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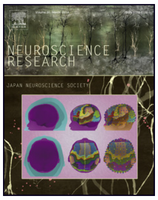

Serotonin 2A receptor regulates microtubule assembly and induces dynamics of dendritic growth cones in rat cortical neurons *in vitro*

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ABSTRACT

Serotonin (5-HT) regulates the development of cerebral cortex, but 5-HT receptors mediating the effects are poorly understood. We investigated roles of 5-HT2A receptor in dendritic growth cones using dissociation culture of rat cerebral cortex. Neurons at embryonic day 16 were cultured for 4 days and treated with 5-HT2A/2C receptor agonist (DOI) for 4 h. DOI increased the size of growth cone periphery which was actin-rich and microtubule-associated protein 2-negative at the dendritic tip. The length increase of the growth cone periphery may be mediated by 5-HT2A receptor, because the 5-HT2A receptor antagonist reversed the effects of DOI. Moreover, the time-lapse analysis demonstrated the increase of morphological dynamics in dendritic growth cones by DOI. Next, to elucidate the mechanisms underlying the actions of 5-HT2A receptor in dendritic growth cones, we examined the cytoskeletal proteins, tyrosinated α-tubulin (Tyr-T; dynamic tubulin) and acetylated α-tubulin (Ace-T; stable tubulin). DOI increased the fluorescence intensity of Tyr-T, while decreased that of Ace-T in the dendritic growth cone periphery. These effects were reversed by the 5-HT2A receptor antagonist, suggesting that 5-HT2A receptor promotes microtubule dynamics. In summary, it was suggested that 5-HT2A receptor induces morphological changes and dynamics of dendritic growth cones through regulation of microtubule assembly.

1. Introduction

Serotonin (5-hydroxytryptamine, 5-HT) is one of the monoamine and acts as a neurotransmitter in the matured brain (Bockaert et al., 2006; Daubert and Condron, 2010). The early embryonic appearance of 5-HT neurons (Lidov and Molliver, 1982a,b; Wallace and Lauder, 1983; Lauder, 1990) suggests that 5-HT has crucial roles in the neural development (Gaspar et al., 2003). In the cerebral cortex, 5-HT is involved in survival and differentiation of neurons (Dooley et al., 1997; Lavdas et al., 1997), dendrite formation (Vitalis et al., 2007) and synaptogenesis (Chubakov et al., 1986; Matsukawa et al., 2003). However, roles of 5-HT receptors mediating these actions in the development of the cerebral cortex are not well understood.

5-HT receptors are classified into 7 families and at least 14 different subtypes (Barnes and Sharp, 1999; Bockaert et al., 2006; Celada et al., 2013). Among these receptors, 5-HT2A receptor appears early in the developing cerebral cortex (Hellendall et al., 1993; Li et al., 2004). A previous study reported that 5-HT2A/2C receptor agonist inhibits the cell death of excitatory neurons (Dooley et al., 1997). In dendrites, it was shown that 5-HT2A/2C receptor agonist promotes spine formation (Jones et al., 2009; Yoshida et al., 2011) of rat embryonic cortical neurons. However, roles of 5-HT2A receptor in the growth cone of dendrites at early stage before the spine formation are not clear.

Microtubule (MT) and actin play crucial roles in axonal growth cones during the axon elongation and guidance (Dehmelt and Halpain, 2004; Geraldo and Gordon-Weeks, 2009; Lowery and Vactor, 2009). The change in the growth direction depends on the reorganization of MTs and actin in axonal growth cones. It was also reported that the individual dynamic MTs run parallel to filamentous actin (F-actin) bundles in axonal growth cones and may act as scaffolding for guidance cue signaling (Dent and Gertler, 2003; Conde and Caceres, 2009; Lowery and Vactor, 2009). These reports suggest that dynamics of MTs are important for the behavior of axonal growth cones. In contrast to axons, roles of cytoskeletons in the dendrite growth cones are less clear. In dendrites, a recent study showed that 5-HT1A receptor reduces dendritic growth cone dynamics by decreasing actin polymerization (Ferreira et al., 2010). In dendritic spines, it was reported that 5-HT2A receptor decreases clusters of F-actin and drebrin (Roppongi et al., 2013). However, roles of MTs in the dendritic growth cone dynamics have not yet been investigated.

