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The 5-Hydroxytryptamine receptor 1F stimulates mitochondrial biogenesis and angiogenesis in endothelial cells

Tess V. Dupre, PhD[1], **Dorea P. Jenkins**[2], **Robin C. Muise-Helmericks, PhD**[2], **Rick G. Schnellmann, PhD**[1,3,*]

[1]Dept. of Pharmacology and Toxicology, University of Arizona, Tucson, AZ 85721

[2]Department of Regenerative Medicine and Cell Biology, Medical University of South Carolina, Charleston, SC 29425

[3]Southern Arizona VA Health Care System, Tucson, AZ

Abstract

A hallmark of acute kidney injury (AKI) is vascular rarefication and mitochondrial dysfunction. Promoting vascular recovery following AKI could facilitate kidney repair as the vasculature is responsible for oxygen and nutrient delivery to extravascular tissues. Little is known about mitochondrial biogenesis (MB) in endothelial cells, and the role of 5-HT_{1F} receptor signaling in MB has only been studied in epithelial cells. Our laboratory has shown that stimulating MB through the 5-HT_{1F} receptor promotes recovery from AKI and that 5-HT_{1F} receptor knockout mice have decreased MB and poor renal recovery. We hypothesized that the 5-HT_{1F} receptor plays a role in vascular homeostasis and mediates MB in renal endothelial cells. 5-HT_{1F} receptor knockout mice had decreased renal vascular content, as evidenced by decreased CD31+ endothelial cells and αSMA+ vessels. Human glomerular endothelial cells (HEC) and mouse glomerular endothelial cells (MEC) expressed the 5-HT_{1F} receptor. Treatment of HEC and MEC with 5-HT_{1F} receptor agonists LY344864 or lasmiditan (0–500 nM) induced MB as evidenced by maximal mitochondrial respiration, a marker of MB. HEC and MEC treated with lasmiditan or LY344864 also had increased nuclear- and mitochondrial-encoded proteins (PGCIa, COX-1, and VDAC), and mitochondrial number, confirming MB. Treatment of HEC with LY344864 or lasmiditan enhanced endothelial branching morphogenesis and migration, indicating a role for 5-HT_{1F} receptor stimulation in angiogenic pathways. We propose that stimulation of 5-HT_{1F} receptor is involved in MB in endothelial cells and that treatment with 5-HT_{1F} receptor agonists could restore stimulate repair and recovery following kidney injury.

Graphical Abstract

*To whom correspondence should be addressed: Rick G. Schnellmann, PhD, College of Pharmacy, University of Arizona, Drachman Hall B307B, 1295 N. Martin, Tucson, AZ 85721. schnell@pharmacy.arizona.edu.

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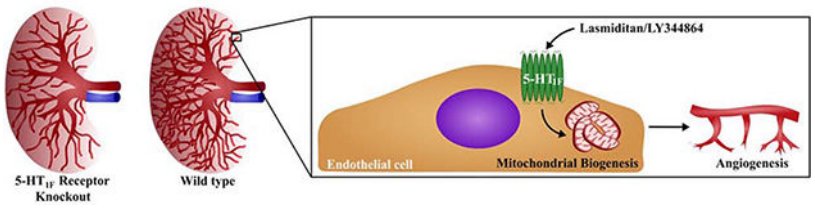

Keywords

Mitochondrial biogenesis; angiogenesis; endothelial cells

1. Introduction

Endothelial cells (EC) are located in capillary beds of organs and are important in the maintenance of many biological processes such as vascular permeability, coagulation, angiogenesis, and inflammation [1]. EC have a unique metabolic profile compared to other cell types, primarily using aerobic glycolysis to produce the majority of their ATP [2, 3]. Aerobic glycolysis occurs when cells utilize glycolysis rather than oxidative phosphorylation under aerobic conditions [2, 3].

Our laboratory focuses on the identification of mitochondrial biogenic agents as a therapeutic target in the treatment of multiple types of diseases and acute organ injury. Mitochondrial biogenesis (MB) is commonly defined as the generation of new, functional mitochondria [4–6]. We have focused on MB in epithelial cells, namely renal proximal tubule cells (RPTC) [4–6]. RPTC are highly metabolic and readily utilize aerobic respiration as their primary source of ATP production [7].

5-hydroxytryptamine (5-HT, serotonin) 1F receptor is a G-protein coupled receptor (GPCR) subtype whose functions are not fully understood [8–10]. Of its known physiological roles, the 5-HT_{1F} receptor mediates the signaling of 5-HT in the central nervous system; little is known about the 5-HT_{1F} receptor in peripheral tissues [8–10]. Interestingly, 5-HT_{1F} receptors were found on RPTC and a 5-HT_{1F} receptor agonist signals MB in RPTC to promote recovery of renal function in mice subjected to ischemia-reperfusion AKI [11, 12]. Finally, knockout of the 5-HT_{1F} receptor in mice potentiates AKI and impairs renal recovery [13].

A hallmark of many models of renal disease and AKI is vascular rarefication and mitochondrial dysfunction [14–17]. Little is known about MB in microvascular endothelial cells, and the role of 5-HT_{1F} receptor in endothelial cells has not been studied. Thus, gaining a better understanding of the role of 5-HT_{1F} receptor in MB in microvascular endothelial cells is novel and may lead to the identification of new therapeutic targets for the treatment of diseases and kidney injury involving microvascular endothelial cells. Here, we present data indicating that 5-HT_{1F} receptor knockout (KO) mice have decreased CD31+ endothelial cells and decreased $aSMA^+$ vessels in the kidney. Human and mouse glomerular endothelial cells express the 5-HT_{1F} receptor and agonism of the 5-HT_{1F} receptor induces MB, endothelial cell branching and increases endothelial cell migration.

2. Materials and Methods

2.1 Animal Studies:

A 5-HT_{1F} receptor KO breeding colony was established from heterozygous mutants (B6N(Cg)-*Htr1f*$^{tm1-1(KOMP)Vlcg}$/*J*, (stock no. 024269)) and age-matched wild-type (WT) male (C57BL/6NJ (stock no. 005304)) purchased from the Jackson Laboratory (Bar Harbor, ME). Mice were housed in temperature-controlled conditions under a light/dark photocycle with *ad libitum* food and water. Tissues were harvested from male 5-HT_{1F} KO and WT mice at 10 weeks of age. Studies were carried out in accordance with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. All protocols were approved by the Institutional Animal Care and Use Committee at the University of Arizona and VA, and all efforts were made to minimize animal suffering.

