

Subtype specific roles of serotonin receptors in the spine formation of cortical neurons in vitro

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Abstract

Dendritic spines are postsynaptic structures which are formed from filopodia. We examined roles of serotonin (5-HT) receptors in the spine formation. Embryonic rat cortical neurons were cultured for 10 or 14 days and treated by 5-HT receptor agonists for 24 h. At 11 days in vitro, 5-HT_{1A} agonist increased filopodia density, whereas 5-HT_{2A/2C} agonist increased the density of puncta and spines. At 15 days in vitro, 5-HT_{1A} agonist decreased the density of puncta and spines, whereas 5-HT_{2A/2C} agonist decreased filopodia density with increase of spines. In conclusion, the present study showed 5-HT receptors have subtype-specific effects on the spine formation.

Serotonin (5-hydroxytryptamine, 5-HT) is a neurotransmitter in the matured brain, and disorders of 5-HT system are associated with various types of mental illness (Charney and Nestler, 2004). Because 5-HT neurons appear early in the embryonic brain and send axons diffusely to wide brain regions including the cerebral cortex (Lidov and Molliver, 1982; Wallace and Lauder, 1983), it was suggested that 5-HT plays various roles in the brain development (for reviews, see Azmitia, 2001; Gaspar et al., 2003). In the cerebral cortex, 5-HT is involved in the dendrite formation (Vitalis et al., 2007, Hayashi et al., 2010) and synaptogenesis and spine formation (Chubakov et al., 1986; Chen et al., 1994; Matsukawa et al., 2003; Jones et al., 2009). In spite of these studies, 5-HT receptors which mediate 5-HT actions have been only partly understood. In the present study, we examined roles of 5-HT_{1A} and 5-HT_{2A/2C} receptors, 5-HT receptors abundant in the cerebral cortex, in the spine formation during development.

Dissociated culture of cortical neurons was performed as previously described (Gandou et al., 2010, Harigai et al., 2011). Briefly, cerebral cortex was excised from rat embryos (Wistar/ST, Nihon SLC, Hamamatsu, Japan) at embryonic day (E) 16. Cells were dissociated by trypsin treatment and were plated on 8-well chamber slides (NUNC, Rochester, NY, USA) at a density of 5×10^4 cells/cm². The cells were cultured for 24 h in the Minimal Essential Medium (MEM, Invitrogen, Carlsbad, CA, USA) supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen). At 1 day after plating, 5 μ M cytosine- β -d-arabinofuranoside (Ara-C, Sigma, St. Louis, MO) was added for 24 h to remove proliferating non-neuronal cells and neuronal progenitors in the basal medium composed of Neurobasal medium (Invitrogen) with 2% B-27 supplement (Invitrogen), 0.5 mM L-glutamine and penicillin and streptomycin. Cortical neurons were then cultured in the basal medium which was changed every 2 days. At 10 days or 14 days *in vitro* (DIV), 100 nM of 5-HT_{1A} agonist (R-(+)-8-hydroxy-dpat hydrobromide, 8-OH-DPAT, Sigma) or 1000 nM of 5-HT_{2A/2C} agonist ((+)-DOI hydrochloride, DOI, Sigma) was added for 24 h. These doses were determined by our pilot study and a previous study (Jones et al., 2009). The cultures were fixed by 4% paraformaldehyde and incubated with rhodamine-phalloidin (1:100 dilution, Invitrogen)

for 30 minutes. Serial optical sections (10-15 sections with 0.1 μm thickness for each dendrite) were captured at X63 with LSM 510 META laser-scanning confocal microscope (Carl Zeiss, Oberkochen, Germany). Dendritic protrusions on the primary dendrites within the 100 μm from the cell bodies (dendritic diameters ranging from 0.6 μm to 2.9 μm) were counted from reconstructed Z-sections. In our dissociation culture, cortical neurons in the layers V and VI may be major components, because we treated E16 cortical cells with Ara-C at 1 DIV when cortical neurons in the layers V-VI are already born (at E16 and E17) and those in the layers II-IV are still under proliferation *in vivo* (Berry et al., 1964; Hayashi et al., 2010). Our previous study showed that most neurons (about 90%) in the present dissociation culture were glutamic acid decarboxylase (GAD)-negative, suggesting that excitatory neurons are dominant in our culture (Hayashi et al., 2010). These neurons extended multipolar dendrites, and dendrites of presumptive pyramidal neurons could not be identified either as apical or basal. Protrusions were classified into three types: spines with a thin neck and a bulbous head, puncta (stubby spine) without neck and 0.5-1.5 μm long, and thin filopodia 1.5-5 μm long. Each experiment was repeated at least 3 times, and 14-16 neurons and 2-4 dendrites of each neuron were examined in each experimental group. All image analysis was performed blind to treatment conditions. Statistical analyses were performed by ANOVA followed by post hoc analysis (Fisher's protected least significant difference test). Differences were considered significant if the probability of error was less than 1%. All the results were expressed as the mean \pm SEM.