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In the present study, we examined roles of 5-HT2A receptor in the morphology and the dynamics of the dendritic growth cones of cortical neurons *in vitro*. In addition, we examined the assembly of MTs as an underlying mechanism for the actions of 5-HT2A receptor in the dendritic growth cones.

2. Materials and methods

All the experiments followed the Guide for the Care and Use of Laboratory Animals described by the National Institutes of Health (USA), and were approved by the Animal Experimentation Committee of University of Tsukuba.

2.1. Dissociated culture of cortical neurons

Wistar/ST rats (Nihon SLC, Hamamatsu, Japan) were used in the present study, and the day of the vaginal plug was considered to be embryonic day (E) 0. Embryos at E16 were removed from pregnant rats under the deep anesthesia by isoflurane (Mylan, Tokyo, Japan). Embryos were then quickly decapitated and the cerebral cortex was excised. After the careful removal of meninges, the cerebral cortex was incubated in 0.05% trypsin–EDTA (Life Technologies, Carlsbad, CA, U.S.A.) for 5 min at 37 °C and cells were dissociated by trituration with a Pasteur pipette. After filtration with 70-μm nylon cell strainer (BD Falcon, San Jose, CA, U.S.A.), dissociated cells were plated on 8-well chamber slides (NUNK, Rochester, NY, U.S.A.) coated with 0.2% polyethyleneimine (Sigma, St. Louis, MO, U.S.A.) at a density of 4×10^4 cells/cm^2. The cells were cultured in Minimal Essential Medium (Life Technologies) supplemented with 10% heat-inactivated fetal bovine serum (Life Technologies), 0.5 mM L-glutamine (Life Technologies), 25 μM glutamate (Wako, Osaka, Japan) and 25 μg/ml penicillin/streptomycin (Sigma) in a humidified atmosphere of 95% air–5% CO_2 at 37 °C. One day after plating, 5 μM cytosine-β-arabinofuranoside (Ara-C; Sigma) was added for 24 h in Neurobasal Medium (Life Technologies) with 2% B-27 supplement (Life Technologies), 0.5 mM L-glutamine and 25 μg/ml penicillin/streptomycin to remove proliferating glial and neuronal progenitors.

2.2. Immunocytochemistry of cytoskeletons and 5-HT2A receptor

Cortical neurons were cultured for 4 days as described above and fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (PB) for 30 min at room temperature. Nonspecific antibody binding was blocked by incubation with 2% normal goat serum and 0.1% Triton X-100 in 0.1 M PB for 30 min. To examine the distribution of cytoskeletons, neurons were incubated overnight at 4 °C with chick anti-microtubule-associated protein 2 (MAP2) antibody (1:4000 dilution, Chemicon, Temecula, CA) and mouse anti-β-tubulin antibody (1:5000 dilution, Promega, Madison, WI). Neurons were then incubated with biotinylated goat anti-chick IgG antibody (1:500 dilution, Vector Laboratories, Burlingame, CA) and Alexa Fluor 488-conjugated goat anti-mouse IgG (1:500 dilution, Life Technologies) for 1 h. They were then incubated with streptavidin-conjugated Pacific Blue (1:500 dilution, Life Technologies) for 1 h and rhodamine-phalloidin (1:100 dilution, Life Technologies) for 30 min which selectively stains F-actin. All the incubation except for the primary antibodies was performed at room temperature. To examine the expression of 5-HT2A receptor, the neurons were incubated overnight at 4 °C with the affinity-purified rabbit anti-5-HT2A receptor antibody (1:1000 dilution) which was raised against the C-terminus of the 5-HT2A receptor (Hamada et al., 1998). Immunostaining of the primary antibody which was adsorbed by the antigen peptide or without the primary antibody yielded no specific staining in the rat brain (Hamada et al., 1998). We also confirmed the specificity of the antibody in the cultured neurons by immunostaining without the primary antibody. After the incubation with the 5-HT2A receptor antibody, the cultured neurons were incubated with Alexa Fluor 488-conjugated goat anti-rabbit IgG antibody (1:500 dilution, Life Technologies) for 1 h at room temperature. Neurons were then incubated with rhodamine-phalloidin for 30 min. X–Y plane or Z-stack images of the stained neurons were taken respectively at 20× or 63× with a confocal laser scanning microscope (LSM 510META ver.3.2, Carl Zeiss, Oberkochen, Germany).

