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A novel regulator of angiogenesis in endothelial cells: 5-hydroxytriptamine 4 receptor

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Abstract

The 5-hydroxytryptamine type 4 receptor (5-HT_4R) regulates many physiological processes, including learning and memory, cognition, and gastrointestinal motility. Little is known about its role in angiogenesis. Using mouse hindlimb ischemia model of angiogenesis, we observed a significant reduction of limb blood flow recovery 14 days after ischemia and a decrease in density of CD31-positive vessels in adductor muscles in 5-$HT_4R^{-/-}$ mice compared to wild type littermates. Our *in vitro* data indicated that 5-HT_4R endogenously expressed in endothelial cells (ECs) may promote angiogenesis. Inhibition of the receptor with 5-HT_4R antagonist RS 39604 reduced EC capillary tube formation in the reconstituted basement membrane. Using Boyden chamber migration assay and wound healing "scratch" assay, we demonstrated that RS 39604 treatment significantly suppressed EC migration. Transendothelial resistance measurement and immunofluorescence analysis showed that a 5-HT_4R agonist RS 67333 led to an increase in endothelial permeability, actin stress fiber and interendothelial gap formation. Importantly, we provided the evidence that 5-HT_4R-regulated EC migration may be mediated by Gα13 and RhoA. Our results suggest a prominent role of 5-HT_4R in promoting angiogenesis and identify 5-HT_4R as a potential therapeutic target for modulating angiogenesis under pathological conditions.

Keywords

angiogenesis; 5-hydroxytriptamine 4 receptor; endothelial cell migration; hindlimb ischemia model

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Disclosures
None

Introduction

Angiogenesis is a process of capillary formation from the existing vessels. Endothelial cells (ECs) play a principal role in angiogenesis, giving rise to vascular extensions by sprouting [1]. Angiogenesis is composed of multiple steps that include proliferation, migration, morphogenesis, and maturation of ECs [1]. Other distinctive features of angiogenesis include increased vascular permeability [2], changes in the adhesion of ECs to the extracellular matrix and induction of protease activities [3]. The main transmembrane receptors that transduce angiogenic signals are G-protein-coupled receptors, tyrosine-kinase receptors and serine-threonine kinase receptors [3]. Angiogenesis is essential during embryonic development, growth, regeneration and wound healing [4]. It is also implicated in many pathological processes including tumorigenesis and tumor malignancy [5,6], macular degeneration, endomethriosis, arthritis [6], and neovascularization of ischemic tissue [7]. Therefore, it is of considerable clinical interest to understand regulatory mechanisms that promote or inhibit angiogenesis.

Serotonin (5-hydroxytryptamine, 5-HT) is a neuromodulator involved in a variety of important physiological functions [8]. Among known 5-HT receptors, 5-HT_3 is an ion channel, whereas all other types (5-HT_1 to 5-HT_7) are G protein-coupled receptors [9]. Besides its well-established function as a neurotransmitter in CNS, the evidence is emerging for diverse functions of 5-HT in numerous physiological processes outside CNS. It has been reported that 5-HT may promote tumor growth [10], which is significantly reduced in 5-HT transporter knockout mice with depleted 5-HT levels [11]. 5-HT has an essential role in the colon cancer growth by suppressing expression of matrix metalloproteinase 12 and thus lowering the production of antiangiogenic peptide angiostatin [12]. Thus, one of the mechanisms of 5-HT's tumorigenesis-promoting effects may be stimulation of angiogenesis. Several reports suggested that 5-HT may play a positive role in angiogenesis [13,14,12]).

Little is known about the receptors and downstream signaling that may contribute to the effects of 5-HT on angiogenesis. To date, 5-HT_1 and 5-HT_2 receptors were implicated in angiogenic effects of 5-HT [15,13,10]. Some evidence became recently available on phosphorylation signaling activated by 5-HT in EC, presumably largely via 5-HT_{1B} receptor [16]. In addition to 5-HT_1 and 5-HT_2 receptors, expression of mRNA for 5-HT_4 and 5-HT_7 receptors was reported in ECs from different vascular beds [17]. The role of 5-HT_4 receptor (5-HT_4R) in angiogenesis has not been investigated. 5-HT_4R is expressed in high-grade prostate cancer and its inhibition reduces tumor growth [18], but whether this effect may involve tumor angiogenesis remains unknown. Mosapride, known as a 5-HT_4R agonist, was reported to have antiangiogenic effects *in vitro* [19].

5-HT_4R is coupled to the Gs protein, leading to activation of adenylyl cyclase [20]. We have previously demonstrated that 5-HT_4R also activates heterotrimeric Gα13 protein and RhoA, leading to neurite retraction and cell rounding in mouse NIE-115 cells [21].

In the present work, we studied the role of 5-HT_4R in regulation of angiogenic properties of ECs. We established an essential role of 5-HT_4R in *in vivo* angiogenesis, using a mouse hindlimb ischemia model. We further investigated the role of 5-HT_4R in ECs. Using several *in vitro* assays, we demonstrated a critical role of endogenous 5-HT_4R in regulation of capillary tube formation, migration, adhesion, and endothelial permeability in human umbilical vein ECs (HUVECs). Finally, we proposed the mechanism of 5-HT_4R-mediated regulation of EC migration. These data demonstrate a prominent role of 5-HT_4R in regulation of angiogenesis in ECs.

Materials and Methods

Materials

Rabbit polyclonal anti-5-HT_4-receptor antibody was obtained from Evgeni Ponimaskin (Universität Göttingen, Germany). pGEX-2T plasmid containing gluthation S-transferase-rhotekin-Rho binding domain, provided by Dr. M.A. Schwartz (Scripps Research Institute, La Jolla, CA). 5-HT_4 receptor agonist, RS 67333 hydrochloride, and antagonist, RS 39604 hydrochloride, were from Tocris (Ellisville, MO). Monoclonal anti-VE-cadherin, anti-RhoA, anti-Hsp90 and polyclonal anti-Gα13 antibodies were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). 5-HT_7 receptor agonist, 5-carboxamidotryptamine maleate salt (5-CT), and serotonin or 5-hydroxytryptamine (5-HT) were purchased from Sigma-Aldrich (St. Louis, MO). Thrombin was purchased from Enzyme Research Laboratories Inc. (South Bend, IN). Rho kinase inhibitor Y-27632 and bovine serum albumin (BSA) was purchased from Sigma-Aldrich (St. Louis, MO). Protein kinase A inhibitor was from EMD (Gibbstown, NJ). Fetal bovine serum (FBS) and Hanks' balanced salt solution (HBSS) were from Invitrogen (Carlsbad, CA). Endothelial growth medium (EGM-2) BulletKit, EGM-2 MV BulletKit and EBM-2 basal medium were purchased from Lonza (Walkersville, MD).

Mice studies

The generation and characterization of the 5-$HT_4R^{-/-}$ mouse is described by Compan et al. [22]. 5-$HT_4R^{-/-}$ mice have pure 129sv background. Wild type (WT) mice and 5-$HT_4R^{-/-}$ mice were age-matched littermates obtained by heterozygous breeding. The colonies were maintained at the University of Illinois College of Medicine Research Building animal facility. The studies involving animal models were approved by the University of Illinois Institutional Animal Care and Use Committee.

Mouse hindlimb ischemia model

Female mice, aged 9–10 weeks were used. Mice were subjected to unilateral hindlimb surgery under anesthesia with intraperitoneal administration of ketamine (100 mg/kg) and xylazine (10 mg/kg). We performed ligation and segmental resection of left superficial and deep femoral vessels followed by physiological and histological analysis as was described previously [23]. The left femoral artery was exposed, ligated both proximally and distally using 6-0 silk sutures and the vessels between the ligatures were excised without damaging the femoral nerve. All arterial branches between the ligations were obliterated using an electrical coagulator. Skin closure was done using 6-0 nylon sutures.

Laser Doppler blood flow analysis

Ischemic (left)/nonischemic (right) limb blood flow ratio was measured using a Laser Doppler Blood Flow (LDBF) analyzer (PeriScan PIM 3 System; Perimed, North Royalton, OH) as was described previously [23]. Before and after surgery, both normal and ischemic feet were scanned while mice were under anesthesia on a warming plate at 37 °C. Blood flow was displayed as changes in the laser frequency, represented by different color pixels. The mean LDBF values were calculated, and hindlimb blood flow was expressed as the ratio of ischemic to nonischemic LDBF.