2.2 Immunohistochemistry

Kidney sections (5 μm thick) were rehydrated in Histoclear followed by an ethanol gradient. Antigen retrieval was performed in citric acid buffer (pH 6.0) at 95°C in a steamer for 30 min. Endogenous peroxidases were blocked with 3% hydrogen peroxide. Slides were then blocked with avidin for 10 min followed by a PBS wash and then biotin for 10 min followed by a wash in PBS (SP-2002, Vector Laboratories, Burlingame, CA). Slides were further blocked with 5% normal horse serum in PBS for 1 h at room temperature. CD31 primary rabbit antibody (Abcam, Cambridge, MA) was added to slides at a 1:100 dilution and allowed to incubate at 4°C overnight in a humidified environment. Slides were washed with PBS for 5 min, three times. Biotinylated goat anti-mouse/rabbit IgG antibody (1: 25,000, BA-1400, Vector Laboratories, Burlingame, CA) was added to each section and incubated for 30 min at room temperature. Slides were rinsed twice with PBS (5 min each). Vector ABC reagent (PK-6200, Vector Laboratories, Burlingame, CA) was added to each section and incubated for 30 min at room temperature. Slides were rinsed two times with PBS followed by the addition of 100 μl of DAB substrate for 5–7 min to detect horseradish peroxidase (SK-4800, Vector Laboratories, Burlingame, CA). Slides were rinsed in distilled water for 5 min, counterstained with modified Mayer's hematoxylin (72804, ThermoFisher, Waltham, MA), and dehydrated in an ethanol gradient to Histoclear followed by mounting with Permount (SP15, Fisher Scientific, Hampton, NH). Positive staining for CD31 was quantified using aperio image analysis positive pixel algorithm software, and the number CD31 positive pixels was calculated in a total of 5 fields, determined, and normalized to area of the image analyzed.

2.3 Immunofluorescence:

Kidney tissue was harvested and fixed in 4% paraformaldehyde and embedded in paraffin. Paraffin embedded whole kidney sections were de-paraffinized using Histo-Clear II (Atlanta, GA) and rehydrated in an ethanol series with increasing amounts of water. Upon hydration, sections were permeablized in 0.1% Triton 100-X for 10 minutes. Following a wash in 1X PBS, sections were blocked for 1 hour in 5% BSA-PBS, and incubated with a Cy5-labeled smooth muscle actin antibody (Sigma Aldrich, St. Louis, MO) overnight at 4°C. Sections were washed in 1X PBS and stained with DAPI nuclear dye (ThermoFisher, Waltham, MA). Fluorogel was applied to each section and a glass cover slip was used to cover the sections

for imaging. All images were acquired on the Zeiss Axio Imager M2 fluorescent microscope. Positive aSMA vessels were enumerated in a total of 5 fields per section, determined, and normalized to area of the image analyzed.

2.4 Cell Culture:

Human renal glomerular endothelial cells (HEC) were purchased from ScienCell Research Laboratories (Carlsbad, CA). HEC were cultured in fibronectin-coated flasks using endothelial cell medium (1001, ScienCell Research Laboratories). C57BL/6 mouse primary kidney glomerular endothelial cells were purchased from Cell Biologics, Inc. (C57–6014G, Chicago, IL). MECs were maintained in gelatin-coated flasks using mouse endothelial cell medium (M1168, Cell Biologics, Inc.) with growth factor supplement additives (Cell Biologics, Inc.) and 5% (v/v) fetal bovine serum (Cell Biologics, Inc.).

Female New Zealand White rabbits (1.5–2.0 kg) were purchased from Charles River Laboratories (Wilmington, MA). Renal proximal tubule cells were isolated using the iron perfusion method previously described [18].

2.5 Analysis of Oxygen Consumption

The oxygen consumption rate (OCR) of MECs and HECs was measured using the Seahorse Bioscience XF-96 Extracellular Flux Analyzer as previously described (Agilent, Santa Clara, CA) [19]. Each 96-well assay plate was treated with vehicle (DMSO; <0.5%, Sigma Aldrich, St. Louis, MO) or the experimental compounds. Basal OCR was measured before injection of carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP; 0.5 μM) (Sigma Aldrich, St. Louis, MO) to measure the uncoupled OCR (FCCP-OCR), a marker of MB [19].

2.6 Reverse Transcriptase- Polymerase Chain Reaction (RT-PCR)

RNA was isolated using TRIzol (15596018, ThermoFisher, Waltham, MA) per the manufacturer's protocol. cDNA was synthesized with an iScript cDNA Kit (1708841, Bio-Rad, Hercules, CA) per the manufacturer's instructions. Transcripts were amplified using Promega PCR master mix (M7502, Madison, WI) per the manufacturers' instructions. The transcripts were quantitated using *homo sapiens* B2M (5'-ACT ACA CTG AAT TCA CCC CCA C-3' and 5'-GCT TAC ATG TCT CGA TCC CAC TT-3'), *homo sapiens* 5-HT_{1F} Receptor (5'-GCT ATA GCT TTG GAT CGG TAT CGA G-3' and 5'-CAA TCC TAC TTG CTT GTC TCT TGT G-3'), *mus musculus* B2M (5'-CTG GTC TTT CTG GTG CTT GTC −3' and 5'-TAT GTT CGG CTT CCC ATT CTC C −3'), *mus musculus* 5-HT_{1F} Receptor (5'- GCC GTG ATG ATG AGTGTG TC-3' and 5'-ATC ATC CGA CTC GCT TGT CT −3'). PCR products were run on a 1% agarose gel to confirm the presence of the product.

2.7 Protein Isolation and Immunoblot Analysis

Protein isolation and immunoblot analysis were performed as previously described [20]. Samples were analyzed via immunoblot using an antibody against PGC-1α (ST1202) (EMD Millipore, Billerica, MA). Primary antibodies TFAM (ab131607), COX-1 (ab14705), VDAC (ab15895), and a-tubulin (ab52866) were purchased from Abcam (Cambridge, MA).

Secondary antibodies include horseradish peroxidase-labeled anti-rabbit and mouse and were from Abcam.

2.8 Transmission electron microscopy

HEC and MEC were fixed and sectioned for transmission electron microscopy as previously described [21]. Images were viewed by FEI Tecnai Spirit microscope (FEI, Hillsboro, OR) operated at 100 kV and captured using an AMT 4 Mpixel camera (Advanced Microscopy Techniques, Woburn, MA). Mitochondrial count and morphology were analyzed using the analyze particles plug-in in ImageJ FIJI.

2.9 Endothelial Branching Morphogenesis

Endothelial tubule formation was performed as previously described [22]. Briefly, HEC were grown to confluence on fibronectin-coated dishes. Cells were then serum starved and treated with either vehicle (<0.5%, DMSO, Sigma Aldrich), LY344864 (300 nM, 2451 Tocris, Bristol, UK), or lasmiditan (100 nM, L22337 Sigma Aldrich) for 24h. Cells were enumerated, then 70,000 cells per treatment were transferred onto matrigel (354230 Corning, Corning, NY) coated dishes and allowed to form tubules for 4h. Tubules were visualized via phase contrast microscopy at 100X final magnification. Five randomly selected fields per treatment were analyzed per treatment group for each experiment. Images were analyzed using the angiogenesis analyzer plug-in in imageJ FIJI.

2.10 Migration assay

Migration was assessed as previously described [23]. Briefly, HEC were treated with vehicle (V), 300nM LY344864 (LY), 100nM Lasmiditan (Las) for 24h. Scratches were then made with 10 μL pipette tip, complete media was replaced, and cells were allowed to migrate for 6h. Images were taken of the scratches at 0h and 6h at 100X final magnification. Images of five randomly selected areas of the scratch were taken for each treatment at each time point. The wound area was analyzed via MRI wound healing plug-in in FIJI imageJ. Briefly, for each time point, the area of each scratch was determined, and an average scratch area was calculated for each treatment group. The percent migration was determined by the following formula: % Area of migration 6h post-scratch= [(scratch area 0h post-scratch - scratch area 6h post-scratch) / (scratch area 0h post-scratch) * 100.

