Serotonin induces the migration of PC12 cells via the serotonin receptor 6/cAMP/ERK pathway

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Abstract. Serotonin (5-HT) functions as a chemoattractant that modulates neural migration during prenatal and early postnatal development. However, its molecular mechanism remains to be elucidated. The effect of 5-HT on neural cell migration was examined using PC12 neuron-like cell line. Transwell migration assay was used to determine the effect of 5-HT on PC12 cell migration. The results demonstrated that 5-HT and nerve growth factor (NGF) induced PC12 cell migration in a dose-dependent manner. Additionally, 5-HT receptor antagonists suggest that 5-HT-induced migration was mediated by serotonin receptor 6 (5-HT_6), a Gs-protein coupled receptor that elevates the intercellular cAMP level. By contrast, antagonists of serotonin receptor 3 (5-HT_3) did not show any effects on PC12 cell migration. Clozapine, an inhibitor of cAMP accumulation mediated by 5-HT_6, significantly reduced the effect of 5-HT on the PC12 cell migration. An inhibitor of extracellular signal-regulated kinase (ERK) also suppressed migration. These results suggest that 5-HT induces PC12 cell migration by activating cAMP/ERK signaling pathways, which is mediated by 5-HT_6 receptor.

Introduction

Neural cell migration is crucial in the formation of highly organized structures of mammalian brain. Abnormal migration in prenatal and early postnatal brain causes various types of psychiatric diseases, including mental retardation, autism, bipolar disorders and schizophrenia (1,2). Various molecules have been identified as inducers and modulators of the migration including growth/neurotrophic factors and certain types of neurotransmitters (3-5).

Serotonin (5-HT) has a critical role in neural migration, which is mainly supplied from placental sources and serotonergic projections from the dorsal raphe nuclei in the fetal brain (6). Depletion of 5-HT by injection of DL-P-chlorophenylalanine (PCPA, an inhibitor of 5-HT synthesis) during the E12-E17 stage inhibited migration and disorganized the positioning of cortical neurons (7). By contrast, cortical slices exposed to high doses of 5-HT (100-400 μM) inhibited the migration of GABergic neurons. The arrested migration was recovered by application of the 5-HT_6 antagonist, SB258585 (8,9). However, the association between 5-HT dose and its effect on migration remains to be elucidated.

It is also unclear as to which serotonin receptor mediates 5-HT signal to modulate neural migration. At least 14 classes of 5-HT receptors were identified that are coupled with various types of G proteins, with the exception of 5-HT_3, a ligand-gated ion channel receptor (10,11). 5-HT_6 receptor coupled with Gs protein is positively linked to adenylate cyclase to increase the cAMP level. Recent studies have demonstrated that 5-HT_3 and 5-HT_6 are differentially expressed in migrating neurons in the cerebral cortex (9,12,13). Vitalis *et al* (13) identified 5-HT_3 and 5-HT_6 as candidates involved in the mediation of the 5-HT signal for migration of the pyramidal neurons in the cortex.

To determine the effects of 5-HT on neural cell migration, we used a PC12 neuron-like cell line that expresses 5-HT_3 and 5-HT_6 in experiments. 5-HT and nerve growth factor (NGF) induced PC12 cell migration via 5-HT_6 but not 5-HT_3, stimulating the cAMP and extracellular signal-regulated kinase (ERK) signaling pathways.

Materials and methods

Materials. 5-HT, NGF, Ondansetron, SB271046, SB258585, Clozapine and PD98059 were obtained from Sigma-Aldrich (St. Louis, MO, USA). MDL7222 was obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA).

Cell culture. PC12 cells (RIKEN Tsukuba Institute, Tsukuba, Japan) were routinely cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich) containing 10% fetal bovine serum (Life Technologies, Gaithersburg, MD, USA), 10% horse serum (Life Technologies), 50 U/ml penicillin and 50 μg/ml streptomycin (Life Technologies). The cells were incubated at 37˚C in a 5% CO_2 atmosphere. For each experiment, $3x10^5$ cells were spread onto a 60 mm dish (Becton Dickinson, Franklin Lakes, NJ, USA). Following incubation for two days, the cells were used for RNA extraction and

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transwell migration assay. To obtain differentiated cells, the medium was changed to NGF-supplemented medium (DMEM containing 100 ng/ml NGF, 1% horse serum, 50 U/ml penicillin and 50 μg/ml streptomycin) and incubated for one day.

