

Research Article

Serotonin 5-HT₇ Receptor Regulates Endothelial Cell Migration via Protein Kinase A

Elena Strekalova^{1,2} and Jasmina Profirovic^{3*}

¹Department of Medicine, University of Wisconsin Carbone Cancer Center, University of Wisconsin School of Medicine and Public Health, USA

²Institute of General Pathology and Pathophysiology, Russian Academy of Medical Sciences, Russia

³Pharmaceutical and Administrative Sciences, St. Louis College of Pharmacy, St. Louis, MO, USA

*Corresponding author: Jasmina Profirovic, Pharmaceutical and Administrative Sciences, St. Louis College of Pharmacy, 4588 Parkview Place, St. Louis, MO, 63110, USA

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Abstract

The 5-hydroxytryptamine type 7 receptor (5HT₇R) regulates many physiological processes, including learning and memory, circadian rhythm, and behavior. Its role is also implicated in psychiatric disorders. Little is known about the 5HT₇R function outside the CNS. Here, we report that 5HT₇R, endogenously expressed in endothelial cells (ECs), may promote cell migration and adhesion. Using Boyden chamber migration assay and wound healing “scratch” assay we demonstrated that stimulation of the receptor with 5HT₇R agonists 5-CT and AS 19 significantly increased EC migration. In addition, 5-CT and AS 19 treatment increased EC adhesion to extracellular matrix. Downregulation of 5HT₇R using specific siRNA significantly inhibited baseline and 5-HT-induced EC migration. Additionally, pretreatment of ECs with PKA inhibitor 14-22 amide significantly reduced 5-CT- or AS 19-induced EC migration, suggesting that PKA is involved in the regulation of EC migration mediated by 5HT₇R. Our results suggest a prominent role of 5HT₇R in promoting cell migration and adhesion and identify 5HT₇R as a potential regulator of physiological and pathophysiological processes involving cell migration and adhesion.

Keywords: 5-hydroxytryptamine type 7 receptor; Endothelial cells; Cell migration; Cell adhesion

Abbreviations

5-HT: 5-hydroxytryptamine; 5HT₇R: 5-hydroxytryptamine type 7 receptor; 5HT₄R: 5-hydroxytryptamine type 4 receptor; 5-CT: 5-carboxamidotryptamine maleate salt; EC: Endothelial Cell; HUVECs: Human Umbilical Vein Endothelial Cells; PKA: Protein Kinase A; PKI: Protein Kinase A Inhibitor; siRNA: Small Interfering RNA; FBS: Fetal Bovine Serum; BSA: Bovine Serum Albumin; PBS: Phosphate-Buffered Saline; HBSS: Hanks’ Balanced Salt Solution; SDS-PAGE: Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis; RT-PCR: Reverse Transcriptase Polymerase Chain Reaction; qPCR: Quantitative Polymerase Chain Reaction; GAPDH: Glyceraldehydes-3-Phosphate Dehydrogenase; EGM-2: Endothelial Growth Medium 2

Introduction

Cell migration is a complex process that involves coordinate changes in cell adhesion, signal transduction and cytoskeletal organization. It is an essential process in the development and homeostatic processes such as angiogenesis, tissue repair and immune responses. Furthermore, it contributes to processes such as vascular disease, chronic inflammatory diseases, tumor formation and metastasis [1]. Therefore, it is of a considerable clinical interest to understand molecular mechanisms involved in regulation of cell migration.

Serotonin (5-hydroxytryptamine, 5-HT) functions as a neurotransmitter that regulates multiple physiological and pathophysiological functions in the human body (reviewed in [2-4]). Although most of body serotonin is found in the intestinal enterochromaffin cells, serotonin is involved in regulation of all major organ system functions [5]. In endothelial cells (ECs), serotonin

promotes cell migration [6], proliferation [7], induces endothelial nitric oxide synthase (eNOS) expression [8] and activation [9].

The actions of serotonin are mediated by over a dozen of cell membrane receptors divided into seven families of receptors that consist of multiple subtypes of receptors with distinct tissue and cell expression and signaling. All the receptors, except for 5-HT₃ that is a ligand-gated ion channel, are G-protein coupled receptors [10].

