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Research Article

Serotonin 5-HT$_7$ Receptor Regulates Endothelial Cell Migration via Protein Kinase A

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Abstract

The 5-hydroxytryptamine type 7 receptor (5HT$_7$R) 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 5HT$_7$R function outside the CNS. Here, we report that 5HT$_7$R, 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 5HT$_7$R 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 5HT$_7$R 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 5HT$_7$R. Our results suggest a prominent role of 5HT$_7$R in promoting cell migration and adhesion and identify 5HT$_7$R 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; 5HT$_7$R: 5-hydroxytryptamine type 7 receptor; 5HT$_4$R: 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-HT$_7$R 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-HT$_7$R 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-HT$_7$R 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-HT$_7$Rs was demonstrated in ECs [22], their role in these cells has not been understood.

Our previous data indicate that another serotonin receptor, 5-HT$_4$ receptor (5-HT$_4$R), 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-HT$_7$R 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-HT$_7$R in regulation of migration. Additionally, our results suggested that 5-HT$_7$R is essential for adhesion as well. Finally, we proposed the mechanism of 5-HT$_7$R- mediated regulation of migration involving

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protein kinase A (PKA). These data suggest a prominent role of 5-HT_7R in promoting cell migration and adhesion of ECs.

Materials and Methods

Reagents

Rabbit polyclonal anti-5-HT_7R antibody was obtained from Imgenex (Imgenex Corporation, San Diego, CA). 5-HT_7R 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-HT_7R antibodies.

Reverse transcription-polymerase chain reaction (RT-PCR)

Detection of 5-HT_7R 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-HT_7R 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, 1µmol/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-HT_7R 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 x 10^3 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 (1×10^5 cells per well) and incubated in the presence of 5- HT_7 R 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-HT_7R. The non-silencing control siRNA was from Qiagen (Valencia, CA). The effects of siRNAs were confirmed by real-time qPCR 24 h after transfection.

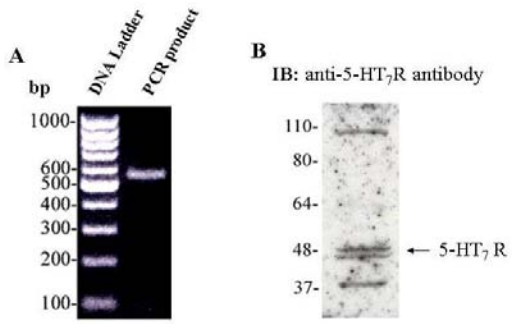

Figure 1: 5-HT$_7$R is expressed in HUVECs. A: RT-PCR analysis of the 5-HT$_7$R expression in HUVECs. Gene-specific cDNA primers for 5-HT$_7$R were used as described in "Materials and Methods". **B:** Detection of 5-HT$_7$R by Western blot analysis of the HUVEC lysates. Confluent HUVECs were lysed and cleared cell lysates were subjected to 9% SDS-PAGE and immunoblotting with anti-5-HT$_7$R antibody.

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-HT$_7$R 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 5$_7$°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-HT$_7$R promotes EC migration and adhesion

We confirmed the expression of 5-HT$_7$R in HUVECs using two approaches: (I) detection of 5-HT$_7$R mRNA by RT-PCR, and (II) detection of 5-HT$_7$R protein expression by immunoblotting (Figure 1A and B). These findings were in line with the previous report that 5-HT$_7$R mRNA is expressed in HUVECs [22].

We employed two methods to assess the effects of 5-HT$_7$R 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-HT$_7$R using two different 5-HT$_7$R 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-HT$_7$R stimulation on EC migration, we performed Boyden chamber migration assay to quantify HUVEC migration through a porous membrane [25]. Stimulation of 5-HT$_7$R 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-HT$_7$R 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-HT$_7$R 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-HT$_1$ and 5-HT$_2$ receptors [7,29,30]. Our previous study has demonstrated that 5- HT$_4$R 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-HT$_7$R, which promotes processes of EC migration and adhesion.

Serotonin 5-HT$_7$R is expressed in the brain [11,12],

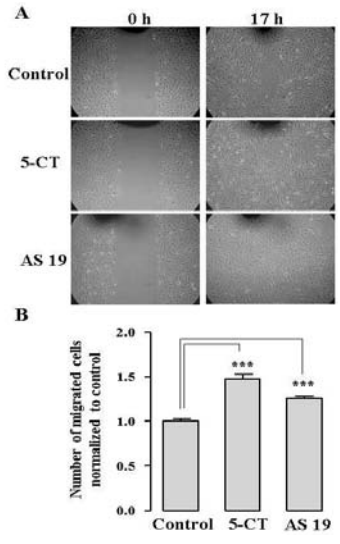

Figure 2: 5-HT$_7$R agonists, 5-CT and AS 19, promote wound healing in ECs. A: Representative phase-contrast images of the cell monolayers taken at the time of wounding and 17 h after wounding. **B:** Cell migration was quantified by counting the number of cells within the wounded region. The data represent mean number of migrated cells normalized to control ± S.E. ($n = 3$, $^{***}P < 0.001$).

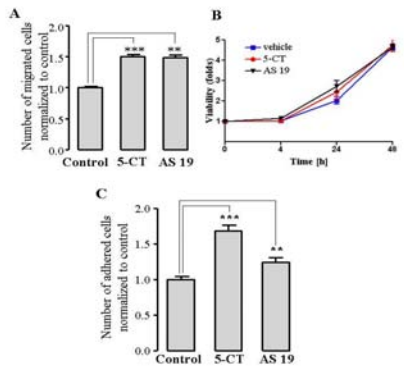

Figure 3: 5-HT$_7$R agonists, 5-CT and AS 19, promote EC migration in a Boyden chamber migration assay and enhance EC adhesion. A: Cell migration was quantified by counting the migrated cells in 10 fields of each well. The data represent mean number of migrated cells normalized to control of three experiments done in quadruplicates ± 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 ± S.E. C, 5-CT and AS 19 enhance EC adhesion. HUVECs were incubated with 1 µmol/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 ± S.E. (** P< 0.01,***P < 0.001).