RNA extraction and RT-PCR. Total RNAs were extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), then cDNAs were synthesized with ReverTra Ace (Toyobo, Osaka, Japan) according to the manufacturer's instructions. PCRs were performed with a denaturation step at 95˚C for 5 min, followed by 35 (Fig. 2A), 29 (Fig. 2B) or 35 cycles (Fig. 2C) of denaturation at 95˚C for 30 sec, primer annealing at 55˚C for 30 sec, and primer extension at 72˚C for 30 sec. TATA binding protein (TBP) was used as a control. The primers used were: 5-HT$_{1A}$ (forward) 5'-CTCTGTTGCTGGGTACTCTCATT/(reverse) 5'-AGTCTATAGGGTCGGTGATAGCC-3', 5-HT$_{1B}$ 5'-3'-, 5-HT$_{2A}$ 5'-TGTACGTGAACCAAGTCAAAGTG-3'/5'-GTAGATGATGGGGTTGATGAGAG-3', 5-HT$_{2A}$ 5'-ATGCTGAAAACAGAACCAACCT-3'/5'-ACATCCAGGTAAATCCAGATCG-3', 5-HT$_{2B}$ 5'-TCGTCAAGATTACGGTGGTATG-3'/5'- CACCATCTTTTCTGGTGATGAA-3', 5-HT$_{2C}$ 5'-ATAGGGGGCAACATTCTTGTTAT-3'/5'-ACAGGGATAGGAACTGAAACTCC-3', 5-H$_{T3}$, 5'-GGAAGTCTCCAAGCATTCCTTAT-3'/5'-ACGTAGAACTTCATTTCCGCATA-3', 5-HT$_4$ 5'-CCAATATTGTGGACCCTTTCATA-3'/5'-GACTGGCTTCTTTTCAAGCTACA, 5-HT$_{5A}$ 5'-AAGATTTACAAGGCTGCGAAGTT-3'/5'-ACTGATGAGCTCCGTAACAAAGA-3', 5-HT$_{5B}$ 5'-CTGGATCGCTACTGGACTATCAC-3'/5'-GTGA ATACCGTCTCAGACTCCTG-3', 5-HT$_6$ 5'-CTGGGAATGTTCTTTGTCACCT-3'/5'-GAAGCGGAGTCTGAATCTGA GTT-3', 5-HT$_7$ 5'-ACTTCTTCTGCAACGTCTTCATC-3'/5'-GCGGCCTTGTAAATCTGATAGTA-3', TBP 5'-TGCTGGCGGTTTGGCTAGGTTTCTGC-3'/5'-GGTCAGAGTTTGAGAATGGAAGAGTT-3'.

Transwell cell migration assay. PC12 cell suspension containing 2x10^5 cells in DMEM was applied to each upper well of the transwell chamber (Becton Dickinson), which was previously coated with type I collagen (50 μg/ml, Becton Dickinson) on both sides. In the bottom well, DMEM with or without motogen, NGF (100 ng/ml) and/or 5-HT (0.1-10 μM) were applied to allow the cells to migrate across filters (8 μm pore size). Ondansetron (1 μM, 5-HT$_3$ antagonist), MDL7222 (1 μM, 5-HT$_3$), SB271046 (1 μM, 5-HT$_6$), SB258585 (1 μM, 5-HT$_6$) and PD98059 (20 μM, ERK inhibitor) were also applied in some of the experiments. The transwell migration assay was performed at 37˚C for 5 h. After removal of the remaining cells on the top side of filters using cotton swabs, the filters were fixed with 4% PFA/PBS for 15 min. After incubation with Hoechst 33258 (Nacalai Tesque, Kyoto, Japan) at room temperature for 5 min, the cells on the bottom side of transwell inserts were washed three times with PBS and examined by fluorescence microscopy (IX83; Olympus, Tokyo, Japan). Ten images were captured randomly for one experiment and the number of nuclei in a 600x600 μm in each image was counted. Image J was used for counting as previously described (14). Relative percentages of the cell number to the average of the cell number in the control experiments (DMEM only in bottom well) were plotted on the graphs.