5-HT₇R was the newest addition to the serotonin receptor family, cloned in 1993 [11-15]. It is expressed in several regions the CNS as well as in peripheral tissues, particularly in gastrointestinal tract and vascular smooth muscle cells [13]. The 5-HT₇R is coupled to Gs protein, leading to activation of adenylyl cyclase [13,16] and to G12 protein, leading to Cdc42-mediated filopodia formation [17]. A great number of studies have suggested that 5-HT₇R regulates many physiological and pathophysiological processes, including learning and memory [18,19], sleep and circadian rhythm [12], body temperature [20] and development of migraine [21]. Although the expression of 5-HT₇Rs was demonstrated in ECs [22], their role in these cells has not been understood.

Our previous data indicate that another serotonin receptor, 5-HT₄ receptor (5-HT₄R), promotes migration and adhesion of ECs. It also stimulates angiogenesis both in ECs and in mouse model of ischemia [23]. The present study was aimed to characterize the role of 5-HT₇R in EC migration and adhesion. Using specific agonists and siRNA-mediated downregulation of 5-HT₇R in two different migration assays, we demonstrated a critical role of endogenous 5-HT₇R in regulation of migration. Additionally, our results suggested that 5-HT₇R is essential for adhesion as well. Finally, we proposed the mechanism of 5-HT₇R-mediated regulation of migration involving

protein kinase A (PKA). These data suggest a prominent role of 5-HT₇R in promoting cell migration and adhesion of ECs.

Materials and Methods

Reagents

Rabbit polyclonal anti-5-HT₇R antibody was obtained from Imgenex (Imgenex Corporation, San Diego, CA). 5-HT₇R agonists, 5-CT salt (5-carboxamidotryptamine maleate) and AS 19 ((2S)-(+)-5-(1,3,5-Trimethylpyrazol-4-yl)-2-(dimethylamino)tetralin) were from Tocris (R&D Systems, Inc., Minneapolis, MN). Serotonin (5-hydroxytryptamine, 5-HT) and bovine serum albumin (BSA) were purchased from Sigma-Aldrich (St. Louis, MO). Protein kinase A inhibitor 14-22 amide, cell-permeable, myristoylated - Calbiochem was from EMD Millipore (Billerica, MA). Fetal bovine serums (FBS), Phosphate-Buffered Saline (PBS) and Hanks' balanced salt solution (HBSS) were from Invitrogen (Life Technologies, Grand Island, NY). The human umbilical vein ECs (HUVECs) and endothelial growth medium (EGM-2) BulletKit were purchased from Lonza (Walkersville, MD).

Cell culture and immunoblotting

HUVECs were cultured in the EGM-2 BulletKit 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 sonication. 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-HT₇R antibodies.

Reverse transcription-polymerase chain reaction (RT-PCR)

Detection of 5-HT₇R mRNA in HUVECs was performed using RT-PCR. Total RNA was isolated from HUVECs using RNeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. Primer sequences for human 5-HT₇R were as follows: sense primer, 5'-GAAGGAGGTGGAAGAGTGTGCAA-3'; antisense primer, 5'-ACAGAAGCTGCATTCCATTCTGC-3'. This primer set produces a PCR product of 516 bp. RT-PCR was performed using Easy-A One-Tube RT-PCR Kit (Agilent Technologies, Santa Clara, CA). 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.

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 TM membrane (Whatman, Clifton, NJ) as described previously [23]. Briefly, HUVECs, grown until confluent on gelatin-coated plate, were incubated in serum free medium containing 0.1% BSA overnight. Next day, the cells were trypsinized, resuspended and placed in the lower wells of the chamber. After 90

min of upside down incubation at 37°C, 1 μmol/L 5-CT or 1 μmol/L AS 19 in 0.1% BSA was applied to the upper wells. After 4 h of incubation at 37°C, the filter was fixed and stained with Diff Quick kit (Dade Behring, Newark, DE). Thereafter, the cells that migrated through the pores to the upper side of the filter were quantified 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

We performed in vitro wound healing assay as described previously [24]. HUVECs seeded onto a 6-well plate were incubated for 24 h and maintained in serum free medium for 8 h. After wounding in a straight line using a sterile 200 μL tip, the cells were washed with PBS and incubated with 5-HT₇R agonist 1 μmol/L 5-CT or 1 μmol/L AS 19 in serum free medium for 17 h. Images were taken at the time of the wounding and at 17 h after wounding using Nikon eclipse TE 300 microscope equipped with 4 × objective and Nikon cool pix 990 digital camera. The number of cells within the wounded region represented the number of migrated cells. In each well, four measurements were taken from four fields. At least three independent experiments were performed.