Immunocytochemical analysis of hindlimb tissues

Mice were euthanized and perfused through the left ventricle with PBS followed by 4% paraformaldehyde. The legs were fixed in 4% paraformaldehyde overnight. After decalcification in 10% EDTA, pH 7.5 for 10 days, thighs were cut into 3 pieces to obtain different levels of muscles and embedded in paraffin. Thereafter, 5-μm sections were

deparaffinized, rehydrated, performed antigen retreaval, stained with anti-CD31 antibody (BD Biosciences, San Jose, CA), followed by Vectastain ABC kit, and visualized by 3,3'-Diaminobenzidine (Vector Laboratories Inc., Burlingame, CA). The numbers of CD31-positive capillaries per fiber or the density were counted in 5 high power fields (magnification 400×)/mouse under light microscopy.

Blood pressure measurement

Baseline systolic blood pressure in WT and $5HT4^{-/-}$ mice was measured using the tail-cuff method (BP2000 Visitech System Inc, Apex, NC) as described previously [24].

Cell culture and immunoblotting

HUVECs and human lung microvascular ECs (HLMVECs) were purchased from Lonza (Walkersville, MD), were cultured in the EGM-2 BulletKit and EGM-2 MV BulletKit, respectively, supplemented with 10% FBS up to 8 passages. Confluent HUVECs were lysed in the lysis buffer containing 25 mmol/L HEPES (pH 7.5), 150 mmol/L NaCl, 10% glycerol, 1% Triton X-100, 0.5% Na-deoxycholate, 0.1% SDS, 5 mmol/L EDTA, 1 mmol/L dithiothreitol (DTT), and 5 μL/mL mammalian protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO) and homogenized by passing through 27 gauge needle. The insoluble material was removed from the lysates by centrifugation at 15,000×g for 10 min. Cleared lysates were subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to polyvinylidene difluoride membrane (PVDF) membrane, and analyzed by immunoblotting with anti-5-HT_4 receptor antibodies.

Reverse transcription-PCR (RT-PCR)

Detection of 5-HT_4 receptor mRNA in HUVECs was performed using RT-PCR. Total RNA was isolated from HUVECs using Micro RNA Isolation Kit (Stratagene, La Jolla, CA) according to the manufacturer's protocol. Primer sequences for 5-HT_4 receptor were as follows: sense primer, 5'-AGCACTCATCGCATGAGGACAG-3'; antisense primer, 5'-GGATGATGAGGAAGGCACGTCT-3', which gives the PCR product of 241 bp. RT-PCR was performed using StratScript™ One-Tube RT-PCR System (Stratagene, La Jolla, CA 92037). RT-PCR reaction was performed for 1 cycle of 15 min at 42 °C and 1 min at 95 °C, followed by 40 cycles of 30 s at 95 °C, 30 s at 58 °C and 2 min at 68 °C, and 1 cycle of 5 min at 68 °C. PCR products were analyzed by 1.5% agarose gel electrophoresis.

Capillary tube formation assay

The Basement Membrane Matrix or Matrigel (BD Bioscience, San Jose, CA) layer was formed by pipetting 400 μL/well ice-cold Matrigel in 24-well plate and allowing it to solidify for 30 min at 37 °C. HUVECs were seeded at 4×10^4 cells/well resuspended in 200 μL of EBM-2 medium supplemented with 0.1% BSA. Thereafter, the 5-HT_4R antagonist was added at a concentration as indicated. After 18 h of incubation, the images were taken using Nikon eclipse TE 300 microscope equipped with 4× objective and Nikon cool pix 990 digital camera. The number of branch points is counted in each well using UTHSCSA ImageTool Version 3.0 program (University of Texas Health Science Center, San Antonio, TX, http://ddsdx.uthscsa.edu/dig/itdesc.html). The experiment was repeated three times with similar results.

Boyden chamber migration assay

Migration assays were performed using a 48-well Boyden chamber (Neuroprobe Inc., Gaithersburg, MD) with 8-μm-pore size polycarbonate Nuclepore™ membrane (Whatman, Clifton, NJ). ECs were grown until confluent on gelatin-coated plate and incubated in serum free medium containing 0.1% BSA overnight. Next day, the cells were trypsinized,

resuspended at concentration 1.8×10^6 cells/mL and 29 μl of cell suspension was placed in the lower wells of the chamber. The chamber was incubated upside down at 37 °C for 90 minutes to allow cell attachment to the membrane. Thereafter, 30 μmol/L RS 39604 (5-HT_4 receptor antagonist) or 100 μmol/L 5-HT in 0.1% BSA was applied to the upper wells. After 4 h of incubation, the filter was fixed and stained with Diff Quick kit (Dade Behring, Newark, DE). The cells that migrated through the pores to the upper side of the filter were counted using light microscopy at magnification of 100×. The average number of migrating cells in 10 fields was taken as a number of migrated cells of the group. Each experiment was performed at least three times, and all samples were tested in quadruplicates.

In vitro wound healing assay

HUVECs were seeded at 3×10^5 cells/well into a 6-well plate, incubated for 24 h and maintained in serum free medium for 18 h. Thereafter, the monolayers of HUVECs were scratch wounded in a straight line using a sterile 200 μL tip and marked at the injury line. After wounding, the cells were washed with PBS and incubated with 10 μmol/L RS 39604 (5-HT_4 receptor antagonist) or 100 μmol/L 5-HT in serum free medium for 11 h. Images were taken at the time of the wounding and at 11 h after wounding using Nikon eclipse TE 300 microscope equipped with 4× objective and Nikon cool pix 990 digital camera. The unhealed area was determined using UTHSCSA ImageTool Version 3.0 Program and expressed as a percentage of the originally wounded area. Four measurements were taken from four fields of each well in at least three independent experiments.

Cell viability assay

A Non-Radioactive Cell Proliferation Assay (MTS assay) was used to analyze the effect of RS 39604 (0–30 μmol/L) or 5-HT (0–100 μmol/L) on cell viability. Cells were cultured in 96-well plates (2.5×10^3 cells/well). Cell viability was assessed after 4 or 24 hours. The number of viable cells was determined in triplicate wells by measuring A490 nm 1 hour after adding the MTS reagent (Promega) as described by the manufacturer. Cell viability was expressed as the percentage of viable cells: Aexp group/Acontrol × 100.

Adhesion assay

We used the method described previously [25] with modifications. Briefly, 24-well plates were coated with gelatin for 1 h at 37 °C. Gelatin solution was removed and the dishes were washed and blocked with 1% BSA for 1 h at 37 °C. HUVECs, starved overnight in the serum free medium containing 1% BSA were seeded onto the 24-well plate (1×10^5 cells per well) and incubated in the presence of 30 μmol/L RS 39604 (5-HT_4 receptor antagonist) or medium alone for 30 min at 37 °C. After three washings with phosphate-buffered saline, attached cells were fixed and stained with 0.5% solution of crystal violet. The adhesion was quantified by counting cell number in 4 fields of each well using light microscopy at magnification of 10×. All adhesion experiments were done in triplicates and repeated at least three times.

Transfection of siRNAs

HUVECs were transfected using siRNA transfection kit (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) according to the manufacturer's protocol. Gα13-specific siRNA was made by Qiagen (Valencia, CA) to target the sequence AAGGAGATCGACAAATGCCTG (94–114) of human Gα13. RhoA-specific siRNA duplexes were purchased from Dharmacon (Thermo Fisher Scientific, Waltham, MA). The non-silencing control siRNA was from Qiagen (Valencia, CA). The effects of siRNAs were confirmed by Western blotting 48 h after transfection. Detection of the Hsp90 served as a control for the equal loading.