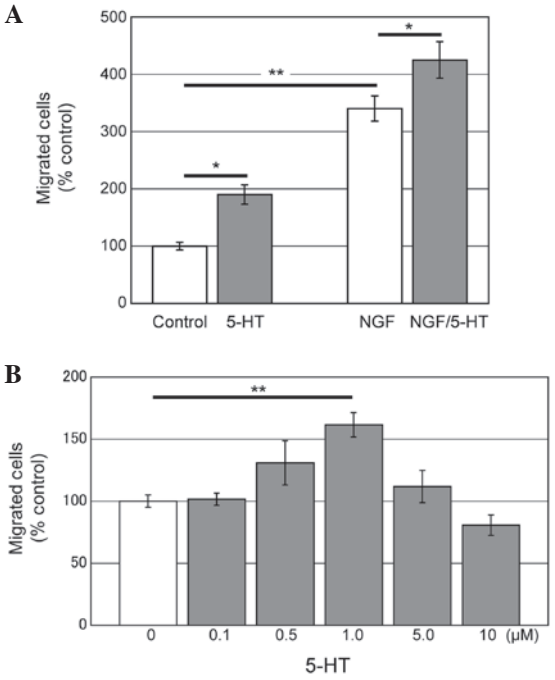

Figure 1. Transwell migration assay reveals serotonin (5-HT) induction of PC12 cell migration. (A) PC12 cells were spread onto the upper side of transwell inserts to allow migration through the membrane. In the bottom well, Dulbecco's modified Eagle's medium with or without chemical cues, serotonin (1 μM) and/or nerve growth factor (NGF) (100 ng/ml) were applied. 5-HT and NGF significantly increased the migrating cells. (B) 5-HT induced migration in a dose-dependent manner. P-values were calculated by two-way or one-way ANOVA followed by Tukey's HSD. *P<0.05; **P<0.01. Data are presented as mean ± standard error. HSD, honestly significant difference.

Statistical analysis. P-values were calculated by one- or two-way ANOVA followed by Tukey's HSD. Data are presented as mean ± standard error.

Results

5-HT induced PC12 cells migration in a dose-dependent manner. To determine the effect of 5-HT on PC12 cell migration, the transwell migration assay was used (15,16). Cells were spread onto the upper side of the transwell insert and the number of cells migrating across the filter was counted. Addition of 5-HT to the bottom well together with DMEM significantly increased the migrating cells (Fig. 1A). 5-HT-induced migration was identified in a dose-dependent manner (Fig. 1B). An amount of 1 μM 5-HT significantly increased cell migration, while 0.1, 0.5, 5.0 and 10 μM 5-HT did not show significant changes compared with the control. NGF is known to induce PC12 cell migration (15,16), thus we added 5-HT together with NGF. NGF and 5-HT induce migration in an additive manner.

5-HT$_6$ receptor mediated 5-HT-induced migration. We examined which serotonin receptor mediates PC12 cell migration induced by 5-HT. Previously, it was reported that 5-HT$_3$ enhanced neurite outgrowth induced by NGF in PC12 cells (17). However, little is known regarding the expression and molecular function of other 5-HT receptors in PC12 cells.

mRNA expression of 5-HT receptors was examined by RT-PCR and 5-HT$_6$ and 5-HT$_3$ were found to be expressed

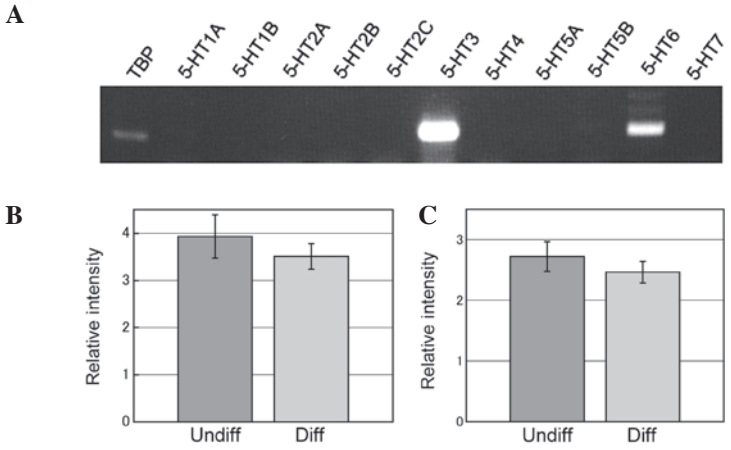

Figure 2. RT-PCR of serotonin (5-HT) receptors. (A) Expression of serotonin receptor 3 (5-HT_3) and 5-HT_6 was observed in undifferentiated PC12 cells that were used for the transwell migration assay. (B and C) Semi-quantitative RT-PCR reveals mRNA expression of 5-HT_3 (B) and 5-HT_6 (C) in undifferentiated (Undiff) and differentiated (Diff; 1 day after application of 100 ng/ml NGF) PC12 cells.

