volume, and ejection fraction were determined by the bi-plane area-length method, using companion software supplied by the vendor (VisualSonics). The heart rate was determined using pulse-wave Doppler interrogation of ventricular inflow.



**FIGURE 2.** Zebrafish embryos exposed to sertraline from 5.5 to 72 hpf or 20 to 72 hpf demonstrated. Smaller ventricular size at 32 and 48 hpf. In situ hybridization depicts diminished expression of *vmhc* in sertraline-treated embryos treated from 5.5 to 72 hpf or 20 to 72 hpf compared with controls. At 32 and 48 hpf indicating reduced or delayed formation of the ventricle with sertraline exposure.

## Analysis

To assess heart development at the various stages, all in situ hybridization images of the same type were acquired using the same settings, processed using the MetaMorph software, and edited and compiled using Adobe Photoshop and Adobe Illustrator software (San Jose, CA). Data were compiled from each individual embryo. To objectively describe data, image quantification was performed using ImageJ software. All quantification analyses show individual measurements and mean  $\pm$  standard error of the mean. Statistical analyses were performed using the Student's *t* test.  $P < 0.05$  was considered significant.

## RESULTS

### Sertraline-Exposed Zebrafish Embryos Demonstrate Decreased Heart Size and Cardiomyocyte Number

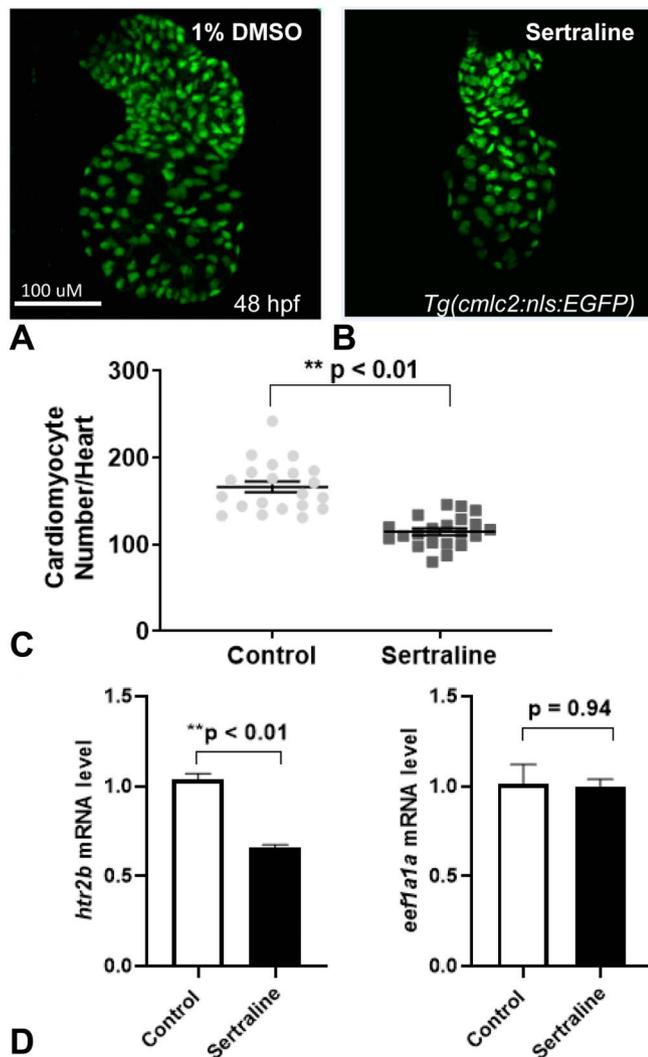
To initially assess whether sertraline exposure influenced development in zebrafish embryos, either sertraline (5–20  $\mu$ M in 1% DMSO) or 1% DMSO (vehicle control) was added to daily E3 medium from 5.5 to 72 hpf (early exposure) or from 20 to 72 hpf (late exposure). Live imaging was performed at 32, 48, and 72 hpf. Initial dose titration curves indicated that sertraline doses of 20  $\mu$ M frequently resulted in death of the embryos by 72 hpf. In addition, no developmental effects were seen with 5  $\mu$ M sertraline dosing at 32, 48, and 72 hpf (data not shown). Pericardial edema, a nonspecific indicator of disrupted development, was observed in bright-field images in sertraline-treated embryos at both the early and late exposure time points (Fig. 1A). This pericardial edema was observed in a dose-dependent manner. However, the phenotype was most notable with early exposure; approximately 60% of early exposure embryos treated with 15  $\mu$ M sertraline developing pericardial edema by 72 hpf (Fig. 1B).