For the analysis of the 5-HT receptor distribution, the neurons were fixed by 4% paraformaldehyde at 10 DIV and 14 DIV. They were incubated with affinity-purified goat anti-5-HT_{1A} antibody (1:100 dilution, C-19, Santa Cruz Biotechnology, Santa Cruz, CA) or rabbit anti-5HT_{2A} antibody (1:1000 dilution, Hamada et al., 1998) overnight at 4°C in combination with rhodamine-phalloidin. They were incubated with FITC-conjugated donkey anti-goat IgG antibody (1:500 dilution, Jackson ImmunoResearch Laboratories, West Grove, PA) or Alexa Fluor 488-conjugated goat anti-rabbit IgG antibody (1:500 dilution, Invitrogen) for 1 h at RT. The specificity of the primary antibodies was confirmed by western blotting analysis and the incubation with

the primary antibodies pre-absorbed by the each antigen (Suppl. Fig. 1, Hamada et al., 1998). All the experiments were approved by the Animal Experimentation Committee of the University of Tsukuba.

We examined roles of 5-HT receptors in the maturation of spines by treating cortical neurons at 10 DIV or 14 DIV for 24 h with 8-OH-DPAT or DOI. We focused on 10 DIV and 14 DIV in order to elucidate the roles of 5-HT receptors in the spine formation during the development. Before 10 DIV, dendrites are elongating but a few dendritic protrusions are formed (data not shown). In contrast, at 21 DIV, many dendritic protrusions are matured spines (Jones et al., 2009). At 11 DIV, the density of filopodia, puncta and spines was 11.1 ± 0.45 , 12.9 ± 0.47 , 7.9 ± 0.34 , respectively, along 100 μm dendrite in the control culture. 8-OH-DPAT increased the filopodia density by 21% (Fig. 1A and B). In contrast, DOI increased the density of puncta and spines by 23% and 21 %, respectively (Fig. 1A and B). DOI also increased the density of total protrusions (filopodia, puncta and spines) by 15%, although 8-OH-DPAT had no effects (Fig. 1A and C).

At 15 DIV, the density of filopodia, puncta and spines was 10.1 ± 0.55 , 14.6 ± 0.67 , 8.8 ± 0.46 along 100 μm dendrite in the control culture (Fig. 1A and D). 8-OH-DPAT decreased the density of puncta and spines by 27% and 28%, respectively (Fig. 1A and D). In contrast, DOI decreased the density of filopodia by 22%, whereas increased the spine density by 33% (Fig. 1A and D). 8-OH-DPAT decreased the density of total protrusions by 17%, whereas DOI had no effect (Fig. 1A and E).

Next, we examined the distribution of 5-HT_{1A} and 5-HT_{2A} receptors in cultured cortical neurons at 10 DIV and 14 DIV (Fig. 2). Similar immunoreactivity was observed between the two stages. 5-HT_{1A} receptor immunoreactivity was observed in cell bodies and dendritic shafts, but not in dendritic protrusions. 5-HT_{2A} receptor immunoreactivity was observed in cell bodies and dendritic shafts. The weak immunoreactivity was observed in some dendritic protrusions.

The present study revealed differential effects of 5-HT_{1A} receptor and 5-HT_{2A/2C}

receptors in the maturation of dendritic spines. In addition, the effects were dependent on developmental stages. Thus, 5-HT_{1A} receptor agonist inhibited the maturation of dendritic spines by maintaining the filopodia at earlier stages and inhibiting the formation of puncta and spines at later stages. In contrast, 5-HT_{2A/2C} receptor agonist promoted the maturation of spines by stimulating the formation of puncta and spines at earlier stages and inhibiting filopodia formation and stimulating spine formation at later stages. A recent study reported that the acute treatment of cortical neurons with DOI at 21 DIV induces the transient increase of spine size without effects on the spine density (Jones et al., 2009). The present study showed that DOI may promote the formation of spines from filopodia. The difference of the DOI effects on the number and size of spines may be derived from the maturation of cortical neurons, because we examined cortical neurons at 10 DIV and 14 DIV, and Jones et al. (2009) examined more matured neurons at 21 DIV. The present study further showed that 5-HT_{1A} receptor has antagonistic effects on the spine formation compared with 5-HT_{2A/2C} receptors. Interestingly, the antagonistic actions of these receptors have been shown in the dendrite formation of cerebellar Purkinje cells (Kondoh et al., 2004).