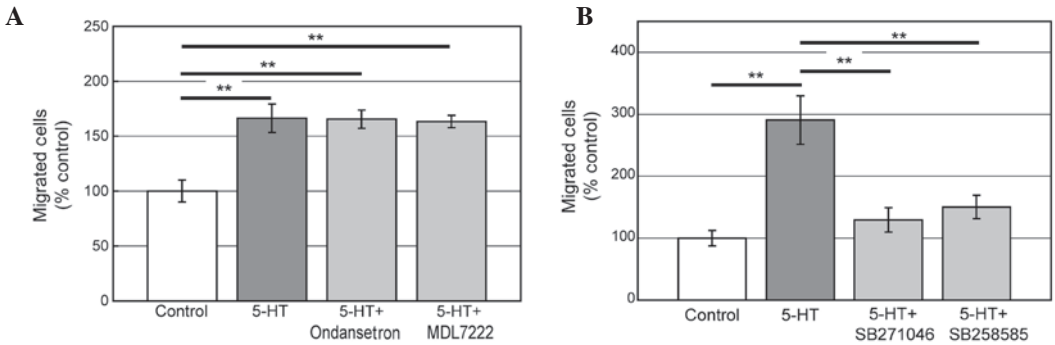

Figure 3. Effects of serotonin receptor antagonists. (A) Serotonin receptor 3 (5-HT_3) antagonists, Ondansetron (1 μM) or MDL7222 (1 μM) did not inhibit migration. (B) 5-HT_6 antagonists, SB271046 (1 μM) or SB258585 (1 μM) significantly reduced PC12 cell migration induced by 5-HT. Data were statistically analyzed by one-way ANOVA followed by Tukey's HSD. **P<0.01. Data are presented as mean ± standard error.

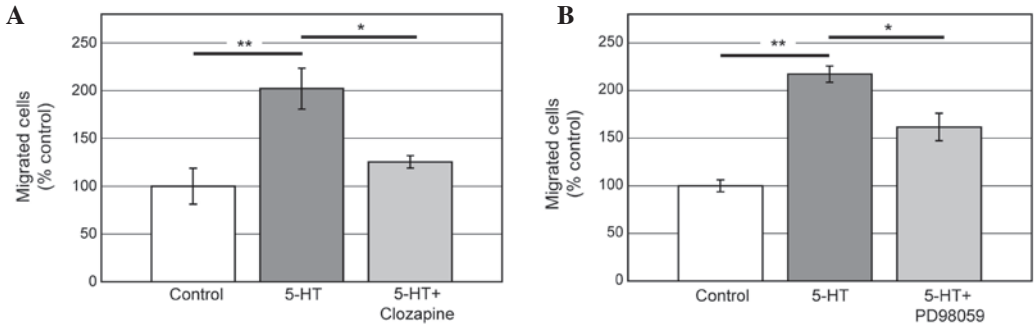

Figure 4. Effect of Clozapine (A) and PD98059 (B) on serotonin (5-HT)-induced PC12 cell migration. Clozapine (1 μM) and PD98059 (20 μM) significantly reduced migrating cells induced by 5-HT. Data were statistically analyzed by one-way ANOVA followed by Tukey's HSD. *P<0.05; **P<0.01. Data are presented as mean ± standard error.

(Fig. 2A) in undifferentiated and differentiated (1 day after application of 100 ng/ml NGF) PC12 cells (Fig. 2B and C).

Using antagonists against 5-HT_3 and 5-HT_6 receptors, we investigated which 5-HT receptor mediates the migration induced by 5-HT. Addition of Ondansetron (5-HT_3 antagonist, 1 μM) or MDL7222 (5-HT_3, 1 μM) with 5-HT did not reveal any significant changes (Fig. 3A) while antagonists of 5-HT_6, SB258585 (1 μM) or SB271046 (1 μM) inhibited the migration induced by 5-HT (Fig. 3A). The data indicate that 5-HT_6 but not 5-HT_3 mediates serotonin-induced PC12 cell migration.