To evaluate the impact of exposure window on cardiac development, we assessed *vmhc* expression at 32 and 48 hpf in embryos treated with 1% DMSO or 15  $\mu$ M sertraline beginning at 5.5–72 hpf or 20–72 hpf. As compared with 1% DMSO, the cardiac expression pattern of *vmhc* was reduced in sertraline-exposed embryo at 32 and 48 hpf, particularly among the embryos that were exposed at 5.5 hpf (Fig. 2). Based on these representative images and the percentage of sertraline-treated embryos with pericardial edema (Fig. 1B), further experiments only used the 5.5 to 72 hpf exposure window.

To evaluate ventricular size and number of cardiomyocytes, hearts were extracted from *Tg(cmlc2:nls-EGFP)*<sup>31</sup> zebrafish embryos treated with either 1% DMSO or 15  $\mu$ M sertraline at 48 hpf. Representative confocal images are shown in Figure 3. Sertraline-exposed embryos had a decrease in heart size (Fig. 3B vs. Fig. 3A) with a significant decrease in the total number of cardiomyocytes compared with controls (sertraline-exposed  $114.8 \pm 3.8$ , control  $166.4 \pm 6.2$ ,  $P < 0.01$ ,  $n = 7$  embryos/group counted by 3 independent, blinded investigators, Fig. 3C). These data indicate that exposure to the SSRI sertraline reduces the cardiomyocyte number of zebrafish embryos, consistent with our prior findings in mice<sup>23</sup> and work from others showing reduced cardiomyocyte proliferation with SSRI exposure.<sup>15,16</sup> Although we did assess cardiomyocyte proliferation in our zebrafish, the overall number of proliferating cardiomyocytes was low in zebrafish embryos treated with 1% DMSO or sertraline (see **Figure 1, Supplemental Digital Content 1**, <http://links.lww.com/JCVP/A835>).

### Expression of *htr2b*, the Gene Encoding the Serotonin Receptor Subunit 5-HT<sub>2B</sub>, Is Decreased in Sertraline-Exposed Zebrafish Embryos

We previously demonstrated that sertraline exposure in pregnant mice leads to decreased cardiac expression of the 5-HT<sub>2B</sub> receptor.<sup>23</sup> The 5-HT<sub>2B</sub> receptor has been



**FIGURE 3.** Sertraline exposure leads to decreased total numbers of cardiomyocytes. Hearts were dissected from *Tg(cmlc2:nls-EGFP)* embryos treated with 1% DMSO or 15  $\mu$ M sertraline beginning at 5.5 hpf. Confocal images at 48 hpf suggested smaller heart size in sertraline-treated embryos (Figs. 1B vs. 1A). GFP-labeled cardiomyocytes were significantly reduced in sertraline-treated embryos (Fig. 1C,  $n = 21$  counts/group; 7 embryos per group counted by 3 blinded, independent reviewers). Expression of *htr2b*, the gene encoding the 5-HT<sub>2B</sub> receptor, quantified by qRT-PCR was decreased in sertraline-exposed embryos. Compared with controls, the housekeeping gene *eef1a1a* was unchanged (Fig. 1D).  $**P < 0.01$  compared with control.

shown to be critical to normal cardiac development because inactivation and overexpression of the 5-HT<sub>2B</sub> receptor affects cardiomyocyte proliferation and cardiac development.<sup>20,21</sup> To determine whether *htr2b* expression was decreased in sertraline-treated zebrafish embryos, we isolated RNA from hearts of sertraline-exposed and control embryos, synthesized cDNA, and then quantified *htr2b* mRNA levels by real-time PCR. Sertraline-exposed zebrafish hearts had decreased cardiac expression of *htr2b*