The 5-HT_{2A} receptor is localized in dendritic shafts of cortical neurons, but the localization in dendritic spines is less clear (Cornea-Hébert et al, 1999; 2002; Miner et al., 2003; Jones et al, 2009). The present study showed that 5-HT_{2A} receptor was localized in dendritic shaft with weak expression in some spines. In contrast, 5-HT_{1A} receptor was localized in dendritic shafts. Considering the localization of these receptors, it may be possible that the actions of the 5-HT receptor agonists may be mediated mainly through dendritic shafts, although the actions of 5-HT_{2A} receptor may need further examination.

The abnormality of dendritic spines is closely associated with neurodevelopmental disorders and mental retardation, although the causal relationship remains to be clarified (Fiala et al., 2002). Considering the present study showing that 5-HT regulates the morphology of spines through 5-HT_{1A} receptor and 5-HT_{2A/2C} receptor, the balance between these receptors may be crucial for the proper maturation of spines.

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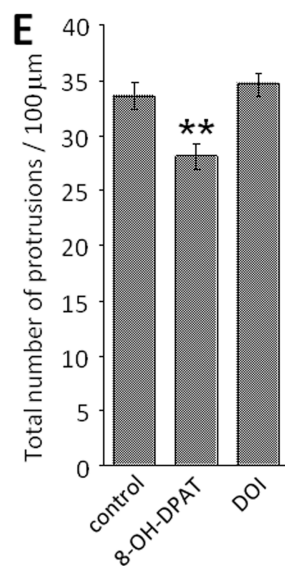
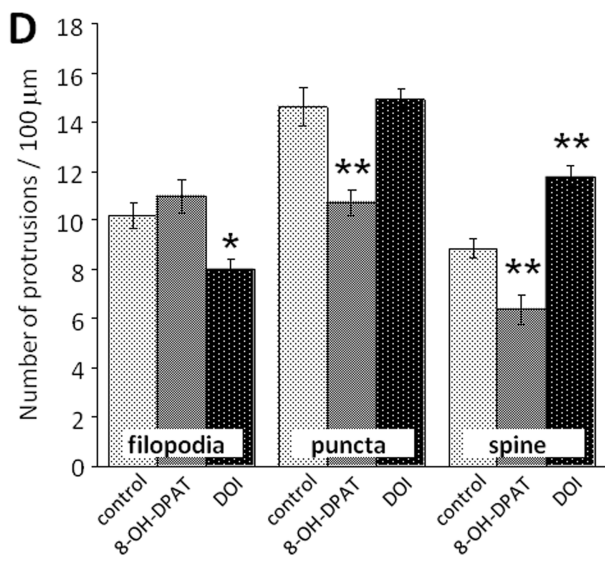
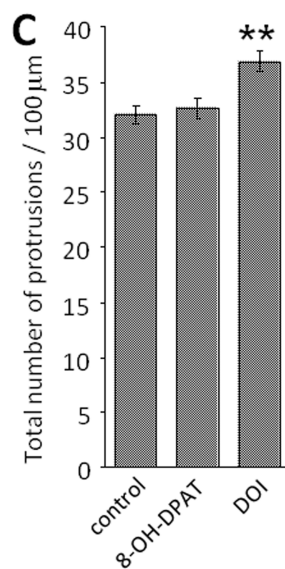
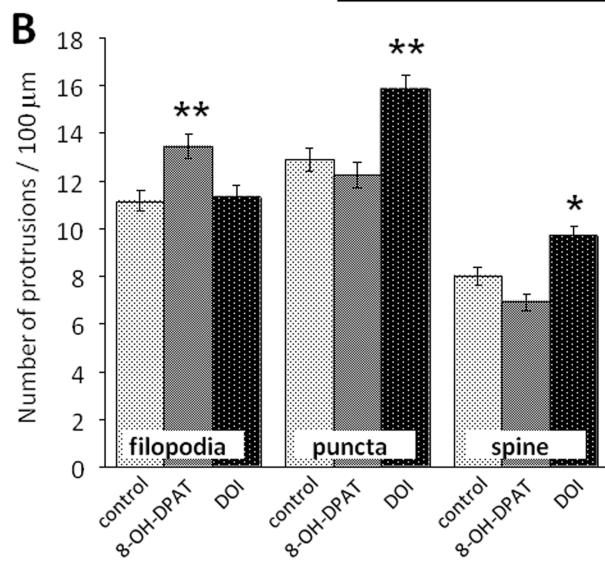
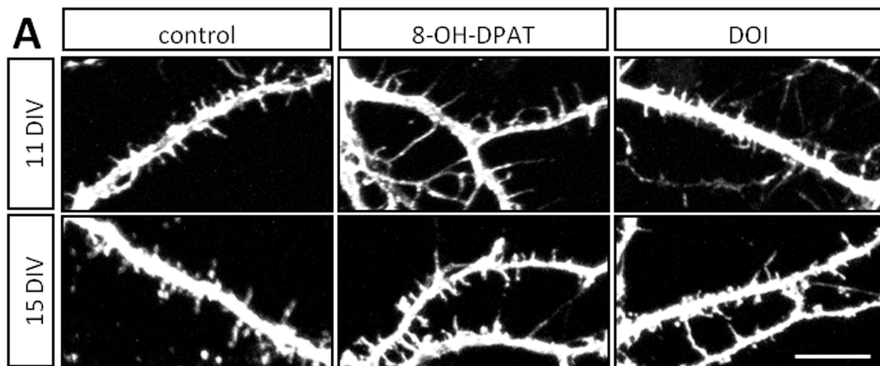
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Figure legends