Cell viability assay

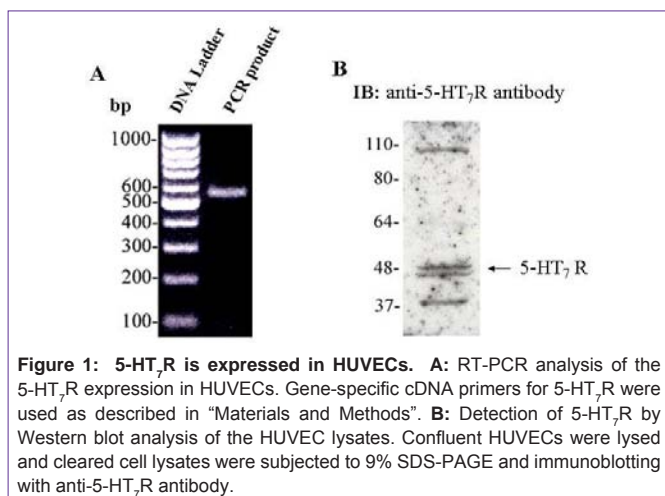
An MTS assay was used to analyze the effect of 5-CT (1 μmol/L) or AS19 (1 μmol/L) on cell viability and assess if these agents affect cell proliferation. Cells seeded at 2.5 × 10³ cells/well in 96-well plates were grown for 4, 24 or 48 hours. The number of viable cells was determined by measuring the absorbance at 490 nm 1 h after addition of the MTS reagent (Promega) as described by the manufacturer. The experiment was performed in triplicate. Cell viability was expressed as the percentage of viable cells: Aexp group/Acontrol × 100.

Adhesion assay

We used the method we described previously [23]. Briefly, gelatin-coated 24-well plates were washed and blocked with 1% BSA for 1 h at 37°C. HUVECs, serum-starved overnight in a medium containing 1% BSA, were seeded onto the 24-well plate (1 × 10⁵ cells per well) and incubated in the presence of 5-HT₇R agonists 1 μmol/L 5-CT, 1 μmol/L AS 19 or medium alone for 2 h at 37°C. Thereafter, the cells were washed three times with PBS, fixed and stained with 0.5% solution of crystal violet. Light microscopy at magnification of 10 × was used to count the number of cells adhered to the extracellular matrix in four fields of each well. Adhesion experiments were done in triplicates and repeated at least three times.

Transfection of siRNAs

HUVECs were transfected using siRNA transfection reagent and medium (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) according to the manufacturer's protocol. siRNA oligonucleotides (SMART pool) were purchased from Dharmacon (Thermo Fisher Scientific, Waltham, MA) to silence human 5-HT₇R. The non-silencing control siRNA was from Qiagen (Valencia, CA). The effects of siRNAs were confirmed by real-time qPCR 24 h after transfection.



Quantitative PCR (qPCR)

Total RNA was isolated from HUVECs using RNeasy Mini kit with DNaseI treatment following the manufacturer's instructions (Qiagen, Valencia, CA). Using random primers and Superscript III transcriptase (Invitrogen, Life Technologies, Grand Island, NY), 1 µg total RNA was converted into cDNA. The housekeeping gene glyceraldehydes-3-phosphate dehydrogenase (GAPDH) was used as a reference gene for quantification. Specific primers for 5-HT₇R and GAPDH primers were synthesized by Integrated DNA Technologies (Coralville, IA). Quantitative PCR was performed with 50 ng cDNA in a 25 µl reaction volume containing a SYBR Green Master Mix (Applied Biosystems, Life Technologies, Grand Island, NY). Amplification was carried in an ABI PRISM 7000 sequence detection system (Applied Biosystems, Life Technologies, Grand Island, NY). Cycling conditions were 50°C for 2 min, 95°C for 10 min followed by a 40-cycle amplification at 95°C for 15 s, and 57°C for 1 min.

Experiments were repeated two times and samples were analyzed in triplicate. qPCR results were presented as Ct values, where Ct is defined as the threshold cycle of PCR at which amplified product was first detected. To compare the different RNA samples, we used the comparative Ct method and compared the RNA expression in samples to that of the control in each experiment.

Statistical analysis

All experiments were carried out at least in triplicates. The results are expressed as the mean value ± SE. The statistical differences between groups were evaluated using Student's t-test. A value of $P < 0.05$ was considered statistically significant.