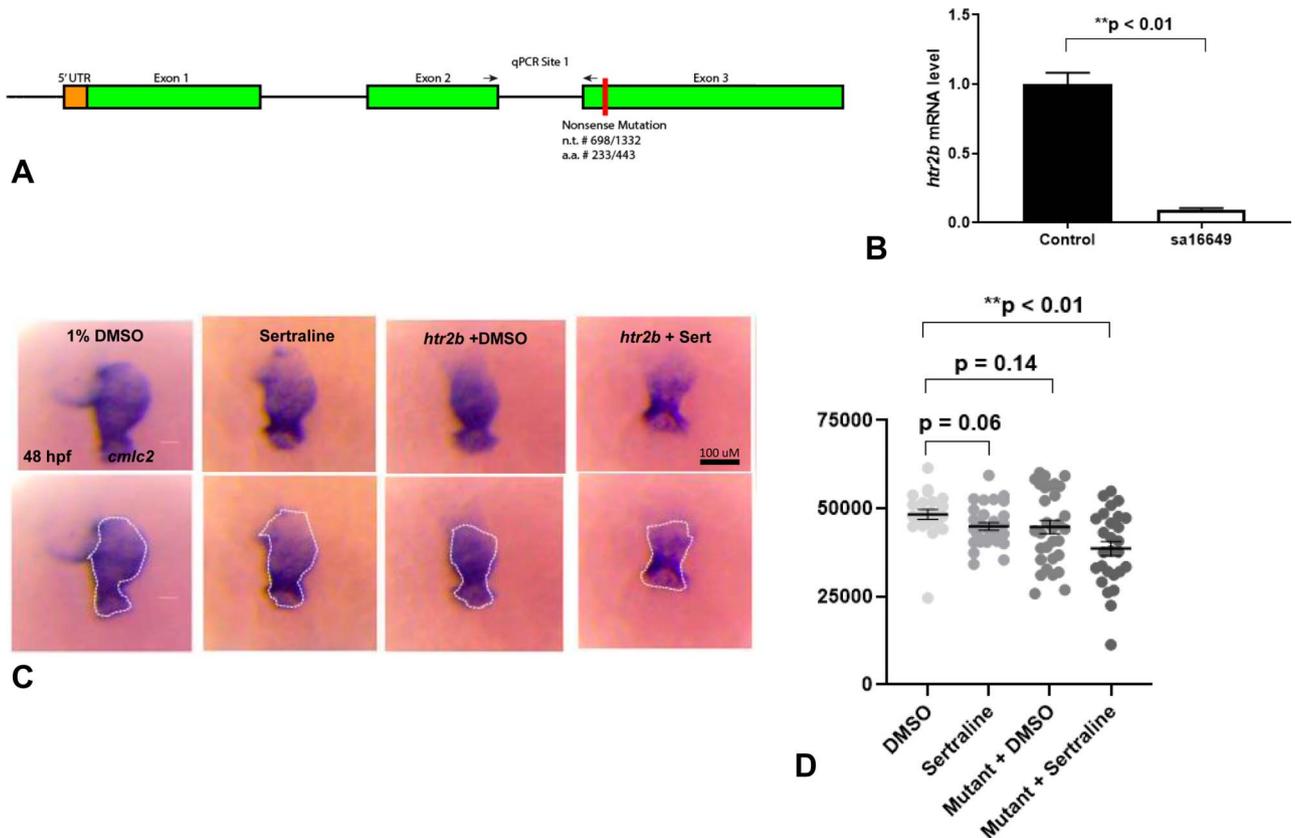
mRNA compared with control embryos ( $n = \sim 100$  embryos/group, Fig. 3D). These results suggest that sertraline exposure decreases expression of the RNA encoding the 5-HT<sub>2B</sub> receptor in zebrafish embryos, paralleling our findings in neonatal mice.