2.11 Statistical Analysis

Data are presented as means±SE. Single comparisons were performed using two-tailed Student's *t*-test, with $p < 0.05$ considered to be a statistically significant difference between means. Where appropriate, multiple comparisons were subjected to one-way ANOVA followed by Dunnetts post-hoc analysis or two-way ANOVA with Sidak post-hoc analysis, with $p < 0.05$ considered to be a statistically significant difference between means. In the figures, different superscripts indicate statistical differences. For *in vivo* studies, an individual mouse represents one n (n=1). For *in vitro* experiments, MEC and HEC isolated from a single passage represented an individual experiment (n = 1) and were repeated until $n = 4–6$ were obtained. At least 2 different lots of cells were utilized.

3. Results

3.1 Mice lacking 5-HT$_{if}$ receptor have decreased renal vasculature.

To determine if the 5-HT$_{1F}$ receptor plays a role in renal vasculature, we assessed the vascular content of 10-week old, male 5-HT$_{1F}$ receptor knockout (KO) and wild type (WT) mice. 5-HT$_{1F}$ receptor KO mice have decreased renal CD31+ endothelial cells and aSMA+ vessels as compared to WT mice (fig 1). CD31 is a pan endothelial cell marker, while aSMA is a marker of arterioles [24, 25]. Taken together, data indicate that loss 5-HT$_{1F}$ receptor plays a role in the maintenance of renal vascular content.

3.2 5-HT$_{1F}$ receptor is expressed in MEC and HEC and endothelial cells respire less than epithelial cells.

Because mice lacking the 5-HT$_{1F}$ receptor have decreased CD31^{+} endothelial cells, we determined if the receptor is expressed in HEC and MEC. MEC (fig 2a) and HEC (fig 2b) expressed 5-HT$_{1F}$ receptor mRNA; suggesting a role for 5-HT$_{1F}$ receptor in the renal microvasculature of both humans and mice.

Previous studies have shown that endothelial cells are highly glycolytic and utilize aerobic glycolysis as their primary source of ATP production [2, 3]. In contrast, epithelial cells are highly oxidative and primarily utilize oxidative phosphorylation as their source of ATP [4, 6]. We compared differences in basal and uncoupled respiration in HEC and MEC to epithelial cells (RPTC). HEC and MEC have a 40–50% decrease in basal oxygen consumption rates (OCR) compared to epithelial cells (RPTC) (fig 2c). FCCP induces maximal OCR, a marker of maximal electron transport chain (ETC) activity. FCCP increased OCR in RPTC 2.5-fold, representing a large reserve of ETC activity. In contrast, FCCP only increased OCR 20–30% in HEC and MEC, demonstrating limited capacity to increase ETC activity. These data demonstrate that HEC and MEC have a marked decreased oxidative capacity compared to RPTC.

3.3 5-HT$_{F}$ receptor agonists increase FCCP-OCR in MEC and HEC.

Our laboratory has reported that exposure of RPTC and mice to the 5-HT1F receptor agonist LY344864 induces MB [11, 12]. We previously developed a phenotypic high-throughput MB assay based on the measurement of FCCP-OCR, i.e. an increase in FCCP-OCR following chemical exposure compared to vehicle treatment [19]. LY344864 and lasmiditan exposure of HEC (24h) and MEC (48h) increased FCCP-OCR in comparison to vehicle controls in a concentration-dependent manner (fig 3). Lasmiditan was approximately 3-fold more potent and 20% more efficacious than LY344864 in MEC (fig 3a and b). In HEC, lasmiditan was 300 times more potent than LY344864 with equal efficacy (fig 3c and d). These data indicate that both LY344864 and lasmiditan induce MB.

3.4 5-HT$_{1F}$ receptor agonists increase MB markers in HECs and MECs.

To further establish that agonism of the 5-HT$_{1F}$ receptor with LY344864 and lasmiditan induces MB in endothelial cells, we assessed the levels of mitochondrial proteins associated with MB, PGCIα, COX-1, and VDAC [4–6]. It should be noted that peroxisome proliferator-activated receptor gamma coactivator-1 alpha (PGC-1α) is thought to be the

master regulator of MB [6]. Cytochrome c oxidase 1 (COX-1) is a mitochondrial DNA encoded protein in complex IV, while voltage-dependent anion channel (VDAC) is a nuclear encoded gene [26]. Treatment of HEC with 300nM LY344864 or 100 nM lasmiditan for 72h increased the expression of COX-1 and VDAC (fig. 4a and b). Treatment of MEC with 300nM LY344864 or 100 nM lasmiditan for 72h increased PGCIα, COX-1, and VDAC (Fig. 4c and d). These results reveal that LY344864 and lasmiditan induce MB in both HEC and MEC.

3.5 Agonism of 5-HT_{1F} receptor increases mitochondrial number in MEC and HEC.

To confirm that agonism of the 5-HT_{1F} receptor with LY344864 and lasmiditan induces MB in HEC and MEC, we enumerated mitochondria. MEC and HEC were exposed to 300nM LY344864 or 100nM lasmiditan for 72h. LY344864 or lasmiditan treatment increased mitochondrial number in both HEC and MEC in comparison to vehicle controls (Fig 5b, d). Total mitochondrial area per field following treatment of HEC with LY344864 or lasmiditan trended upward but was not significantly different from vehicle controls (Fig 5c); however, treatment of MEC with lasmiditan increased mitochondrial area per field (fig 5e). In total, these data show that agonsim of 5-HT_{1F} receptor induces MB.

3.6 Agonism of 5-HT_{1F} receptor increases endothelial branching morphogenesis.

Angiogenesis is an important biological process in endothelial cells that promotes recovery of function [3, 27–29]. MB has been linked to angiogenesis as metabolic needs of endothelial cells increase during angiogenesis [30, 31]. Thus, we performed angiogenesis assays in HEC to assess the branching morphogenesis capacity of these cells following 5-HT_{1F} receptor agonism [22]. Cells were serum starved for 24 hours and concurrently exposed to 300 nM LY344864 or 100 nM lasmiditan, then seeded onto Matrigel basement membrane extract, and allowed to migrate for 4h. Quantitative analysis of the endothelial branches showed that both LY344864 and lasmiditan equally increased the migration of endothelial cells to form branches in comparison to vehicle controls (fig 6). These findings show that agonsim of 5-HT_{1F} receptor with LY344864 or lasmiditan induces an angiogenic phenotype in HEC.

3.7 Agonism of 5-HT_{1F} receptor promotes endothelial cell migration.

An important aspect of microvascular repair following injury is cell migration [32]. Following injury to a vessel, the remaining healthy cells must migrate to the injury site to replace dead or damaged cells and promote vessel recovery [32]. HEC were grown to confluence, serum starved and concurrently treated with vehicle (V), 300nM LY344864 (LY), or 100nM lasmiditan (Las) for 24h. Following serum starvation, scratches were made, media was replaced with complete media, and cells were allowed to migrate for 6h. Figure 7A and B are representative photomicrographs at 0h and 6h post-scratch. Quantitative analysis of the scratch area revealed that vehicle treated HEC underwent ~30% scratch closure, while exposure with either LY344864 or lasmiditan increased scratch closure to ~55% in comparison to vehicle controls (Fig 7c). These data show that treatment of HEC with 5-HT_{1F} receptor agonists enhances the migratory capacity of these cells.