Results and Discussion

Stimulation of endogenous 5-HT₇R promotes EC migration and adhesion

We confirmed the expression of 5-HT₇R in HUVECs using two approaches: (I) detection of 5-HT₇R mRNA by RT-PCR, and (II) detection of 5-HT₇R protein expression by immunoblotting (Figure 1A and B). These findings were in line with the previous report that 5-HT₇R mRNA is expressed in HUVECs [22].

We employed two methods to assess the effects of 5-HT₇R stimulation on HUVEC migration: (I) wound healing "scratch" assay,

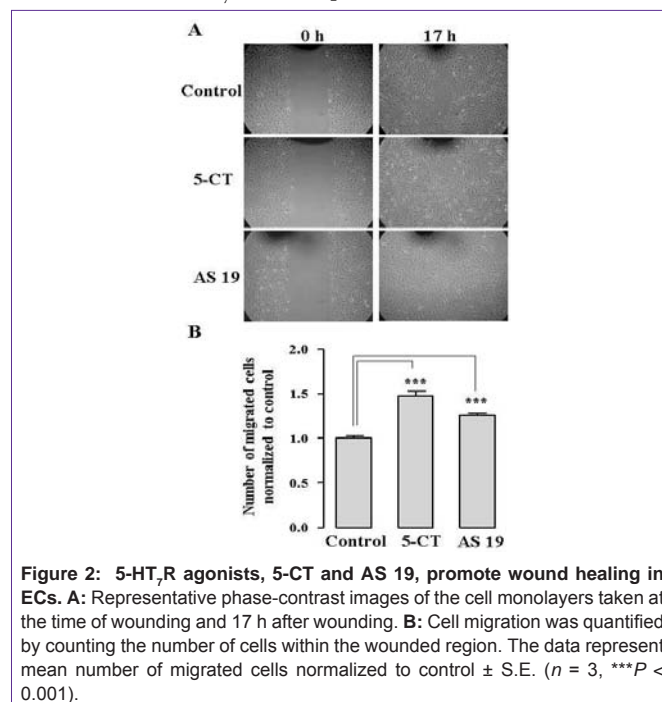
and (II) Boyden chamber migration assay. We used *in vitro* wound healing assay in which cells migrate into a wound produced on a cell monolayer [24]. We stimulated the endogenous 5-HT₇R using two different 5-HT₇R agonists, 5-CT and AS 19. Significantly enhanced healing of the wounded area was observed in the cells treated with either 5-CT or AS 19 in comparison to untreated cells (Figure 2A and 2B). To further confirm the effect of 5-HT₇R stimulation on EC migration, we performed Boyden chamber migration assay to quantify HUVEC migration through a porous membrane [25]. Stimulation of 5-HT₇R with 5-CT or AS 19 significantly increased the number of migrated cells (Figure 3A). Importantly, we did not observe any significant difference in cell viability between the control and 5-CT- or AS 19-stimulated cells 4, 24 and 48 h after cell stimulation in cell viability (MTS) assay (Figure 3B).

Because cell attachment to extracellular matrix is a critical step for the process of cell migration (1), we next investigated if 5-HT₇R activation would affect cell adhesion. Treatment of HUVECs with 1 µmol/L 5-CT or AS 19 for 2 h caused a significant increase in the number of adhered cells compared to untreated cells (Figure 3C). Together, our results suggest that 5-HT₇R activation promotes migration and adhesion of ECs.

Although serotonin has been first isolated and studied in the vasculature [26], many effects of serotonin in the cardiovascular system have not been well understood [27]. It has been shown that serotonin may promote angiogenesis [7, 28]. Several studies have suggested that the angiogenic effects of serotonin may be mediated by 5-HT₁ and 5-HT₂ receptors [7,29,30]. Our previous study has demonstrated that 5-HT₄R promotes angiogenesis both *in vitro* and *in vivo* as well as migration and adhesion of ECs [23].

Our present study identified yet another serotonin receptor, 5-HT₇R, which promotes processes of EC migration and adhesion.