### Ventricle Size Is Reduced by the Deletion of *htr2b* and Treatment With Sertraline

To determine whether deletion of *htr2b* would result in a decreased ventricle size, we acquired from the ZIRC stock center a zebrafish strain with a nonsense mutation near the start of exon 3 of *htr2b* (*htr2b<sup>sa16649</sup>*) (Fig. 4A). Homozygous mutants are viable as adults. To assess the consequence of this mutation to *htr2b* transcript levels, we crossed 2 mutants and assessed *htr2b* mRNA levels in mutant embryos and in wild-type (WT) embryos from a separate clutch at 48 hpf. *htr2b* mRNA levels were at least 8-fold lower in the mutants in comparison with in WT embryos ( $n = \sim 30$  embryos/group, Fig. 4B). This was unexpected because mutations in terminal exons do not traditionally induce non-sense-mediated decay.<sup>39</sup> Of note, the carboxy-terminal region of Htr2b encoded by exon 3 and therefore missing from *htr2b<sup>sa16649</sup>* mutants is typically a crucial component of G-protein-coupled receptors.<sup>40</sup> Based on the low transcript levels and expectation that they encode a nonfunctional protein, we expect that *htr2b<sup>sa16649</sup>* is a null or strong loss-of-function allele. We assessed *cmlc2* expression at 48 hpf in WT embryos treated with 1% DMSO or 15  $\mu$ M sertraline and *htr2b* mutants treated with 1% DMSO or 15  $\mu$ M sertraline. As compared with WT, the cardiac expression pattern of *cmlc2* is reduced in mutants (Fig. 4C). The spectrum of phenotypes observed in each of the 4 groups is demonstrated more clearly in **Supplemental Digital Content 2**, (see **Figure 2**, <http://links.lww.com/JCVP/A836>). Ventricle size was significantly reduced as measured by ImageJ and quantified as total pixel<sup>2</sup> in *htr2b* mutants treated with sertraline [WT + 1% DMSO ( $n = 23$ ):  $48,311 \pm 1406$ , WT + sertraline ( $n = 30$ ):  $44,879 \pm 1061$ , *htr2b* + 1% DMSO ( $n = 31$ ):  $44,751 \pm 1907$ , *htr2b* + sertraline ( $n = 27$ ):  $38,633 \pm 2029$ ;  $**P < 0.01$  compared with WT + 1% DMSO, Fig. 4D].

### Sertraline Exposure Did Not Result in Long-Term Cardiovascular Changes in Volume Status or Function as Measured by Echocardiography

Whereas our previous work in mice showed that sertraline exposure in utero led to decreased shortening fraction and stroke volume in adult mice,<sup>23</sup> sertraline exposure in zebrafish embryos did not affect adult cardiovascular status. At age 4 months, echocardiograms were performed in adult zebrafish with embryonic exposure to 1% DMSO or sertraline. A representative picture of echocardiography images in the long-axis and short-axis for systole and diastole is shown in Figure 5. No differences were observed in any of the measured echocardiography parameters between zebrafish embryos treated with 1% DMSO or sertraline ( $n = 29$  controls, 39 sertraline, Fig. 6). In addition, there was no difference in the overall



**FIGURE 4.** A, Schematic of exon structure of ZIRC *htr2b* mutant. B, qRT-PCR in ZIRC mutants confirmed loss-of-function of the *htr2b* gene. C, *In situ* hybridization of WT and ZIRC mutants treated with 1% DMSO (vehicle control) or sertraline. At 48 hpf, cardiac expression of *cmlc2* is reduced in *htr2b* mutants compared with WT with a more pronounced reduction in ventricular size in mutants exposed to sertraline. Figures demonstrating representative images and outline of ventricular size using ImageJ are shown. D, Results of total pixel<sup>2</sup> as calculated by ImageJ in treated embryos (n = 23 WT + DMSO, n = 30 WT + sertraline, n = 31 mutants + DMSO, n = 27 mutants + sertraline).  $**P < 0.01$  compared with control.