4. Discussion

The 5-HT_{1F} receptor has primarily been studied within the central nervous system (CNS) where 5-HT_{1F} receptor activation reduces migraine-associated pain without CNS vasoconstriction [9, 10, 33]. Little is known about 5-HT_{1F} receptor activation in peripheral tissues and vasculature. We observed that the loss of 5-HT_{1F} receptor reduces renal vascular content *in vivo.* Furthermore, HEC and MEC expressed the 5-HT_{1F} receptor and exposure of HEC and MEC to 5-HT_{1F} receptor agonists induced MB, as evidenced by maximal mitochondrial respiration, increased expression of nuclear- and mitochondrial-encoded proteins and mitochondrial number. Finally, stimulation of 5-HT_{1F} receptor with LY344864 or lasmiditan increased endothelial cell branching morphogenesis and migration, suggesting a role for 5-HT_{1F} receptor in pathways of angiogenesis and MB, and reparative vascular function.

Multiple 5-HT receptor subtypes play a role in angiogenesis and MB. The 5-HT_{1B} and 5-HT_4 receptors have been shown to stimulate angiogenesis [34, 35]. In addition, our laboratory has shown that 5-HT_{2A} and 5-HT_{1F} receptor stimulates MB [11–13, 36]. Importantly, treatment of RPTC and mice with LY344864 induced MB *in vitro* and *in vivo* and accelerated the recovery of renal function following AKI in mice [11–13]. Following AKI, 5-HT_{1F} receptor KO mice had impaired renal recovery and increased mitochondrial dysfunction [13]. Thus, results provide evidence that the 5-HT_{1F} receptor has a number of roles in the regulation of MB, kidney injury, and recovery of renal function.

The pathogenesis of AKI involves a number of parallel and converging pathways, and endothelial cells are implicated in multiple pathways [12, 15, 37–40]. Immediately following an ischemic event, capillary loss is observed in the kidneys [37]. In addition, loss of capillary density leads to decreased renal blood flow and increased renal vasoconstriction, resulting in the extension phase of AKI where continued reduction in blood flow and ongoing hypoxia are observed [15, 41]. Thus, identifying therapeutic agents that can increase vascular repair via angiogenesis, could prove useful in developing agents to promote renal repair following AKI.

Here we present a role for the 5-HT_{1F} receptor in the peripheral vasculature, as HEC and MEC expressed the 5-HT_{1F} receptor and mice lacking 5-HT_{1F} receptor have decreased $CD31^+$ endothelial cells and αSMA^+ blood vessels in the kidney. CD31 is a pan endothelial cell marker [24], and αSMA is only expressed in arteries and arterioles [42]. Similar to WT mice, 5-HT_{1F} receptor KO mice have normal kidney function [13], suggesting that the decreases in vascular content of 5-HT_{1F} receptor KO mouse kidneys is likely not a secondary to kidney dysfunction. Thus, 5-HT_{1F} receptor may play a role in pathways of vascular homeostasis and/or angiogenesis.

A link between MB and angiogenesis has been established [3, 30, 31, 43, 44]; however, the exact role of MB in endothelial cells is currently unclear. Endothelial cells primarily utilize aerobic glycolysis as their source of energy production [3]. Our findings show that while HEC and MEC have a lower respiratory capacity than epithelial cells (RPTCs) (fig 3), it is possible to stimulate their oxidative respiration as evidenced by an increase in FCCP-OCR

in comparison to basal-OCR. The observed decrease in the respiratory capacity of the endothelial cells in comparison to the epithelial cells is likely because the total mass of an epithelial cell is ~30–44% mitochondria [7], while the cytoplasmic volume of an endothelial cell is ~2–6% mitochondria [3].

Previous work found that vascular endothelial growth factor stimulates MB in endothelial cells via an AKT3 dependent pathway, and that stimulation of this pathway induces angiogenesis [30, 31]. Thus, stimulation of vascular MB may be important in vascular recovery following AKI. Our data show that agonism of 5-HT_{1F} receptor induces MB as evidenced by increased OCR, suggesting an increase in the number of functional mitochondria. Importantly, the induction of MB requires tight regulation of both nuclear and mitochondrial encoded genes and proteins, as the mitochondrial genome encodes 1% of mitochondrial proteins, while the nuclear genome encodes the remaining 99% of mitochondrial proteins [26]. Our results indicate that upregulation of both nuclear- and mitochondrial- encoded proteins occurred following LY344864 or lasmiditan treatment, as evidenced by increased expression of mitochondrial DNA encoded COX-1 and nuclear DNA encoded VDAC (fig 5) [4–6].

MB is commonly defined as the generation of new mitochondria [6]. Thus, it is important that 5-HT_{1F} receptor agonism increased mitochondrial number in HEC and MEC (fig 6). One would expect an increase in mitochondrial number to result in an increase in total mitochondrial surface area; however, our data do not reveal a statistically significant increase in mitochondrial surface area under most circumstances. However, treatment of MEC with lasmiditan increased total mitochondrial surface area, suggesting that 5-HT_{1F} receptor agonism with lasmiditan increases both mitochondrial area and size.

The loss of 5-HT_{1F} receptor reduces renal vasculature, suggesting a role for 5-HT_{1F} receptor in pathways of angiogenesis and/or vascular homeostasis. Under normal physiological conditions, angiogenesis is a process that occurs in adults during wound healing, skeletal growth, pregnancy, or the menstrual cycle, thus stimulating angiogenesis following organ injury or disease is important to maintaining organ function [2, 45–47]. Importantly, a key aspect of angiogenesis is the rapid migration and proliferation of endothelial cells [48, 49], wherein cells form angiogenic sprouts at a rate of several millimeters per day [22]. In addition, angiogenic sprouts form branch points in order to revascularize damaged tissue [27, 49]. Our findings show that 5-HT_{1F} receptor is involved in endothelial branching morphogenesis as agonism of 5-HT_{1F} receptor increases endothelial cell branching.

Migration is also an important aspect of angiogenesis and vessel repair as endothelial cells must migrate to the site of injury in order to replace dead or dying cells [49]. Importantly, results reveal that agonism of 5-HT_{1F} receptor increases HEC migration, as treatment with LY344864 or lasmiditan increased wound closure in comparison to vehicle treated controls. Ausprunk and Folkman showed that increased endothelial branching morphogenesis in conjunction with increased migration is indicative of endothelial cells obtaining an angiogenic phenotype [49].

It should be noted that pharmacological agents commonly have an affinity for other biological targets. In this case, LY344864 and lasmiditan are potent (nM) and specific agonists for the 5-HT_{1F} receptor [50, 51]. Lasmiditan has more than 450 times the affinity for the 5-HT_{1F} receptor than other 5-HT receptor subtypes [52], while LY344864 has more than 80 times the affinity for 5-HT_{1F} receptor [53]. Thus, it is unlikely that LY344864 and lasmiditan are exerting their effects through other 5-HT receptor subtypes.

In conclusion, agonism of 5-HT_{1F} receptor induces MB, angiogenesis, and migration pathways. Mitochondrial dysfunction and vascular rarefication are hallmarks of many types of kidney injury. Thus, targeting 5-HT_{1F} receptor following kidney injury could serve as a novel therapeutic target to promote organ recovery. Importantly, lasmiditan has been submitted to the FDA for approval in the clinical treatment of migraines. Thus, lasmiditan could be repurposed to promote vascular recovery following kidney diseases and injury.