2.3. Analysis of the morphology of dendritic growth cones and protrusions

Neurons were cultured for 4 days and treated acutely with 5-HT2A/2C agonist ((±)-2,5-demethoxy-4-iodoamphetamine hydrochloride, DOI, Sigma) or 5-HT (Sigma) at concentrations of 1, 10 and 100 nM for 4 h. To examine the specific contribution of 5-HT2A receptor to the effect of DOI, 5-HT2A receptor antagonist (ketanserin, Tocris Bioscience, Bristol, UK) was added in combination with DOI at concentrations of 1000 nM. After fixation with 4% paraformaldehyde, they were incubated overnight at 4 °C with chick anti-MAP2 antibody and mouse anti-glutamate decarboxylase 65/67 (GAD65/67) antibody (1:1000 dilution, Chemicon). Neurons were then incubated with a mixture of biotinylated goat anti-chick IgG antibody and Alexa Fluor 488-conjugated goat anti-mouse IgG for 1 h at room temperature. They were then incubated with streptavidin-conjugated Pacific Blue for 1 h and rhodamine-phalloidin for 30 min at room temperature. Z-stack images of the stained neurons were taken at 63× with the confocal laser scanning microscope. To examine the effects of DOI on the dendrite morphology, we focused on three parameters of F-actin-rich and MAP2-negative structures. Thus we measured the length of “growth cone periphery” at the dendritic tips, the number of “dendritic shaft protrusions” per unit dendrite length which are short protrusions emerging directly from dendritic shafts and the number of “somatic protrusions” per neuron which are short protrusions emerging directly from the cell body. The length of growth cone periphery, number of dendritic shaft protrusions, and number of somatic protrusions may be related to the dendrite elongation, dendrite branching, and formation of primary dendrites, respectively. In addition to the length, the width and the area of the growth cone periphery were also measured. The width index was determined by the ratio of head width (the width of the most spread portion of F-actin-rich and MAP2-negative dendrite tip) and neck width (the width of the thinnest portion of the same dendrite tip). The measurement of the area was performed by ImageJ software (National Institutes of Health, Bethesda, MD). In this analysis, only GAD65/67-negative neurons were analyzed. The numbers of growth cones for the analysis of the length, width and area of growth cone periphery, the number of dendrites for the analysis of the density of the dendritic shaft protrusions, and the number of neurons for the analysis of the somatic protrusions are shown in Table 1.

2.4. Time-lapse analysis

Cortical neurons were cultured in Neurobasal Medium with 2% B-27 supplement, 0.5 mM L-glutamine and 25 μg/ml penicillin/streptomycin (the basal medium) at a density of 2×10^4 cells/cm^2 in glass-based 35 mm dish (Asahi Glass Co., Tokyo, Japan). At 4 days *in vitro* (DIV), the culture dish was set in a heat stage (37 °C, 5%CO_2) of the confocal laser scanning microscope. Z-stack images of the cultured neurons were captured at 40× using differential interference contrast (DIC) microscopy. Fig. 4A shows the experimental paradigm. During the pre-treatment period, cortical neurons were cultured in the basal medium for 30 min. Then