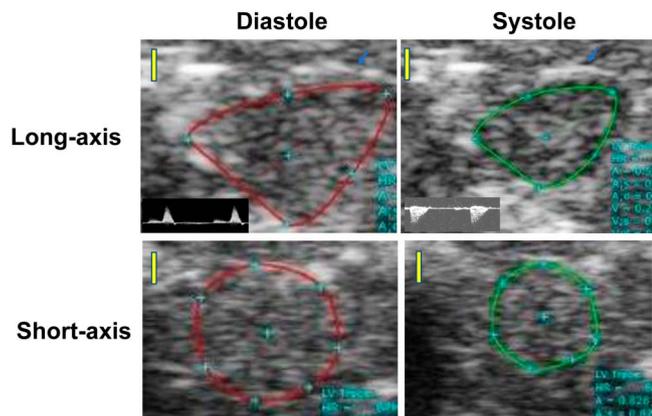
heart rate (Fig. 6). It is important to note that because embryos exposed to sertraline doses of 15  $\mu$ M did not survive after the exposure window ended (after 72 hpf), we modified our dosing regimen to 5–10  $\mu$ M during the 5.5–72 hpf exposure window. For most experiments in this article, sertraline doses of 15  $\mu$ M were used. After this modification, there were no differences in the number of zebrafish deaths between the sertraline and DMSO exposure groups from the end of the exposure window (72 hpf) to age 4 months (data not shown). All 3 sertraline dosing groups were combined into one group for the purpose of comparison because of the small number in each individual dosing group. However, when evaluated by individual concentrations, a dose-dependent trend toward decreased ejection fraction was observed with increased doses of sertraline [ejection fraction—5  $\mu$ M sertraline (n = 9):  $56.3 \pm 5.1\%$ , 7.5  $\mu$ M sertraline (n = 13):  $54.7 \pm 4.2\%$ , 10  $\mu$ M sertraline (n = 17):  $44.67 \pm 3.7\%$ ].

### DISCUSSION

The use of SSRIs to treat depression during pregnancy has increased over the past decade, and SSRIs are now

prescribed to 10% of pregnant women in the United States.<sup>1–4</sup> The association between SSRIs and congenital heart disease continues to be difficult to interpret in the setting of maternal depression<sup>7–12</sup>; thus, preclinical studies play an important role in answering the question of whether SSRIs exert independent effects on cardiac development. To the best of our knowledge, this is one of the first studies investigating the cardiac effects of sertraline exposure in zebrafish embryos, a valuable model system for the study of heart development.

The major finding of this study is that sertraline-exposed zebrafish embryos have decreased total cardiomyocyte number and ventricular size. These data are consistent with our prior work demonstrating reductions in ventricular size in an animal model of in utero SSRI exposure and a small cohort of term infants exposed to SSRIs in utero.<sup>23,41</sup> SSRI exposure has been shown to decrease cardiomyocyte proliferation in vitro and in vivo.<sup>15,16,23</sup> In this model, the reduction in heart size was suggested with exposure beginning before and after myocardial precursor cell differentiation and migration to midline. With sertraline exposure starting at 5.5 or 20 hpf, the hearts fused to form tubes in the midline suggesting that cardiac migration is not a contributing factor to the small ventricle size. Although data suggest that the small ventricle