Rho GTPase activation assay

Determination of Rho GTPase activation was performed as described previously [21]. DH5 bacterial cells were transformed with the pGEX-2T plasmid containing gluthation S-transferase-rhotekin-Rho binding domain. After induction with isopropyl-1-thio-D-galactopyranozide (1 mmol/L), gluthation S-transferase-rhotekin-Rho binding domain (GST-rhotekin-Rho binding domain) protein was purified from DH5 bacteria using Gluthation Sepharose 4B beads (Amersham Bioscience, Piscataway, NJ). Confluent HUVECs grown on the 100-mm dishes were serum-starved for 2 h and stimulated with the agonists for 15 minutes as indicated. Thereafter, the cells were quickly washed with the ice cold PBS and lysed in the lysis buffer containing 50 μmol/L This pH 7.5, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecylsulphate, 500 mmol/L NaCl, 10 mmol/L $MgCl_2$, and 5 μL/mL protease inhibitor cocktail for mammalian cells. The cell lysates were centrifuged at 15,000×g at 4 °C for 2 min, and equal volumes of cell lysates were incubated with GST-rhotekin-Rho binding domain beads at 4 °C for 90 min. Subsequently, beads were isolated from the lysates by centrifugation at 1,000×g at 4 °C for 2 min, washed four times with the washing buffer containing 50 mmol/L This pH 7.5, 1% Triton X-100, 150 mmol/L NaCl, 10 mmol/L $MgCl_2$, and 5 μL/mL protease inhibitor cocktail for mammalian cells and boiled in the Leammli sample buffer. The total lysates and samples eluted from the beads after boiling were subjected to 12% SDS-PAGE and RhoA protein was detected by immunoblotting with monoclonal anti-RhoA antibody. The amount of the RhoA bound to the GST-rhotekin-Rho binding domain beads was normalized to the total amount of Rho in the cell lysates. Fold increase in RhoA activation was assessed using ImageJ program (U. S. National Institutes of Health, Bethesda, MD, http://rsb.info.nih.gov/ij/).

Transendothelial electrical resistance (TER) measurement

Determination of the electrical impedance characteristics of EC monolayer grown on gold electrode allows for monitoring of EC shape change and transendothelial permeability in real time. The electrical resistance across the EC monolayer was measured using an electric cell substrate impedance sensing (ECIS) 1600R [26–28](Applied BioPhysics, Troy, NY). Briefly, ECs, grown on the small gold electrode until they reached confluence, were serum-starved for 2 h before the experiment. Thereafter, the electrodes were connected to a phase-sensitive lock-in amplifier and a constant current of 1 μA was applied between the small electrodes and the large counter electrode using a 1-V, 4,000-Hz AC signal supplied through a 1-MΩ resistor. The voltage changes were recorded every 2 minutes over the period of 5 h, stored and processed by ECIS software. The cells were stimulated with 100 μmol/L RS 67333 where indicated. The data are presented as the change in the in-phase voltage normalized to its initial value [27].

Immunostaining and confocal microscopy

HUVECs were maintained in the EGM-2 medium supplemented with 10% FBS. Two hours before the stimulation, the cells were maintained in serum-free EBM-2 medium. After stimulation with 100 μmol/L RS 67333 for 15 min, the cells were washed with HBSS, fixed with 2% paraformaldehyde and permeabilized with 0.1% Triton X-100. Nonspecific binding was blocked with blocking solution containing 0.2% fish skin gelatin (Sigma-Aldrich, St. Louis, MO) and 1% BSA in HBSS followed by incubation with mouse anti-VE-cadherin antibody followed by anti-mouse Alexa Fluor 488 (green fluorescence) antibody to detect the localization of endogenous VE-cadherin and phalloidin Alexa Fluor 594 (red fluorescence) to detect actin fibers. Thereafter, the coverslips were mounted using ProLong antifade kit (Molecular Probes, Invitrogen, Carlsbad, CA). Images were taken by laser scanning confocal microscopy on a Zeiss LSM 510 microscope equipped with 63× water-immersion objective and laser excitations at 488 and 543 nm.

Statistical analysis

All experiments were carried out at least in triplicates. The results are expressed as the mean value ± SE. The statistical differences between two groups were evaluated using Student's t-test. The flow recovery data from hindlimb ischemia model were analyzed by two-way ANOVA. A value of $P < 0.05$ was considered statistically significant.

Results

5-HT$_4$R plays a critical role in neovascularization in ischemic muscle

To assess the role of 5-HT$_4$R in angiogenesis *in vivo*, we used a mouse hindlimb ischemia model [23]. Wild type (WT) or 5-HT$_4$R$^{-/-}$ mice were subjected to unilateral hindlimb surgery under anesthesia followed by ligation and segmental resection of left femoral vessels. Ischemic (left)/nonischemic (right) limb blood flow ratio was measured using a laser Doppler blood flow analyzer. The 5-HT$_4$R$^{-/-}$ mice showed significant reduction of limb blood flow recovery 14 days after ischemia compared to WT littermates (Figure 1A and 1B). Histological analysis revealed that capillary density of CD31-positive vessels in adductor muscles was markedly increased after femoral artery ligation, which was significantly inhibited in the 5-HT$_4$R$^{-/-}$ mice. There was no difference in the number of the CD31-positive vessels in non-ischemic limb in WT and 5-HT$_4$R$^{-/-}$ mice (Figure 1C and 1D). Systolic blood pressure was similar in WT and 5-HT$_4$R$^{-/-}$ mice, eliminating the possibility that systolic blood pressure change affected these differences (Figure 1E). Thus, these data suggest that 5-HT$_4$R plays a critical role in capillary formation in ischemic muscles, thereby regulating reparative neovascularization.

5-HT$_4$R regulates capillary tube formation

The expression of 5-HT$_4$R in HUVECs was confirmed by detecting its mRNA using RT-PCR, and protein expression using immunoblotting (Figure 2A and 2B). Because our data demonstrated that 5-HT$_4$R is essential for capillary formation in ischemic muscle, we further investigated the role of 5-HT$_4$R in angiogenic responses of ECs. We examined the role of 5-HT$_4$R in formation of endothelial tube-like structures on reconstituted basement membrane, Matrigel, an *in vitro* assay for angiogenesis. To obtain an *in vitro* setting closest to our *in vivo* knock-out model and to validate the results on human endothelial cells, we initially attempted to downregulate 5-HT$_4$R in ECs using siRNA. However, we could not observe any significant decrease in either mRNA (RT-PCR) or protein (WB) expression using a pool of four commercially available 5-HT$_4$R siRNAs under several conditions (data not shown). Therefore, we chose to downregulate 5-HT$_4$R function pharmacologically. It should be noted that 5-HT$_4$R has considerable constitutive activity, which makes it possible to use its antagonists without any additional receptor activation, such as by its agonist 5-HT [21,29]. Our results showed that treatment of HUVECs with 5-HT$_4$R antagonist RS 39604 significantly inhibited capillary tube formation in a dose-dependent manner (1 μmol/L and 10 μmol/L, Figure 2C). Since the formation of tube-like structures is an important step in angiogenesis, our data indicate a prominent role of 5-HT$_4$R in angiogenesis *in vitro*.

5-HT$_4$R regulates EC migration and adhesion

To investigate the role of 5-HT$_4$R in HUVEC migration, we employed two approaches: i) Boyden chamber migration assay, commonly used for quantification of the cell migration through the porous membrane towards the compartment with the agent of interest [30], and ii) *in vitro* wound healing "scratch" assay that mimics cell migration into an artificial wound produced on a cell monolayer [31]. In the Boyden chamber assay, treatment with 5-HT$_4$R antagonist RS 39604 significantly reduced the number of migrated cells both under basal conditions and after 5-HT stimulation (Figure 3). Similar results were obtained using human

lung microvascular ECs, HLMVEC (data not shown). In a wound healing "scratch" assay, treatment with RS 39604 inhibited the healing ability of HUVECs at 11 h (Figure 4A and 4B) and 17 h after wounding (data not shown). We also tested the effect of 5-HT (i.e. broad specificity agonist) on cell migration in both assays. In the Boyden chamber assay, the effect of 5-HT varied from no effect to a considerable increase in migration shown in Figure 3 (though replicates within each experiment were consistent). In the "scratch" assay, 5-HT treatment had no significant effect (Figure 4A and 4B). Variability of the effects of 5-HT might be due to 5-HT_4R internalization in the presence of agonists [32] and to a longer duration of the "scratch" assay vs. Boyden chamber assay. Whether 5-HT was present or not, the effects of RS 39604 were similar (Figure 4A and 4B).