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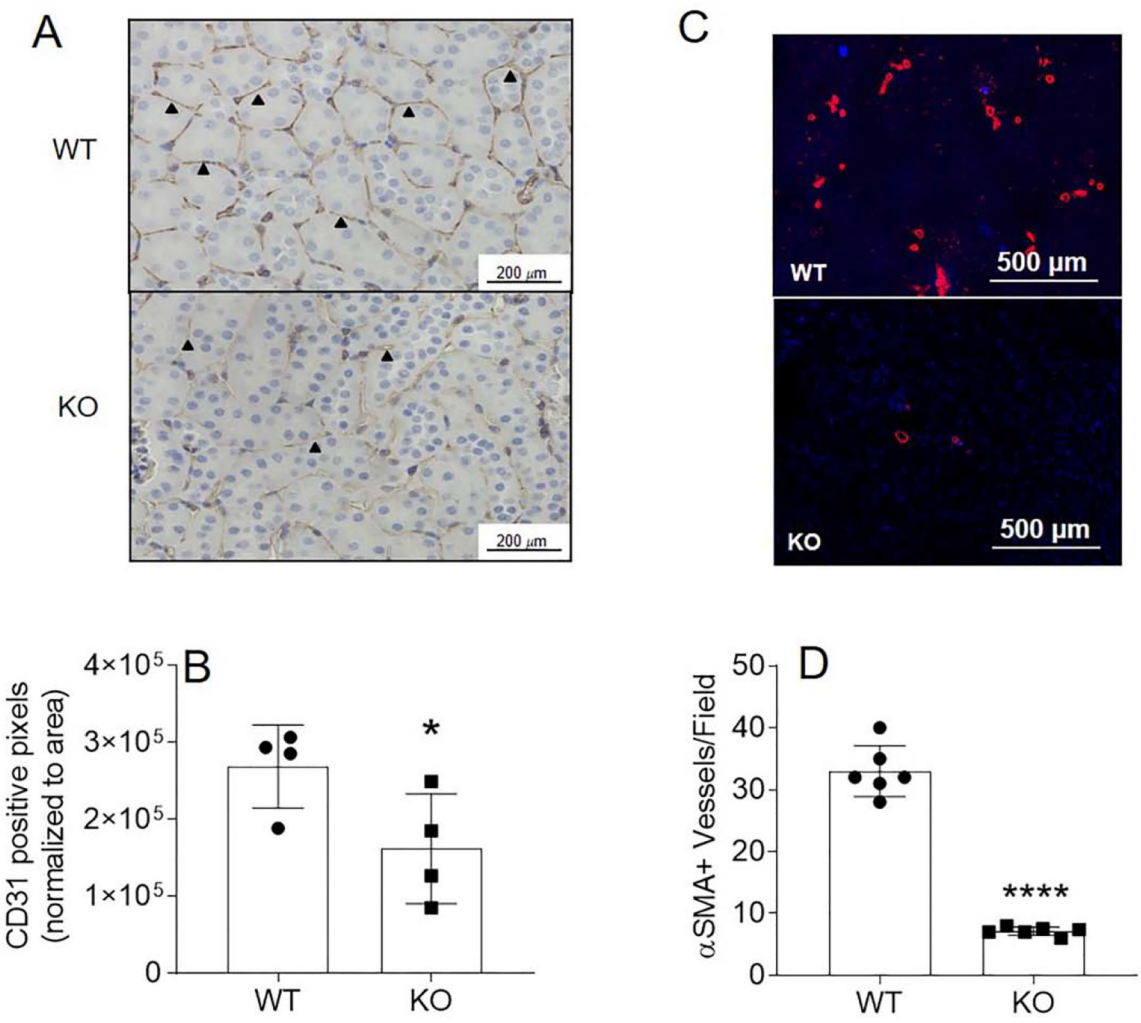

Figure 1. Mice lacking 5-HT_{1f} receptor have decreased renal vasculature.

(A) Immnunohistochemical (IHC) analysis of CD31 (Brown) was assessed in male 10-wk old 5-HT_{1F} receptor knockout (KO) and wild type (WT) mouse kidneys (200X magnification). (B) CD31 positive pixels were normalized to the total area per field of view (mm^2). (C) Immunofluorescent analysis of αSMA (Red) was assessed in male 10-wk old 5-HT_{1F} receptor KO and WT mouse kidneys (200X magnification). (D) Enumeration of aSMA positive vessels per field. Data are reported as means ± SD, * signifies differences from WT, two-tailed unpaired t-test was utilized to determine statistical differences *p < 0.05, **p< 0.01, and ***p < 0.001. CD31, n= 4 ; αSMA, n=6

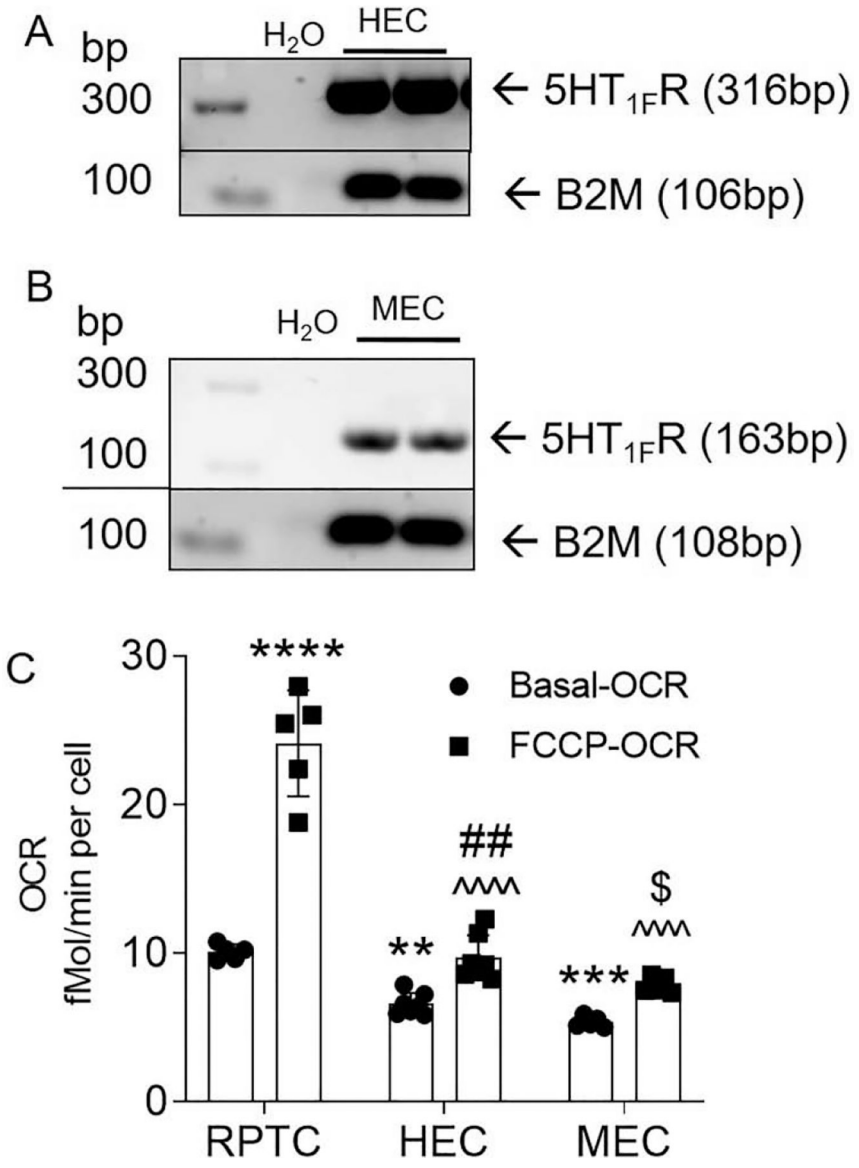

Figure 2. 5-HT$_{1F}$ receptor is expressed in MEC and HEC endothelial cells and respire less than epithelial cells.