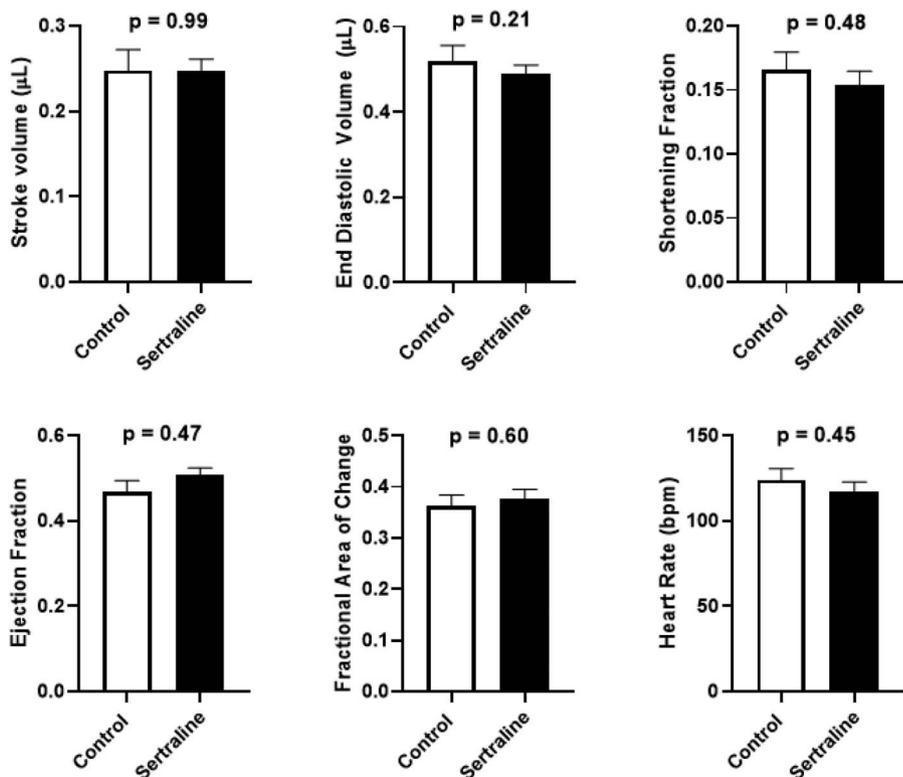


**FIGURE 5.** Echocardiography in a zebrafish treated with vehicle (1% DMSO). Diastolic (red) and systolic (green) epicardial silhouettes were identified visually, in long-axis and short-axis planes, assisted by video playback, which distinguished epicardium from pericardium (blue arrows). Spongiform architecture of ventricular myocardium prevents identification of fluid-endocardial interfaces. Pulse-wave Doppler interrogation of ventricular inflow (inset, diastole) and ventricular outflow (inset, systole) served to confirm the location of the atrioventricular plane and to calculate heart rate. Yellow scale bars = 200  $\mu$ M.

size may be a result of proliferation, our results were inconclusive because the total number of proliferating cardiomyocytes was very low.

Another important finding in this study is the reduced expression of cardiac *htr2b* in sertraline-treated zebrafish embryos. The *htr2b* gene encodes the 5-HT<sub>2B</sub> receptor that is expressed on the cell surface of cardiomyocytes and is critical for normal cardiac development.<sup>20</sup> Inactivating the 5-HT<sub>2B</sub> receptor in mice leads to increased embryonic and neonatal death.<sup>20</sup> Furthermore, mice with inactivated 5-HT<sub>2B</sub> receptors that survive embryogenesis have reduced cardiomyocyte proliferation and develop cardiomyopathy.<sup>20</sup> Previously, we demonstrated decreased cardiac expression of the 5-HT<sub>2B</sub> receptor in offspring of pregnant mice treated with sertraline.<sup>23</sup> Working with zebrafish, we again demonstrated that *htr2b* levels were significantly decreased in hearts dissected from sertraline-exposed embryos compared with those dissected from controls. Because these embryos can survive even in the presence of severe cardiac defects, zebrafish serve as an important model system to better understand the impact of disrupted 5-HT signaling on cardiac development.

Studying zebrafish embryos allows us to further evaluate the link between SSRIs and the 5-HT<sub>2B</sub> receptor. We found that both sertraline exposure and disruption of the 5-HT<sub>2B</sub> receptor affected ventricle development. Notably, this effect on ventricle development was most prominent when the *htr2b* mutant was also treated with sertraline. Because ventricle size was significantly reduced in embryos lacking *htr2b* treated with sertraline, this indicates there is a relationship between SSRIs and the 5-HT<sub>2B</sub> receptor. It has been proposed that the 5-HT<sub>2B</sub> receptor is critical to the action of SSRIs.<sup>42,43</sup> Diaz et al<sup>42</sup> noted long-term behavioral and



**FIGURE 6.** Echocardiograms were performed in sertraline and 1% DMSO-treated embryos at age 4 months (n = 29 control, n = 39 sertraline). No differences were observed in any of the echocardiography measurements between the 2 groups.