Cell viability (MTS) assays showed that RS 39604 and 5-HT did not affect cell viability in the concentrations and duration of treatment used under our experimental conditions (Figure 4C and 4D). We observed reduced viability after 24 h treatment with 30 μM RS 39604, but these conditions were not used in our migration experiments.

Because attachment of ECs to extracellular matrix is a critical step for the process of angiogenesis that affects cell migration [25], we investigated if 5-HT_4R inactivation would influence cell adhesion to gelatin-coated tissue-culture dishes. Treatment of HUVECs with RS 39604 caused a significant decrease in the number of adhered cells compared to untreated cells (Figure 4E). Taken together, our results suggest that 5-HT_4R is required for migration and adhesion of ECs.

Gα13 and RhoA, but not PKA, mediate 5-HT_4R-dependent migration

We examined the specific mechanisms, which endogenous 5-HT_4R employs to regulate EC migration. As described above, we found that the effects of 5-HT_4R antagonist RS 39604 on EC migration are more consistent as compared to 5-HT. Therefore, we used inhibition of EC migration by RS 39604 as a read-out in the following experiments; using an antagonist also allowed us to closer recapitulate conditions of our *in vivo* studies (i.e. the absence of 5-HT_4R signaling).

Our previous studies showed that 5-HT_4R is coupled directly to Gα13 [21]. Therefore, we used Gα13-specific siRNA, which reduced the expression of endogenous Gα13 by approximately 70% as detected by Western blot analysis (Figure 5A, **inset**), and examined cell migration in Gα13-depleted cells with or without 5-HT_4R antagonist. The Boyden chamber assay showed that the basal level of migration was significantly reduced in Gα13-depleted cells (Figure 5A). Treatment with 5-HT_4R antagonist RS 39604 resulted in a small, not statistically significant, further reduction of migration in Gα13-depleted cells in comparison to Gα13-expressing cells (Figure 5A). These results suggest that depletion of Gα13 abolishes the ability of RS 39604 to reduce EC migration, indicating that Gα13 is required for EC migration mediated by 5-HT_4R.

RhoA is involved in the downstream signaling initiated by Gα13 [21,33]. Rho proteins play essential roles in several stages of angiogenesis, including migration [34]. To address whether RhoA is involved in 5-HT_4R-mediated cell migration, we examined the effects of RhoA protein knockdown with specific siRNA [35]. The basal level of migration was significantly reduced in HUVECs depleted of RhoA. After treatment with RS 39604, the migration was slightly, but not significantly, reduced in RhoA siRNA-transfected cells compared to control siRNA-transfected cells (Figure 5B). These results suggest that depletion of RhoA abolished the ability of RS 39604 to reduce EC migration, indicating that RhoA is required for EC migration mediated by 5-HT_4R.

The Rho kinases, ROCKI and ROCKII, have been identified as downstream effectors of RhoA [36,37]. To determine if ROCK is essential for 5-HT_4R-mediated migration, cells were treated with RS 39604 and a specific ROCK inhibitor Y-27632, followed by analysis of cell migration. In the presence of Y-27632, migration of cells treated with RS 39604 was slightly, but not significantly inhibited, suggesting that inhibition of ROCK abolished the ability of RS 39604 to inhibit EC migration (Figure 5C). These results indicate that the RhoA-ROCK pathway may be involved in regulation of 5-HT_4R-induced EC migration.

5-HT_4R is coupled not only to Gα13, but also to Gαs, which can activate PKA [20,21,38], and PKA activity was implicated in angiogenesis [39,40]. Therefore, we next addressed whether PKA is involved in 5-HT_4R-mediated cell migration. Inhibition of PKA with specific PKA inhibitor 14–22 amide (PKI) increased cell migration compared to control. Pretreatment of HUVECs with PKI followed by treatment with RS 39604 significantly reduced EC migration when compared to the cells treated with PKI only (Figure 5C). This result suggests that PKA does not change the ability of RS 39604 to reduce EC migration indicating that PKA is not involved in the regulation of EC migration mediated by 5-HT_4R.

Stimulation of endogenous 5-HT_4R activates RhoA and induces intercellular gap formation and increased endothelial permeability

We next tested if inhibition of endogenous 5-HT_4R by RS 39604 may reduce activation of RhoA in HUVECs using affinity-precipitation of active RhoA with Rho-binding domain of the RhoA effector, rhotekin. However, we were unable to detect a significant difference (data not shown), likely because the assay measures total cellular RhoA activity, while 5-HT_4R-dependent RhoA activity would only constitute a small fraction. Therefore, we employed 5-HT and a more specific 5-HT_4R agonist RS 67333 to directly assess RhoA activation. Both 5-HT and RS 67333 stimulation of HUVECs resulted in a 2-fold activation of RhoA, whereas stimulation with thrombin, used as a positive control, induced a 3-fold activation (Figure 6A and 6B). No RhoA activation was observed after treatment with 5-CT, a 5-HT_7R agonist, indicating that this effect is specific for 5-HT_4R (Figure 6A and 6B). This result indicates that the endogenous 5-HT_4R activates RhoA in ECs.

It is well established that activation of RhoA results in actin stress fiber formation and cell retraction [41]. Therefore, we analyzed the effect of 5-HT_4R stimulation on actin fibers and EC morphology using immunofluorescence. Whereas control cells had low level of polymerized actin, RS 67333 increased actin stress fiber formation and induced discontinued cell junctions with interendothelial gap formation (Figure 6C, arrows). RS 39604, which might be expected to reduce stress fiber formation, did not produce any noticeable effects (data not shown), probably due to the low level of stress fibers in control cells.

Since 5-HT_4R stimulation induced formation of interendothelial gaps, we next examined the role of 5-HT_4R in regulation of endothelial permeability by monitoring TER after treatment with RS 67333. Stimulation of 5-HT4R with RS 67333 induced transient increase in endothelial permeability as evidenced by decreased TER (Figure 6D). RS 67333 was added 45 min after the beginning of the recording and maximum permeability increase was observed 30 min after addition of agonist (corresponding to 75 min from the initiation of the recording). The TER returned to a baseline value about 135 min after addition of agonist (corresponding to 190 min from the initiation of the recording). We observed similar changes in TER in response to RS 67333 stimulation in HLMVEC (data not shown).

Discussion

In the present study, using 5-$HT_4R^{-/-}$ mice, we have demonstrated that 5-HT_4R plays an important role in postnatal angiogenesis in response to hindlimb ischemia. Moreover, we

have confirmed these findings *in vitro* by demonstrating that 5-HT_4R expressed endogenously in ECs is essential in regulating capillary tube formation, EC migration, adhesion, and endothelial permeability, indicating that it is a critical factor in angiogenic responses of EC.

Using 5-$HT_4R^{-/-}$ mice models, several groups have demonstrated the critical role of 5-HT_4R in learning and memory [42,43], as well as sensitivity to novelty and stress [22]. However, a role of 5-HT_4R in angiogenesis has not been investigated. In this study, we used mouse hindlimb ischemia model [23], a well-established assay to assess angiogenesis *in vivo*, which involves responses related to the pathophysiological states such as infarction and ischemic cardiovascular disease. We demonstrated that 5-$HT_4R^{-/-}$ mice showed a significant reduction in blood flow recovery at 14 days post-surgery as well as a decrease in capillary density relative to WT, suggesting that the 5-HT_4R may promote angiogenesis. Notably, the differences we observed in the flow recovery between WT and 5-$HT_4R^{-/-}$ mice were not caused by the differences in systolic blood pressure as these were not significantly different between the two strains. Our data identified for the first time a critical role for 5-HT_4R in capillary formation in ischemic muscles and in reparative neovascularization in response to tissue ischemia. Importantly, this observation correlated well with our *in vitro* data.