Figure 1 Differential effects of 5-HT_{1A} agonist and 5-HT_{2A/2C} agonist on the spine formation. (A) E16 cortical neurons were cultured for 10 days or 14 days in the basal medium and then treated with 5-HT_{1A} agonist (8-OH-DPAT) and 5-HT_{2A/2C} agonist (DOI) for 24 h. Neurons were labeled by rhodamine-phalloidin. Scale bar: 10 μ m. (B) At 11 days in vitro (DIV), 8-OH-DPAT increased the density of filopodia (f), whereas DOI increased the density of puncta (p) and spines (s). (C) At 11 DIV, DOI increased the total number of protrusions (filopodia, puncta and spines). (D) At 15 DIV, 8-OH-DPAT decreased the density of puncta and spines, whereas DOI decreased the filopodia density and increased the spine density (D) At 15 DIV, 8-OH-DPAT decreased the total number of protrusions. Mean \pm SEM. * P <0.01, ** P <0.001.

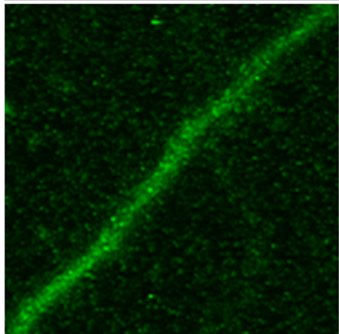
Figure 2 Localization of 5-HT_{1A} receptor and 5-HT_{2A} receptor in cultured cortical dendrites. E16 cortical neurons were cultured for 14 days in the basal medium and double-stained with rhodamine-phalloidin (red) and antibodies against 5-HT_{1A} receptor (green) or 5-HT_{2A} receptor (green). The immunoreactivity of 5-HT_{1A} and 5-HT_{2A} receptors was observed in dendritic shafts. Some dendritic protrusions expressed weak 5-HT_{2A} receptor immunoreactivity (arrows). Scale bar: 5 μ m

Supplemental Figure 1 Specificity of the anti-5-HT_{1A} antibody. (A) Staining of E16 cortical neurons cultured for 14 days. Neurons were stained with the anti-5-HT_{1A} antibody pre-absorbed with the antigen peptide (C-19P, Santa Cruz Biotechnology, left panel), rhodamin-phalloidin (middle panel) and merged figure (right panel). Scale bar: 2 μ m. (B) Western blotting of the adult rat cerebral cortex with the anti-5-HT_{1A} receptor antibody. A single band was recognized at around 67kDa.

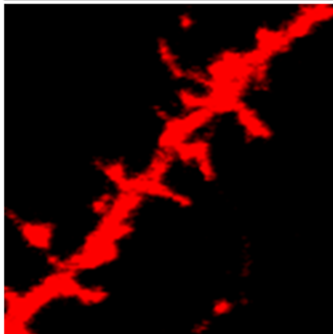


14 DIV

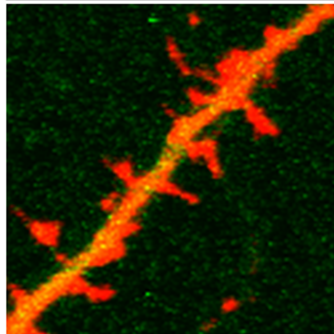
5-HT_{1A}



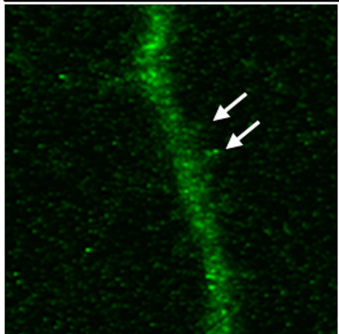
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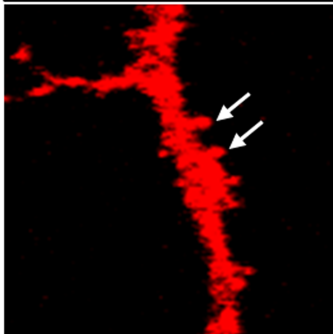
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5-HT_{2A}



phalloidin



merge