Serotonin 5-HT₇R is expressed in the brain [11,12],



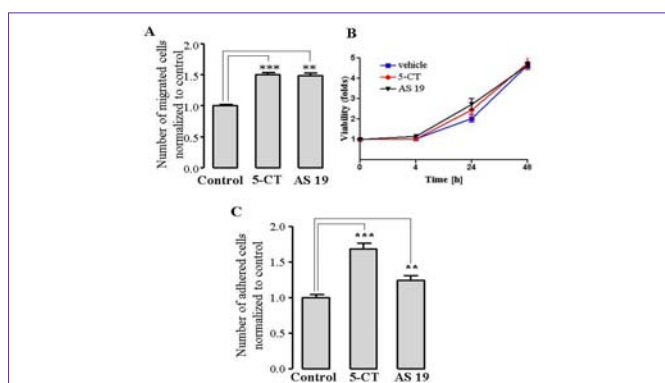


Figure 3: 5-HT₇R agonists, 5-CT and AS 19, promote EC migration in a Boyden chamber migration assay and enhance EC adhesion. **A:** Cell migration was quantified by counting the migrated cells in 10 fields of each well. The data represent mean number of migrated cells normalized to control of three experiments done in quadruplicates \pm S.E. (*** P < 0.001). **B:** Cell viability (MTS) assay of HUVECs treated with 5-CT or AS 19. MTS assay was performed according to manufacturer's instructions. The data represent mean \pm S.E. **C:** 5-CT and AS 19 enhance EC adhesion. HUVECs were incubated with 1 μ mol/L 5-CT or AS 19 for 2 h at 37°C. The data represent mean number of adhered cells normalized to control of three experiments performed in duplicates \pm S.E. (** P < 0.01, *** P < 0.001).

gastrointestinal tract and blood vessels [13]. The role of 5-HT₇R in the CNS has been extensively studied and some of the CNS functions are better understood. Despite the fact that 5-HT₇R expression in ECs was demonstrated almost two decades ago [22], the role of 5-HT₇R in ECs remained unknown. For the first time, the present study demonstrates that 5-HT₇R may serve as a positive regulator of EC migration and adhesion, and presumably the other processes in which migration and adhesion are part of.

Downregulation of endogenous 5-HT₇ receptor in ECs

In our previous experiments, we used pharmacological stimulation of 5-HT₇R to examine its effects on EC migration. We next used small interfering RNA (siRNA)-dependent gene downregulation to knock down the expression of 5-HT₇R. HUVECs were transfected with control or 5-HT₇R-specific siRNA. Expression of mRNA was examined using qPCR, whereby GAPDH, the housekeeping gene, was used as a reference gene for quantification. Twenty-four hours after transfection, 5-HT₇R mRNA level was decreased by 85% in 5-HT₇R siRNA-transfected cells compared to control siRNA-transfected cells (Figure 4A).

Because pharmacological tools used to modulate the receptor function may have known and/or unknown off-target actions, to corroborate our findings obtained by using 5-HT₇R agonists 5-CT and AS 19, we used siRNA-dependent gene expression silencing. We tested if downregulation of 5-HT₇R affects EC migration. HUVECs were transfected with control or 5-HT₇R siRNA and used in wound healing assay. Downregulation of 5-HT₇R significantly inhibited HUVEC migration (Figure 4B). Similar results were obtained from Boyden chamber migration assay in which depletion of 5-HT₇R resulted in significantly reduced migration of ECs compared to control siRNA-transfected cells (Figure 4C). Furthermore, serotonin-induced migration was abrogated in 5-HT₇R siRNA-transfected cells (Figure 4C), supporting the important role of this receptor in cell migration.