Numerous *in vitro* assays have been developed to test each step of angiogenesis. In most cases they are two-dimensional and thus have limitations in representing situation *in vivo*. Yet, such assays may be useful, especially if correlated with the *in vivo* data [44]. Matrigel capillary tube formation assay, which tests the ability of ECs to form three-dimensional tubes in the reconstituted basement membrane, is considered as one of the most specific tests for angiogenesis [45]. Pharmacological inhibition of 5-HT_4R reduced tube formation, thus supporting the critical role of this receptor in angiogenesis. Using two different tests for EC migration (Boyden chamber and a “scratch” assay), we also convincingly demonstrated diminished cell migration after pharmacological inhibition of endogenous 5-HT_4R. Thus, the results of our studies demonstrated that inhibition of 5-HT_4R inhibits EC capillary tube formation, migration and adhesion. Taken together, our findings *in vivo* and *in vitro* are consistent with a conclusion that 5-HT_4R is a positive regulator of angiogenesis.

Our data also reveal some details of the molecular mechanisms that mediate the effect of 5-HT_4R on EC migration. Our previous study demonstrated that 5-HT_4R is coupled to Gα13, and that expression of 5-HT_4R activates RhoA [21]. Gα13 deficiency and endothelial-specific deletion of Gα13 in mice impair EC migration associated with angiogenesis, thus resulting in embryonic lethality [46,47]. RhoA signaling is essential for angiogenesis [48]. Increased expression of RhoA results in enhanced vessel branching and sprouting [49]. The inhibition of RhoA/ROCK signaling reduces cell migration [50], capillary tube formation in the Matrigel assay and angiogenesis in the chick embryo [51]. Our results showed that Gα13 or RhoA depletion, or inhibition of ROCK abolished the ability of RS 39604 to inhibit HUVEC migration, thus indicating that these proteins may be required for 5-HT_4R-mediated cell migration. Our finding that the basal level of migration was reduced in HUVECs depleted of RhoA was consistent with previous reports that inhibition of Rho signaling disrupts EC migration [52,53].

Potentially, 5-HT_4R might also regulate EC migration via PKA, since (i) PKA activity potently contributes to regulation of EC migration [39,40]; (ii) 5-HT_4R couples not only to Gα13, but also to Gαs, which activates PKA via a well-established pathway involving elevation of cAMP [20,21,38]; (iii) Gα13 may also activate PKA in a cAMP-independent manner[54,55,35]. However, our data suggest that PKA is not involved in 5-HT_4R-mediated EC migration, since PKA inhibitor was unable to abolish the ability of RS 39604 to inhibit

cell migration. Together, our data indicate that 5-HT_4R may mediate EC migration and thus angiogenesis via Gα13-RhoA-ROCK pathway.

Increase in vascular permeability is one of the essential features of angiogenesis associated with tumors, wounds and other pathological conditions [2,56,57]. Stimulation of 5-HT_4R with its agonist RS 67333 activated RhoA, induced actin stress fiber and interendothelial gap formation, and resulted in an increase in endothelial permeability in HUVECs. We also observed increased permeability upon 5-HT_4R stimulation in HLMVECs, suggesting that this effect may be similar in ECs from different vascular beds. These data suggest that 5-HT_4R may contribute to 5-HT-induced angiogenesis in part by increasing the endothelial permeability. We also demonstrated that stimulation of 5-HT_7R does not lead to an increase in RhoA activity in HUVECs. Further investigation is needed to test if RhoA (and Gα13) is involved in other steps of angiogenesis mediated by 5-HT_4R.

The 5-HT_4R exhibits high level of agonist-independent constitutive activity [21,29]. Constitutive activity of the G-protein-coupled receptors has been reported for numerous receptors in native and heterologous expression systems and is characterized by their oscillation between an inactive and active state. Treatment with an agonist may further shift equilibrium towards an active state. On the other hand, around 85% of G-protein-coupled receptor antagonists known to date have inverse agonist activity [58]. Of considerable interest are our observations that 5-HT_4R antagonist RS 39604 has an inverse agonist activity because it was able to reduce the constitutive activity of 5-HT_4R. Similar findings were reported for 5-HT_4R antagonist ML 10375 that may also act as an inverse agonist [29].

In summary, we reported the novel function of 5-HT_4R as a stimulator of angiogenesis in ECs, revealed using a knock out mouse model and primary human ECs. Thus, this study identified 5-HT_4R as a potential target for development of pharmacological agents to modulate angiogenesis under physiological and pathological conditions. Further investigations should address several areas of particular physiological significance: (i) the cross-talk between signaling of 5-HT_4R and other angiogenesis-regulating factors, such as VEGF [6]; (ii) the roles of other 5-HT receptors in angiogenesis, which may affect the net effect of 5-HT; (iii) the role of 5-HT_4R in the context of pathologically changed blood vessels in the tumor tissue.

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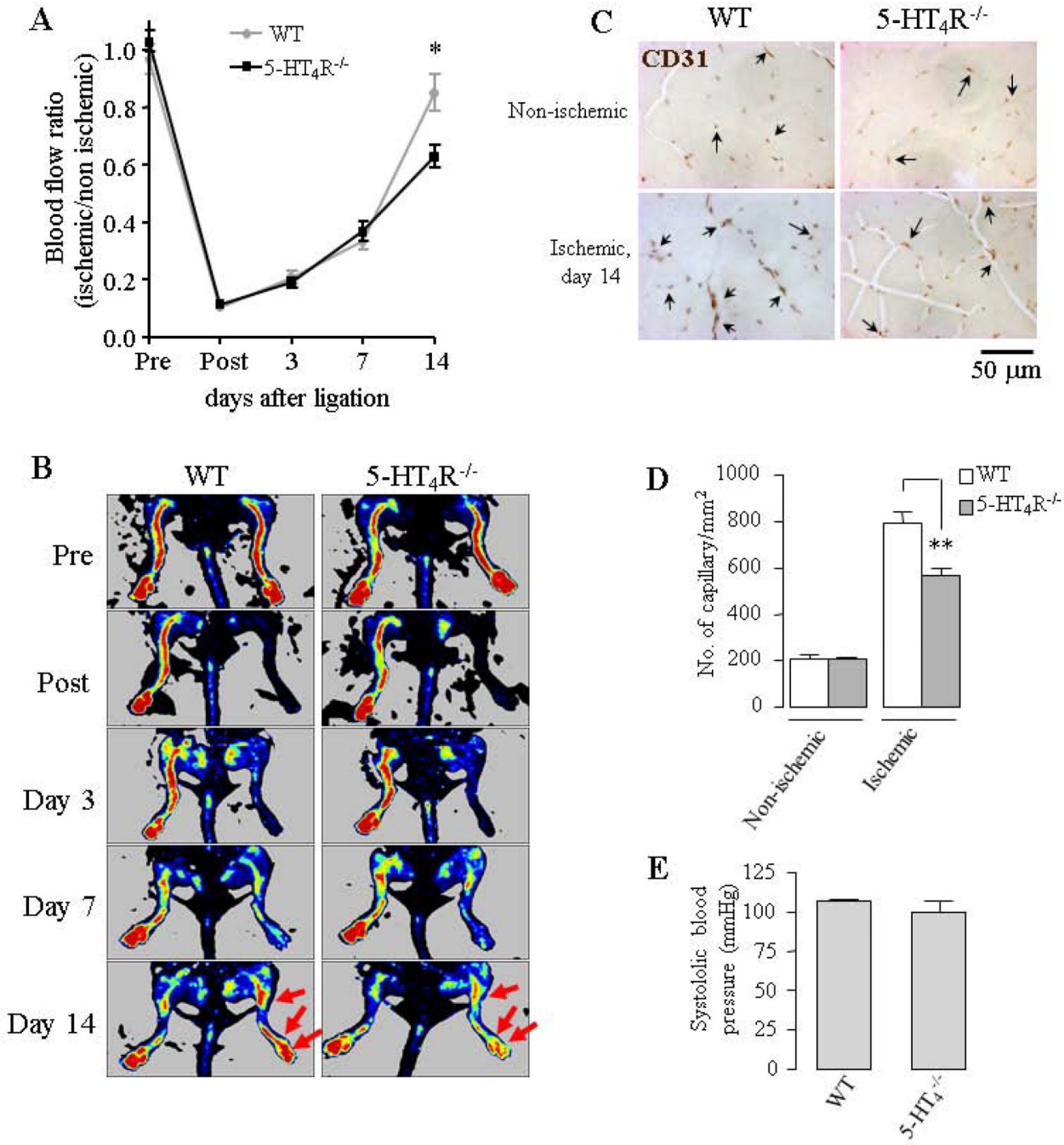