mRNA expression of 5-HT$_{1F}$ receptor in HEC (A) and MEC (B). p_2-macroglobulin (B2M) was utilized as a housekeeping gene. (C) Basal- and FCCP- OCR in RPTC, MEC, and HEC were measured using Seahorse XF96 analyzer. Data are reported as means ± SD. * signifies differences from RPTC Basal- OCR, ^ signifies differences from RPTC FCCP-OCR, # signifies differences from HEC Basal-OCR, and $ signifies differences from MEC Basal-OCR, two-way ANOVA with Sidak post-hoc analysis was utilized to determine statistical differences, $*p < 0.05$, **p< 0.01, and ***p < 0.001. (A and B) n=2; (C) RPTC n=5, MEC n=6, HEC n=6.

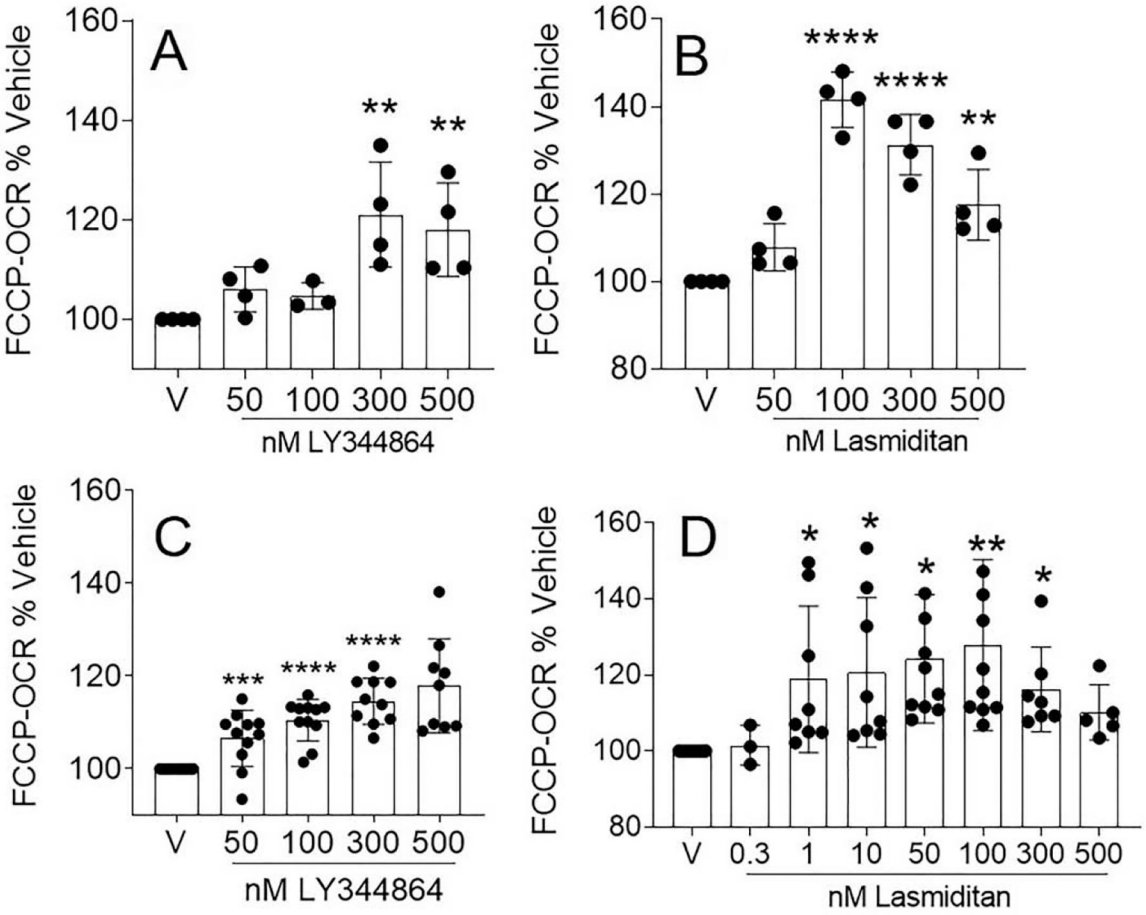

Figure 3. 5-HT$_{1F}$ receptor agonists increase FCCP-OCR in MEC and HEC.

MEC and HEC were treated with either (A, C) LY344864 (0–500 nM) or (B, D) lasmiditan 0–500 nM) for 48h (MEC) or 24h (HEC). After LY344864 or lasmiditan treatment FCCP-OCR, uncoupled mitochondrial oxygen consumption rates were measured using Seahorse XF96 analyzer. Data are reported as means ± SD, * signifies differences from vehicle (v) two-tailed student's t-test was utilized to determine statistical differences, $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$. MEC, n=4; HEC, n=3–10