neurogenic SSRI effects were abolished after inactivation of 5-HT<sub>2B</sub> receptors in rats causing them to postulate that the 5-HT<sub>2B</sub> receptor may positively modulate serotonergic activity required for the therapeutic actions of SSRIs. Our findings suggest that SSRIs do not act exclusively through the 5-HT<sub>2B</sub> receptor to affect heart development because *htr2b* mutants were affected by treatment with sertraline. The serotonergic system is complex with several serotonin receptors important in ventricular development and cardiovascular regulation, particularly 5-HT<sub>2A</sub>, 5-HT<sub>4</sub>, and the serotonin transporter.<sup>19</sup> In this study, we specifically focused on 5-HT<sub>2B</sub> receptor and pharmacologic inhibition of the serotonin transporter, but SSRIs could function through different receptors. There are 7 different 5-HT receptors and at least 15 subpopulations. Notably, the 5-HT<sub>2A</sub> receptor is expressed in the heart and mediates a hypertrophic response to 5-HT.<sup>44</sup> In addition, the 5-HT<sub>4</sub> receptor is expressed in fetal ventricles, and this receptor mediates the inotropic response to 5-HT late in development.<sup>45</sup> Finally, a recent review by Hertz et al<sup>46</sup> suggests the importance of other signaling pathways such as glucose and glycogen metabolism, glutamate and GABA signaling, and arachidonic acid in examining the relationship of chronic SSRI exposure to the 5-HT<sub>2B</sub> receptor. These additional serotonin receptors and signaling pathways may be important to better understand the cardiac effects observed in models of SSRI exposure with disruptions to the 5-HT<sub>2B</sub> receptor.

An alternative hypothesis for our findings is that sertraline exposure delays development. Fraher et al<sup>32</sup> noted that the SSRIs sertraline and citalopram influenced bone development during embryogenesis by affecting osteoblast maturation and mesenchymal stem cell differentiation. Recently, there has been an increase in data suggesting the delayed development influences cardiovascular health. Animal models of adverse intrauterine environments have demonstrated that impaired myocardial development is associated with reductions in postnatal growth and cardiac performance.<sup>23,47,48</sup> Furthermore, cardiomyocyte proliferation is essentially complete at birth in humans,<sup>49,50</sup> and people born prematurely have cardiac dysmorphology.<sup>51–57</sup> Although global delays in the zebrafish embryos were not observed in our bright-field images, we cannot exclude this as a possibility for our findings. The phenotype of reduced ventricular size in sertraline-exposed embryos was not corrected at 72 hpf, but we did not observe later time points. Although no differences were observed in volume status by echocardiography at age 4 months, our findings are confounded because we had to reduce our sertraline dosing regimen for embryos to survive long enough to obtain echocardiograms.

Our study has several important limitations. First, the dosing regimen for zebrafish embryos may exceed clinical dosing regimens.<sup>32</sup> However, pharmacokinetics and drug distribution in zebrafish embryos are not well established, and similar findings were observed in a preclinical mouse model with sertraline levels consistent with fetal exposure.<sup>23</sup> Second, we did not differentiate between atrial and ventricular myocytes and did not perform timing assays to determine whether early or late cardiomyocytes were affected. Third, we looked at development through the first 72 hours but not at later time points. Finally, the ZIRC mutant is an established mutant resulting from premature stop codon in exon 3. Working with a mutation in exon 3

raises the question of whether any *htr2b* translation may have important implications in cardiac development. It may be important to investigate a larger loss-of-function mutation in the *htr2b* gene.

## CONCLUSIONS

We have demonstrated that sertraline exposure in zebrafish embryos leads to decreases in cardiomyocyte number, reduces the expression of cardiac *htr2b* mRNA levels, and reduces ventricular size. Importantly, in our previous study using mouse models, we found reduced cardiomyocyte number, cardiomyocyte proliferation, and stroke volumes after sertraline exposure during development. In addition, both models suggest an important role of the 5-HT<sub>2B</sub> receptor in SSRI-associated cardiac defects. However, this model indicates that the SSRI sertraline does not act exclusively through the 5-HT<sub>2B</sub> receptor. Zebrafish serve as an ideal model system to study cardiac development for multiple reasons including external development apart from mother, easy visualization, and easy pharmacologic and genetic manipulation. We can now focus on the mechanistic pathways for these associations and other serotonin receptor-mediated pathways. In pursuing this research, we furthermore hope to provide new insights into the impact of maternal depression treatment on offspring cardiac development.

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