Fig. 1. Impaired blood flow recovery and capillary formation following hindlimb ischemia in 5-HT$_4$R$^{-/-}$ mice

A, Quantification of blood flow recovery, as determined by the ischemic/nonischemic limb perfusion ratio by laser Doppler imaging in WT and 5-HT$_4$R$^{-/-}$ mice (n=4–8 for each time point, *P<0.05). **B**, Representative image of LBDF analysis of ischemic (right) and nonischemic (left) limbs before (Pre) and at different time points after ligation of femoral artery in WT and 5-HT$_4$R$^{-/-}$ mice. Arrows indicate delayed blood flow recovery. **C**, Representative pictures of immunostaining of CD31-positive cells, obtained from WT and 5-HT$_4$R$^{-/-}$ mice in non-ischemic limb and in ischemic limb 14 days after ligation of femoral artery. **D**, Quantitative analysis of capillary density in ischemic adductor muscle at day 14,

expressed as a number of capillaries per mm^2 in each group. The data represent mean ± S.E. (n=4, $^{**}P<0.01$). **E**, Systolic blood pressure in WT and 5-$HT_4R^{-/-}$ mice (n=3 mice in each group). *WT*, wild type.

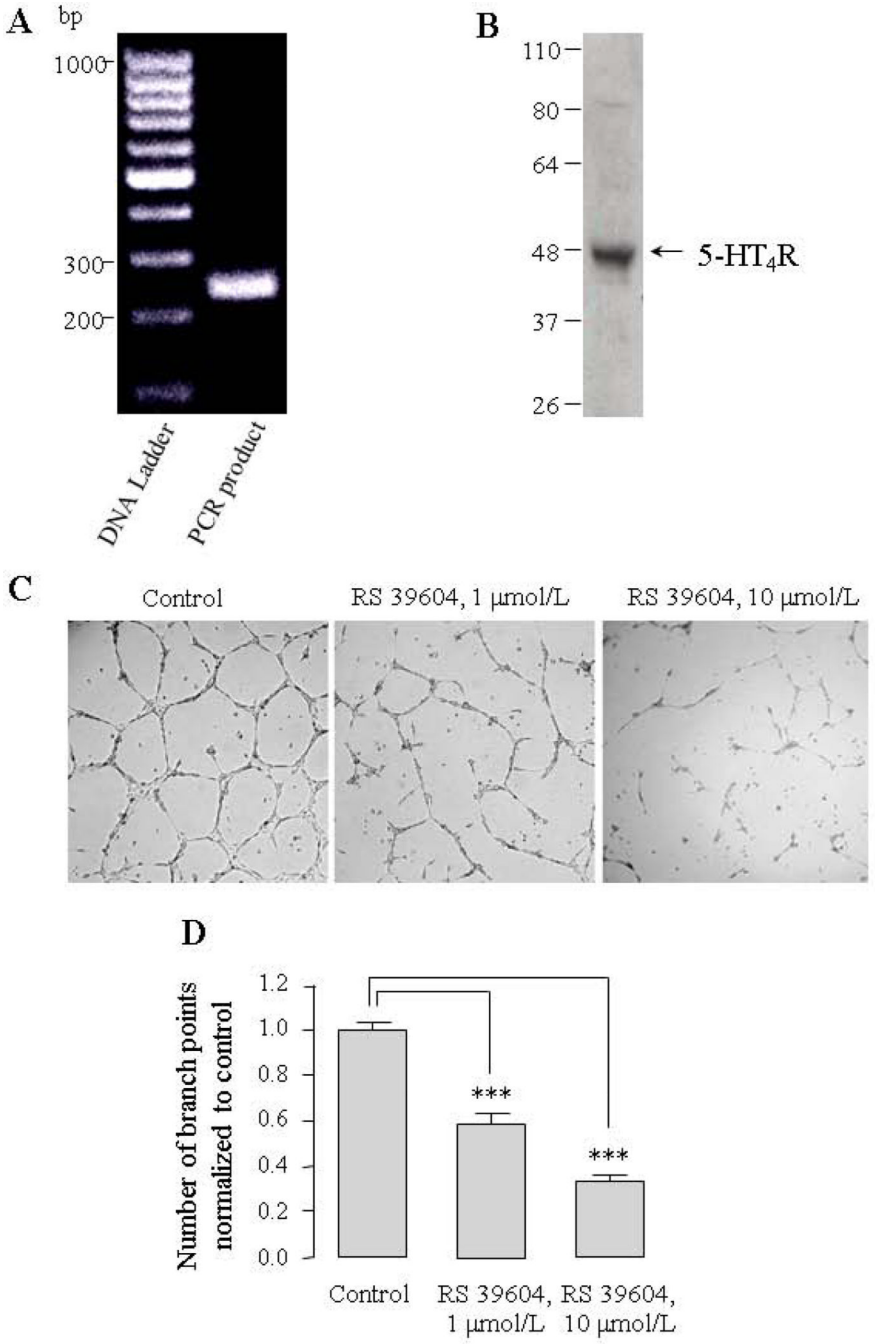

Fig. 2. 5-HT$_4$ receptor antagonist, RS 39604, inhibits capillary tube formation in HUVECs

A, RT-PCR analysis of the 5-HT$_4$R expression in HUVECs. **B**, Detection of 5-HT$_4$ receptor by Western blot analysis of the HUVEC lysates. **C**, HUVECs seeded onto Matrigel were treated with RS 39604 in the concentration as indicated. **D**, RS 39604 significantly reduced the number of branch points formed by HUVECs. The number of branch points in each well was normalized to that in the control well. The data represent mean of three experiments ± S.E. (***P< 0.001).