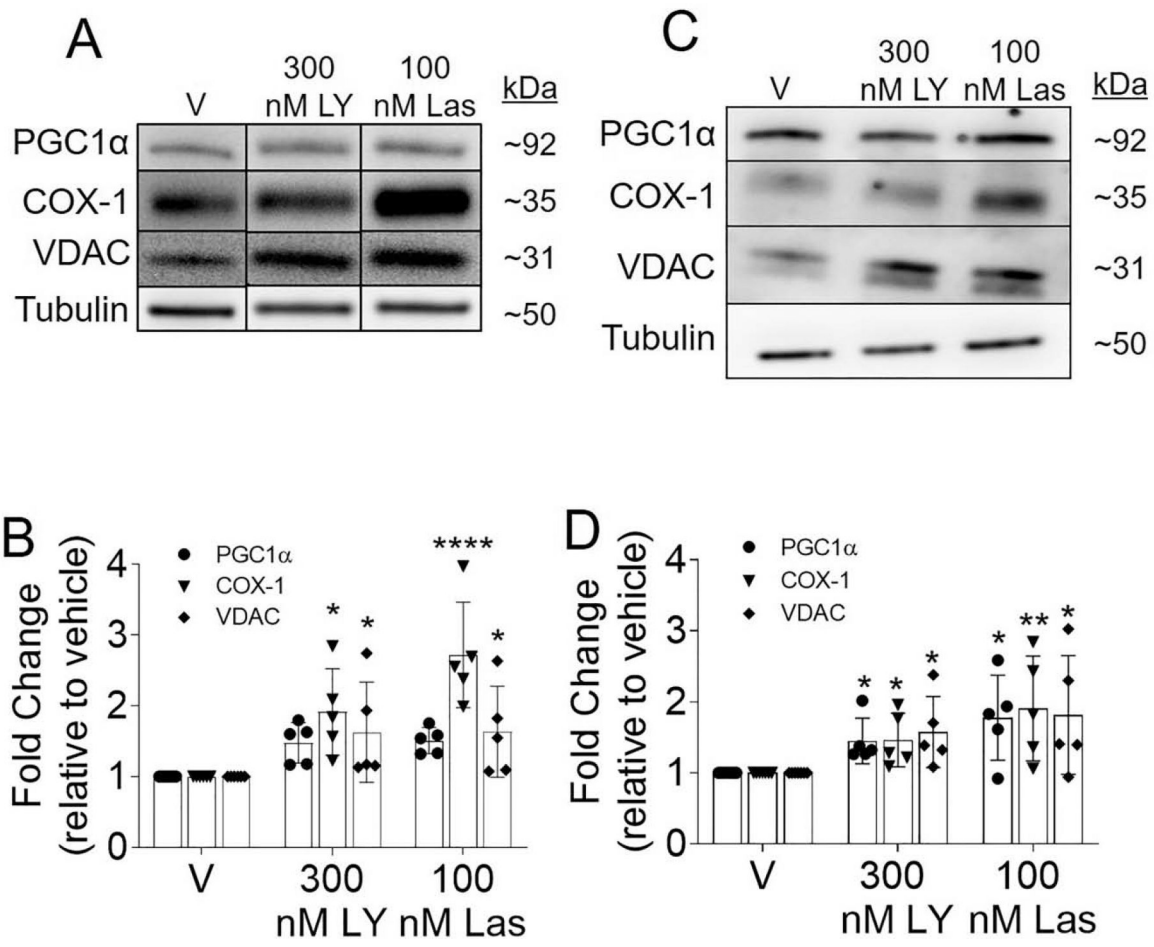

Figure 4. Agonism of the 5-HTiF_receptor increases MB markers in HEC and MEC.

HEC (A,C) and MEC (B,D) were treated with either LY344864 (300 nM) or lasmiditan (100nM) for 72h. Protein expression of PCGIα, COX1, VDAC, and tubulin were assessed, normalized to tubulin and subjected to densitometry analysis. Data are reported as means ± SD, n=5. * Signifies differences from vehicle control, one-way ANOVA with Dunnett post-hoc analysis was utilized to determine statistical differences, $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$.

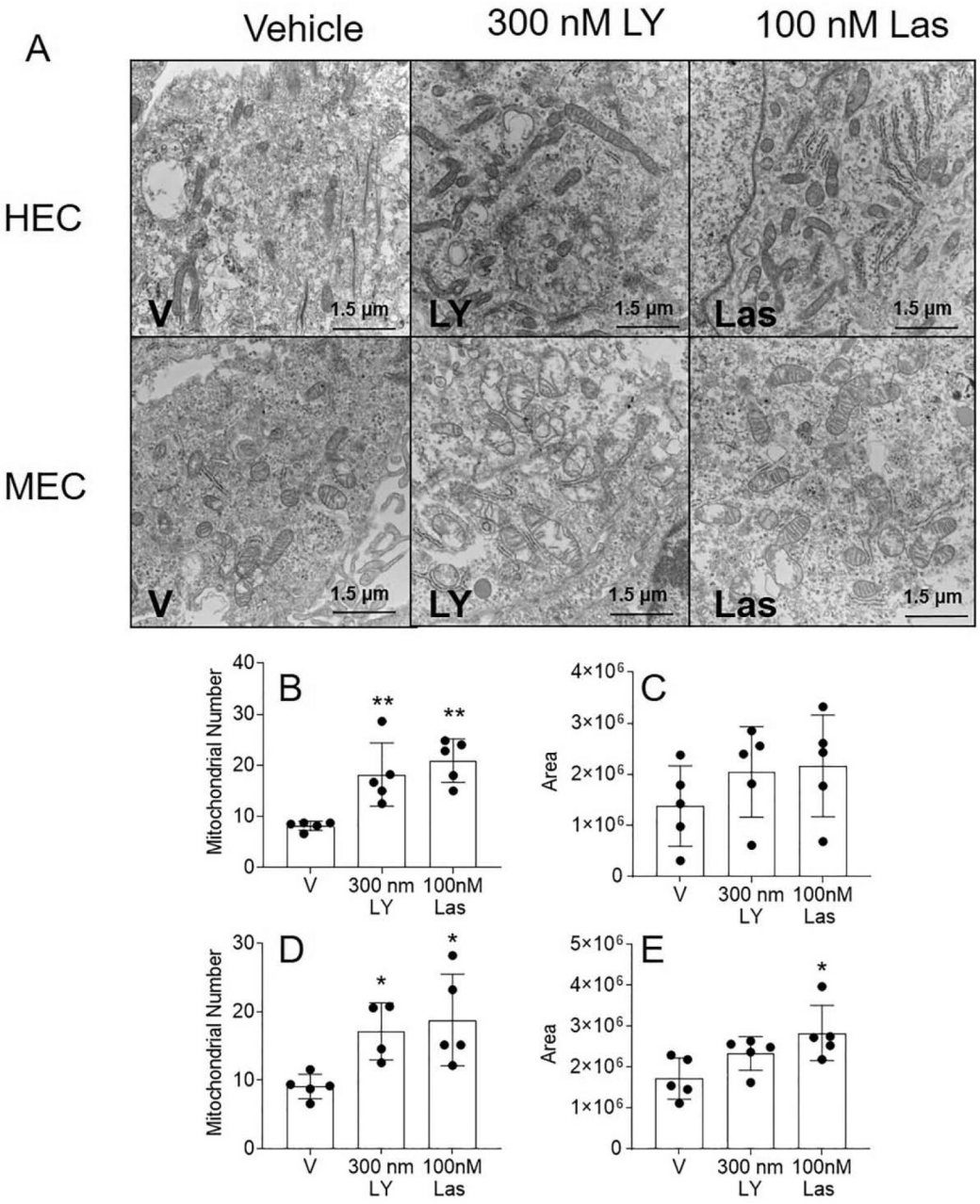

Figure 5. Agonism of 5-HTiF/eceptor increases mitochondrial number in MEC and HEC.

HEC and MEC were treated with Vehicle (V), LY344864 (LY) or Lasmiditan (Las) for 72h. Cells were then fixed and processed for imaging via TEM. (A) Representative photomicrographs of HEC and MEC (16,500X magnification). Mitochondrial number/field of 5 cells in each treatment group were enumerated in (B) HEC and (D) MEC. Average mitochondrial area was calculated in HEC (C) and MEC (E). Data are reported as means ± SD, n=5. * Signifies differences from vehicle (V) one-way ANOVA with Dunnnett post-hoc analysis was utilized to determine statistical differences, $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$, n= 5