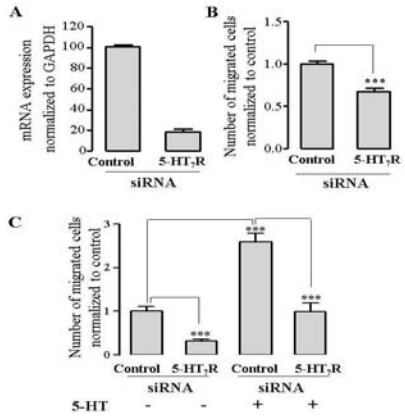

Figure 4: Downregulation of endogenous 5-HT$_7$R in HUVECs by siRNA inhibits EC migration. A: HUVEC were grown to confluence and transfected with 60 nmol/L either 5-HT$_7$R siRNA or control siRNA. 24 h after transfection with siRNA, mRNAs were quantified using qPCR. Expression was normalized to GAPDH expression (100%). **B,C:** Downregulation of 5-HT$_7$R inhibits EC migration in a wound healing assay *(B)*, or a Boyden chamber migration assay *(C)*. HUVECs were transfected with either 5-HT$_7$R siRNA or control siRNA and used in wound healing assay, *(B)* or in Boyden chamber migration assay with or without stimulation with 100 µmol/L serotonin (5-HT) *(C)*. The data represent mean number of migrated cells normalized to control ± S.E. (n = 3, ***P<0.001). 5-HT, serotonin.

gastrointestinal tract and blood vessels [13]. The role of 5-HT$_7$R in the CNS has been extensively studied and some of the CNS functions are better understood. Despite the fact that 5-HT$_7$R expression in ECs was demonstrated almost two decades ago [22], the role of 5-HT$_7$R in ECs remained unknown. For the first time, the present study demonstrates that 5-HT$_7$R 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-HT$_7$ receptor in ECs

In our previous experiments, we used pharmacological stimulation of 5-HT$_7$R to examine its effects on EC migration. We next used small interfering RNA (siRNA)-dependent gene downregulation to knock down the expression of 5-HT$_7$R. HUVECs were transfected with control or 5-HT$_7$R-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-HT$_7$R mRNA level was decreased by 85% in 5-HT$_7$R 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-HT$_7$R agonists 5-CT and AS 19, we used siRNA-dependent gene expression silencing. We tested if downregulation of 5-HT$_7$R affects EC migration. HUVECs were transfected with control or 5-HT$_7$R siRNA and used in wound healing assay. Downregulation of 5-HT$_7$R significantly inhibited HUVEC migration (Figure 4B). Similar results were obtained from Boyden chamber migration assay in which depletion of 5-HT$_7$R resulted in significantly reduced migration of ECs compared to control siRNA-transfected cells (Figure 4C). Furthermore, serotonin-induced migration was abrogated in 5-HT$_7$R siRNA-transfected cells (Figure 4C), supporting the important role of this receptor in cell migration.

These results provide evidence that 5-HT$_7$R deficiency decreases both baseline and agonist-induced EC migration. The finding that 5-HT$_7$R deficiency alone may significantly reduce EC migration may suggest that 5-HT$_7$R 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-HT$_7$R

Because 5-HT$_7$R 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-HT$_7$R-dependent EC migration may be mediated by PKA. We addressed the question whether PKA is essential for 5-HT$_7$R-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-HT$_7$R 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-HT$_7$R activation. These data suggest that 5-HT$_7$R promotes EC migration via PKA pathway.

Interestingly, using the same assay in our previous study, we demonstrated that PKA is not involved in 5- HT$_4$R-dependent EC cell migration despite 5-HT$_4$R's coupling to Gs protein [23]. Unlike 5-HT$_7$R, which has been demonstrated to couple to Gs in bovine ECs [32], 5-HT$_4$R 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-HT$_7$R has been shown to couple to G12

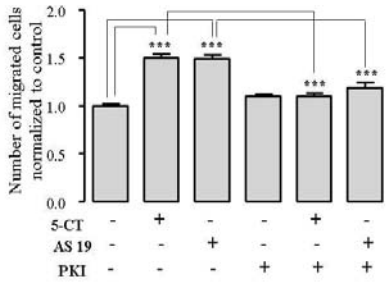

Figure 5: PKI mediates 5-HT$_7$R–dependent EC migration in a Boyden chamber assay. HUVECs were stimulated with 1 μmol/L 5-CT or 1 μmol/L AS 19 alone or together with 10 μmol/L PKI and used in Boyden chamber migration assay. The data represent mean number of migrated cells normalized to control ± S.E. (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-HT$_7$R- dependent EC migration and adhesion.

Conclusion

While the expression of 5-HT$_7$R 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-HT$_7$R, in our present study we have demonstrated that endogenously expressed 5-HT$_7$R promotes EC migration in two different migration assays. We have also shown that 5-HT$_7$R is essential for EC adhesion to extracellular matrix. Finally, we have proposed that 5-HT$_7$R-mediated regulation of migration depends on activation of PKA. For the first time, these data represent the evidence that 5-HT$_7$R may play a critical role in EC function. Based on our data, 5-HT$_7$R 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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