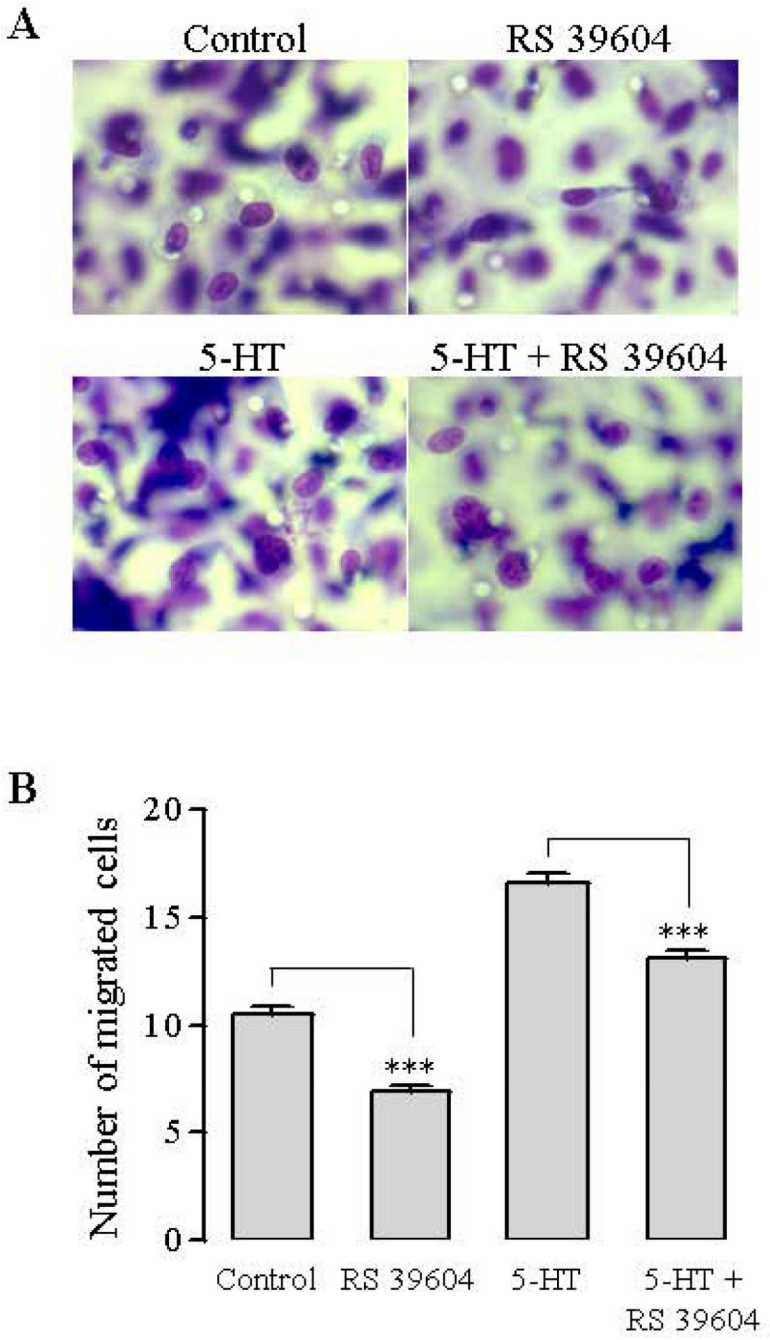

Fig. 3. 5-HT$_4$ receptor antagonist, RS 39604, inhibits EC migration in Boyden chamber migration assay

A, Representative phase-contrast images of the migrated cells. **B**, Cell migration was quantified by counting the migrated cells in 10 fields of each well. The data represent mean ± S.E. of three experiments done in quadruplicates (***$P < 0.001$).

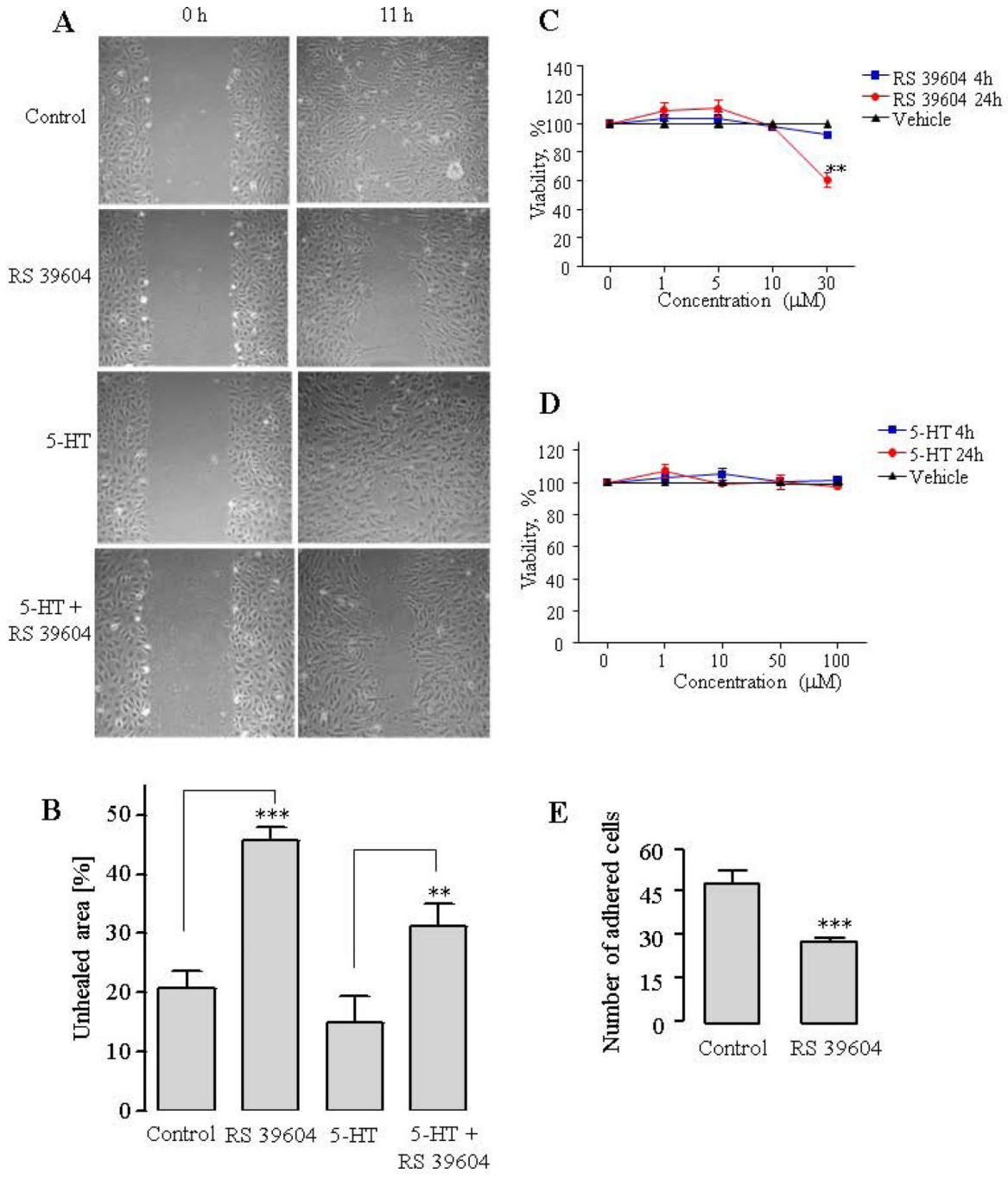

Fig. 4. 5-HT$_4$ receptor antagonist, RS 39604, inhibits wound healing in ECs and suppresses EC adhesion

A, Representative phase-contrast images of the cell monolayers taken at the time of wounding and 11 h after wounding. **B**, Cell migration was quantified by quantifying the unhealed area within the wounded region and expressing it as the percentage of wounded area at zero time point. The data represent mean ± S.E. ($n = 3$, $^*P < 0.05$). **C,D**, Cell viability (MTS) assay of HUVECs treated with RS 39604 (*c*) or 5-HT (*d*) for 4 h or 24 h in the concentrations as indicated. MTS assay was performed according to manufacturer's instructions. The data represent mean ± S.E. (n=3, $^{**}P<0.01$). The experiment was performed three times with similar results. **E**, RS 39604 suppresses EC adhesion. HUVECs

were incubated with 30 μmol/L RS 39604 for 30 minutes at 37 °C. The data represent mean of three experiments performed in duplicates ± S.E. (*$P < 0.05$).