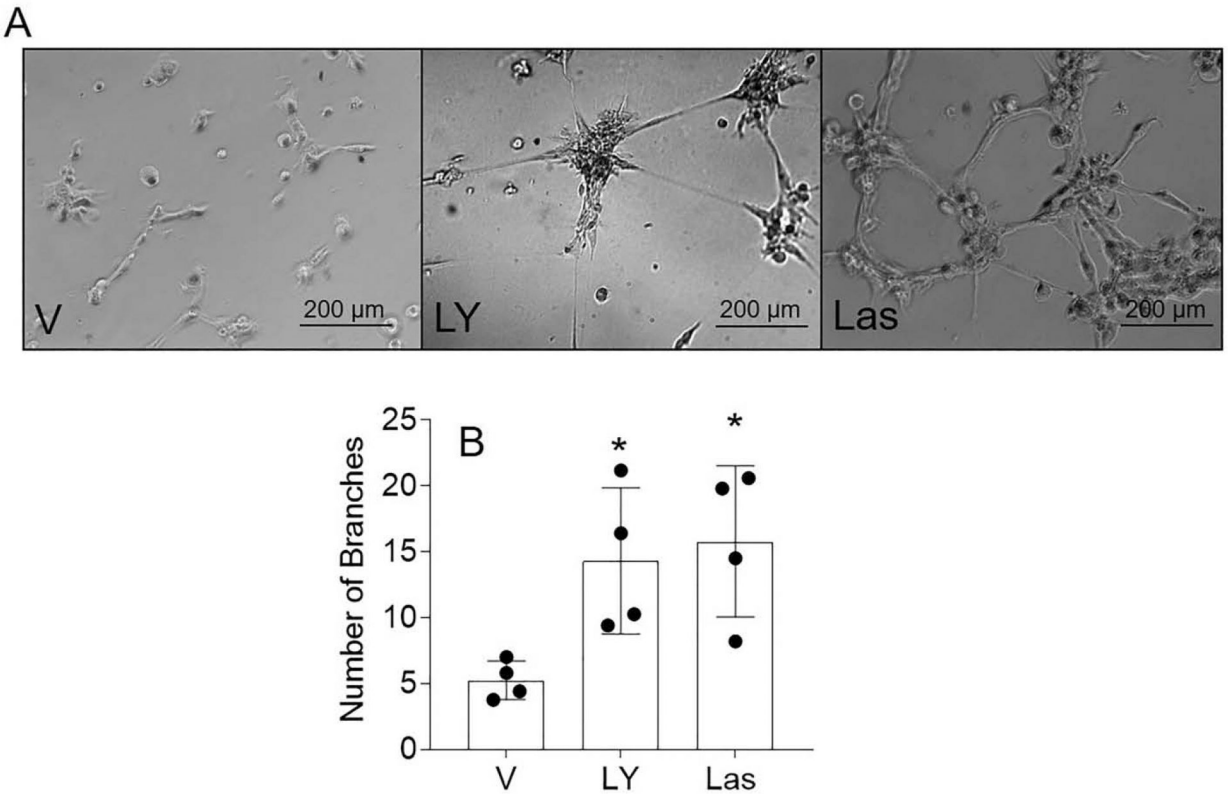

Figure 6. Aqonism of 5-HT$_{1F}$ receptor increases endothelial branching morphogenesis.

HEC were grown to confluence, and concurrently serum-starved and treated with vehicle (V), 300nM LY344864 (LY), or 100nM Lasmiditan (Las) for 24h. Cells were then seeded on matrigel and allowed to migrate for 6h to assess branching morphogenesisi formation. (A) Representative photomicrographs of HEC taken at 100X magnification. (B) Quantification of endothelial branching was determined with the ImageJ angiogenesis analyzer plug-in. Data are reported as means ± SD, n=4. * Signifies differences from vehicle (V), one-way ANOVA with Dunnnett post-hoc analysis was utilized to determine statistical differences, $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$.

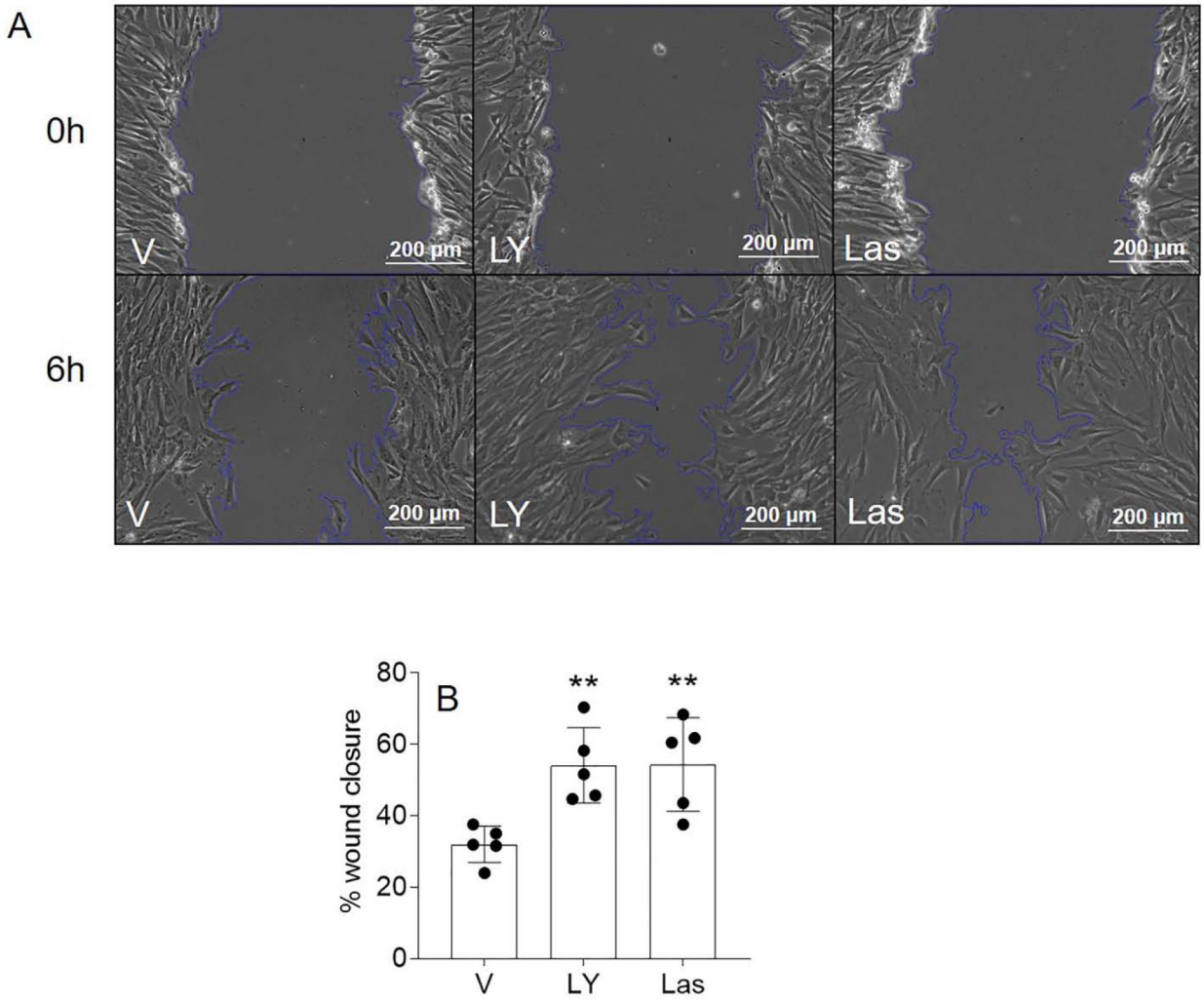

Figure 7. Agonism of 5-HTiF receptor in HEC increases endothelial cell migration.

HEC were grown to confluence and then concurrently serum-starved and treated with vehicle (V), 300nM LY344864 (LY), or 100nM Lasmiditan (Las) for 24h. The cell monolayer was scratched with a 10 uL pipette tip, and the media was replaced with complete media. (A) Representative photomicrographs at 0h post-scratch (100X magnification). (B) Representative photomicrographs at 6h post-scratch (100X magnification). (C) Quantitative analysis of percent wound (scratch) closure. Data are reported as means ± SD, n= 5. * Signifies differences from vehicle (v), one-way ANOVA with Dunnnett post-hoc analysis was utilized to determine statistical differences, $p<0.05$.