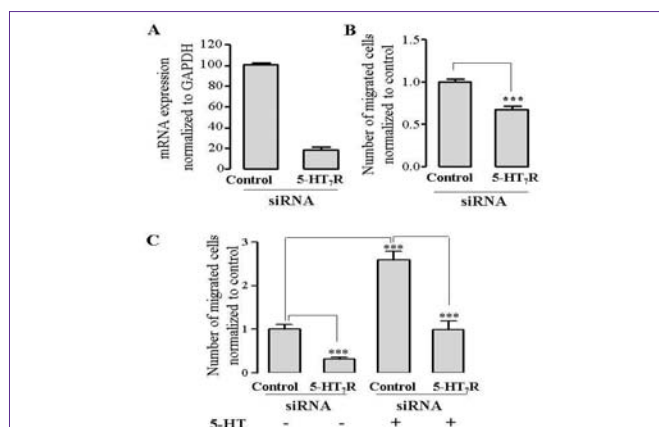


Figure 4: Downregulation of endogenous 5-HT₇R in HUVECs by siRNA inhibits EC migration. **A:** HUVEC were grown to confluence and transfected with 60 nmol/L either 5-HT₇R siRNA or control siRNA. 24 h after transfection with siRNA, mRNAs were quantified using qPCR. Expression was normalized to GAPDH expression (100%). **B,C:** Downregulation of 5-HT₇R inhibits EC migration in a wound healing assay (**B**), or a Boyden chamber migration assay (**C**). HUVECs were transfected with either 5-HT₇R siRNA or control siRNA and used in wound healing assay, (**B**) or in Boyden chamber migration assay with or without stimulation with 100 μ mol/L serotonin (5-HT) (**C**). The data represent mean number of migrated cells normalized to control \pm S.E. (n = 3, *** P < 0.001). 5-HT, serotonin.

These results provide evidence that 5-HT₇R deficiency decreases both baseline and agonist-induced EC migration. The finding that 5-HT₇R deficiency alone may significantly reduce EC migration may suggest that 5-HT₇R exerts constitutive receptor activity in the absence of an agonist, which is in line with the previous studies [17,31].

PKA is involved in regulation of EC migration mediated by 5-HT₇R

Because 5-HT₇R is coupled to Gs protein leading to activation of adenylyl cyclase and accumulation of cAMP in COS-7 cells [13,16], and ECs [32], which may promote cell migration [33], we hypothesized that 5-HT₇R-dependent EC migration may be mediated by PKA. We addressed the question whether PKA is essential for 5-HT₇R-induced migration by using specific cell-permeable PKA inhibitor 14-22 amide (PKI), which acts as PKA pseudosubstrate [34]. Under our experimental conditions, inhibition of PKA with PKI, did not significantly affect cell migration compared to control. Pretreatment of HUVECs with PKI followed by treatment with 5-HT₇R agonists 5-CT or AS 19 significantly reduced EC migration when compared to the cells treated with 5-CT or AS 19 alone (Figure 5) indicating that PKA inhibition abolished EC migration mediated by 5-HT₇R activation. These data suggest that 5-HT₇R promotes EC migration via PKA pathway.

Interestingly, using the same assay in our previous study, we demonstrated that PKA is not involved in 5-HT₄R-dependent EC cell migration despite 5-HT₄R's coupling to Gs protein [23]. Unlike 5-HT₇R, which has been demonstrated to couple to Gs in bovine ECs [32], 5-HT₄R has not been shown to couple to Gs in ECs of any kind yet although this was shown in neuronal cells [35] and insect Sf.9 cells [36].

In addition to Gs, 5-HT₇R has been shown to couple to G12

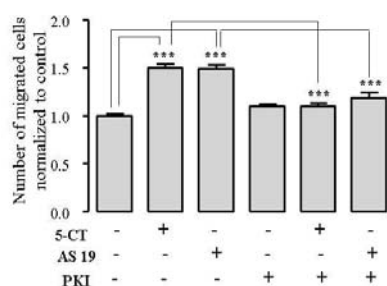


Figure 5: PKI mediates 5-HT₇R-dependent EC migration in a Boyden chamber assay. HUVECs were stimulated with 1 μmol/L 5-CT or 1 μmol/L AS 19 alone or together with 10 μmol/L PKI and used in Boyden chamber migration assay. The data represent mean number of migrated cells normalized to control ± S.E. (n = 3, ***P < 0.001).

protein, leading to Cdc42-mediated filopodia formation in NIH3T3 cells and possibly neurite elongation in mouse hippocampal neurons [17]. The question remains if this pathway may function in ECs as well and if it may contribute to 5-HT₇R-dependent EC migration and adhesion.

Conclusion

While the expression of 5-HT₇R in ECs was demonstrated almost twenty years ago [22], its role in ECs was not studied. Using pharmacological stimulation and siRNA-mediated downregulation of 5-HT₇R, in our present study we have demonstrated that endogenously expressed 5-HT₇R promotes EC migration in two different migration assays. We have also shown that 5-HT₇R is essential for EC adhesion to extracellular matrix. Finally, we have proposed that 5-HT₇R-mediated regulation of migration depends on activation of PKA. For the first time, these data represent the evidence that 5-HT₇R may play a critical role in EC function. Based on our data, 5-HT₇R has a prominent role in promoting cell migration and adhesion of ECs and may serve as a potential molecular target for pharmacological interventions intended to modulate the processes of migration and adhesion, and possibly other complex processes such as angiogenesis, in which migration and adhesion are critical steps.

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