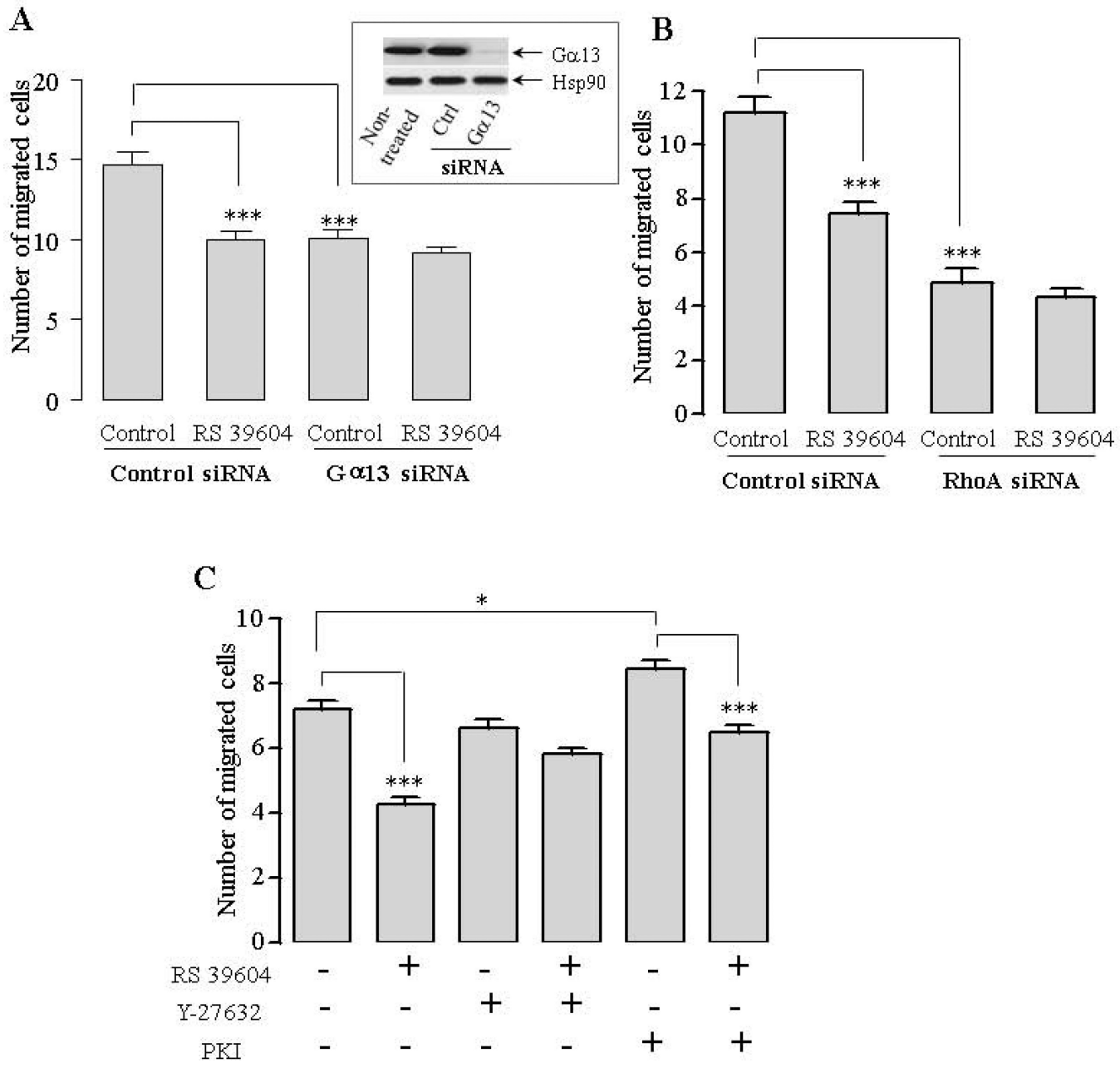

Fig. 5. Ga13-RhoA-ROCK axis, but not PKA mediate 5-HT$_4$R-depedent EC migration

A, Gα13 mediates the 5-HT$_4$R-dependent EC migration as determined by Boyden chamber migration assay. The data represent mean of ± S.E. (n = 3, ***P<0.001). **A, inset**, Downregulation of endogenous Gα13 protein in HUVECs by siRNA. HUVEC were transfected with 60 nmol/L either Gα13 siRNA or control siRNA. **B**, RhoA mediates 5-HT$_4$R–dependent EC migration. HUVEC were transfected with 60 nmol/L either RhoA siRNA or control siRNA and used in Boyden chamber migration assay. **C**, ROCK but not PKA mediates 5-HT$_4$R–dependent EC migration in Boyden chamber assay. The cells were stimulated with 30 μmol/L RS 39604 alone or together with 30 μmol/L PKI or 100 μmol/L Y-27632. The data represent mean of ± S.E. (n = 3, ***P < 0.001).

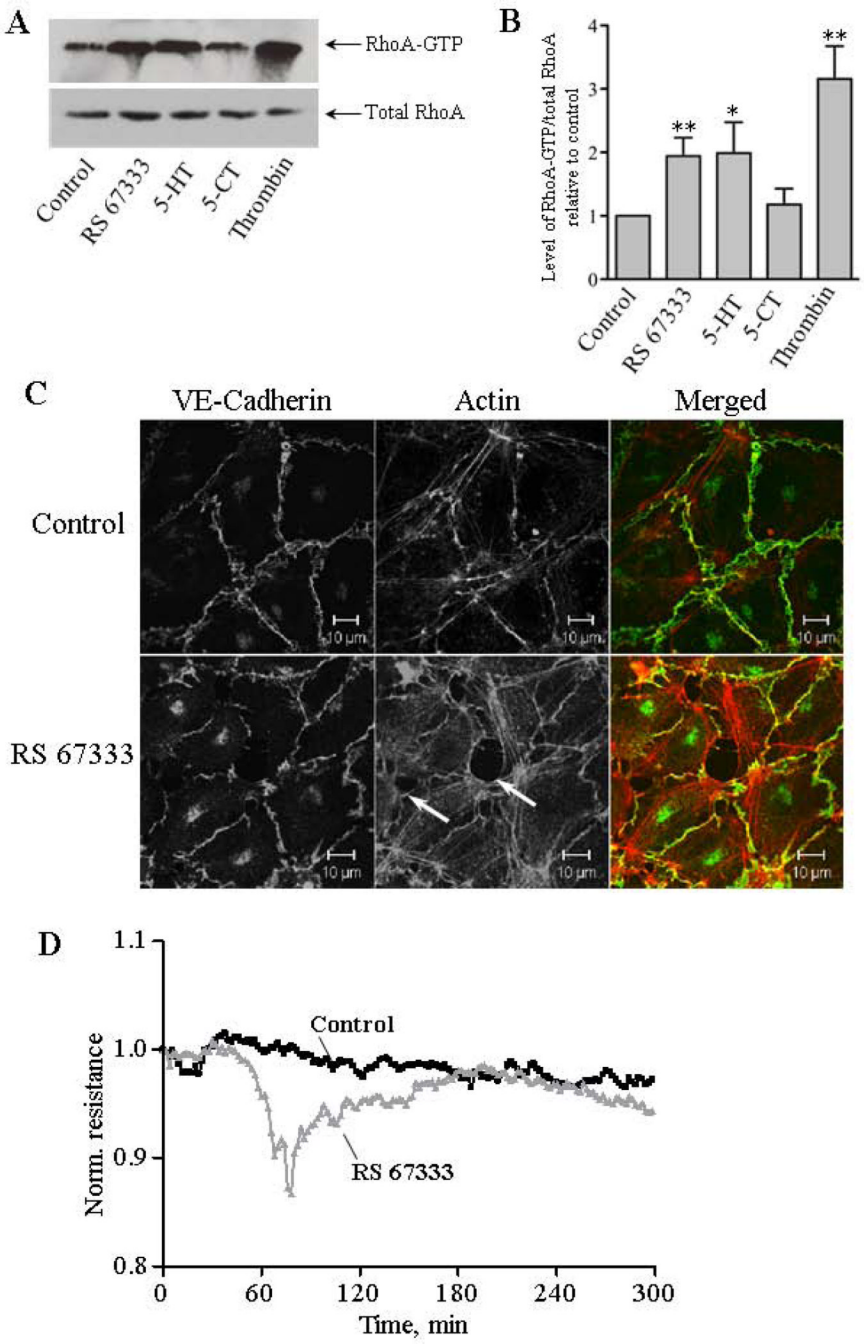

Fig. 6. 5-HT4 receptor agonist, RS 67333, stimulates RhoA and induces intercellular gap formation and increases endothelial permeability

A, Stimulation of 5-HT_4 receptor activates RhoA as measured by Rho GTPase activation assay. The HUVECs were stimulated with 100 μmol/L RS 67333, 100 μmol/L 5-HT, 30 μmol/L 5-CT (5-HT_7 agonist) or 25 nmol/L thrombin for 15 minutes before lysis. The experiment was repeated three times with similar results. **B**, Quantitative analysis of the protein band densities expressed as mean ± SD of the level of RhoA-GTP over total RhoA normalized to control (n=3, ** P<0.01, * P<0.05 compared to non-stimulated control cells). **C**, RS 67333 stimulation induces gap formation in the HUVEC monolayer as demonstrated by immunofluorescence studies. HUVECs were stimulated with 100 μmol/L RS 67333 for

15 minutes, fixed and stained with anti-VE-cadherin antibody and phalloidin Alexa Fluor 594 to detect actin fibers. Arrowheads are pointing to the gaps. *Scale bars*, 10 μm. **D**, HUVECs, cultured on the electrodes, were stimulated with 100 μmol/L RS 67333 and the changes in TER were recorded. The experiment was repeated three times and the representative ECIS experiment is shown. *Norm.*, normalized.