Involvement of cAMP and ERK in 5-HT_6-mediated signaling pathways. 5-HT_6 receptor is known to be coupled with Gs protein which mediates the accumulation of cAMP (18). To examine whether cAMP pathway is involved in 5-HT_6 signaling in order to induce migration, Clozapine, which was reported to inhibit cAMP accumulation mediated by 5-HT_6,

was used (19). Clozapine (1 μM) significantly reduced the effect of 5-HT on migration (Fig. 4A). Increasing of cAMP activates ERK through protein kinase A-Rap1 (20). Thus, we examined whether the inhibition of ERK activation affects 5-HT-induced PC12 cell migration. Application of ERK inhibitor, PD98059 (20 μM), significantly suppressed the cell migration induced by 5-HT (Fig. 4B). These data indicate that cAMP and ERK are involved in the 5-HT$_6$-mediated signaling pathways in order to induce PC12 cell migration.

Discussion

5-HT induced PC12 cell migration in a dose-dependent manner. In the present study, we have demonstrated that 5-HT induced PC12 cell migration in a transwell migration assay in a dose-dependent manner (Fig. 1A). A number of studies have indicated that 5-HT affects proliferation of neural cells (7,21-23) as well as neurite outgrowth (17,24-26), however, its effect on neural migration remains to be clarified. Findings of previous studies suggest 5-HT affects cortical neuron migration during prenatal development (7-9,13). Depletion of 5-HT by injection of DL-P-chlorophenylalanine (PCPA, an inhibitor of 5-HT synthesis) during the E12-E17 stage arrested migration and disorganized the positioning of cortical neurons (7), suggesting a positive effect of 5-HT on migration. By contrast, cortical slices exposed to high doses of 5-HT (100-400 μM) arrested the migration of GABergic neurons (8,9). Although findings of those studies reported negative effects of 5-HT, the concentration of 5-HT was considerably higher than that of another study focusing on the prenatal cortex (100-200 fmol/mg) (7). The effect of 5-HT on neural migration therefore remains to be elucidated. A positive effect of 5-HT at the concentration of 1 μM was observed, which is similar to the *in vivo* results obtained in that study.

5-HT induced migration via 5-HT$_6$ receptor-cAMP pathway independently from NGF-ERK. Blocking of 5-HT stimulation by 5-HT$_6$ antagonists, SB271046 and SB258585 (Fig. 3B) indicate that 5-HT requires 5-HT$_6$ receptor but not 5-HT$_3$ to induce PC12 cell migration (Fig. 3A). 5-HT$_6$ coupled with Gs-protein is known to elevate the cAMP level that stimulates ERK via protein kinase A (19,26). Inhibition of cAMP accumulation by Clozapine (19) and ERK by PD98059 (27) reduced the 5-HT effect on the migration (Fig. 4).

Previous studies suggest that NGF showed a positive effect on PC12 cell migration by activating ERK signaling (15,16). EGF and cAMP pathways activate ERK independently in order to promote PC12 cell differentiation (5,28,29). Similarly, it is possible that the 5-HT$_6$-cAMP activates an independent pathway from the NGF-ERK signal to yield an additive effect on PC12 cell migration (Fig. 1A).

Insensitivity of 5-HT$_3$ in undifferentiated PC12 cells. Antagonists of 5-HT$_3$ did not show any effects on PC12 cell migration in our experiment (Fig. 3A) although 5-HT was expressed (Fig. 2A), suggesting independence of 5-HT$_3$ signaling pathway from cAMP or ERK. Another possibility is that 5-HT$_3$ is not sensitive to a 5-HT induction in the experiments of this study. Homma *et al* (17) suggest a difference of 5-HT sensitivity between differentiated (pretreated with NGF for 3 days) and undifferentiated PC12 cells to enhance neurite outgrowth mediated by 5-HT$_3$. In their experiment, 50 μM 5-HT were required to enhance neurite outgrowth of undifferentiated PC12 cells whereas 5 μM of 5-HT were sufficient for differentiated cells. In our experiment, undifferentiated PC12 cells were treated with 1 μM 5-HT together with 5-HT$_3$ antagonists (Fig. 3A). Higher concentrations of 5-HT may be required to activate the 5-HT$_3$-dependent pathway in order to modulate PC12 cell migration.

5-HT$_3$ and 5-HT$_6$ are proposed as candidates to modulate neural migration (30). Although we observed an effect of 5-HT$_6$ but not 5-HT$_3$ on PC12 migration, it is possible 5-HT$_3$ also modulated the PC12 cell migration in different conditions, such as in the presence of NGF. Transwell migration assay with PC12 cells is useful in the study of molecular mechanisms of the neural migration induced by 5-HT or by NGF *in vitro*.

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