Roles of 5-HT_{1A} receptor in the expression of AMPA receptor and BDNF in developing mouse cortical neurons

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Highlights

1. 5-HT_{1A} receptor upregulates BDNF and AMPA receptor expression *in vitro*.
2. 5-HT_{1A} receptor downregulates BDNF expression in developing cortex *in vivo*.
3. 5-HT_{1A} receptor activation has different effects between *in vitro* and *in vivo*.

Abstract

The possible interactions between serotonergic and glutamatergic systems during neural development and under the pathogenesis of depression remain unclear. We now investigated roles of 5-HT_{1A} receptor in the mRNA expression of AMPA receptor subunits (*GluR1* and *GluR2*) and brain-derived neurotrophic factor (*BDNF*) using primary culture of cerebral cortex of mouse embryos. Neurons at embryonic day 18 were cultured for 3 days or 14 days and then treated with 5-HT_{1A} receptor agonist (8-OH-DPAT) for 3 hours or 24 hours. In neurons cultured for 3 days, 8-OH-DPAT treatment for both 3 hours and 24 hours increased the mRNA levels of *BDNF* and *GluR1*, but not *GluR2*. In neurons cultured for 14 days, however, 8-OH-DPAT had no effects on these mRNA levels. Next, we examined *in vivo* roles of 5-HT_{1A} receptor by administration of 8-OH-DPAT to newborn mice. Twenty-four hours after the oral administration of 8-OH-DPAT, the mRNA expression of *BDNF* was decreased in the frontal cortex, but had no effects on the mRNA expression of *GluR1* and *GluR2*. Taken together, the present study suggests that 5-HT_{1A} receptor activation modulates mRNA expression of AMPA receptor subunit and BDNF in cortical neurons, and the effects are different between *in vitro* and *in vivo*.

Key words: serotonin; AMPA receptor; BDNF; cerebral cortex; dorsal raphe

1. Introduction

Serotonin (5-hydroxytryptamine, 5-HT) is a monoamine with multiple physiological functions. The early appearance of 5-HT neurons and 5-HT receptors in the embryonic brain (Lidov and Molliver, 1982a,b; Wallace and Lauder, 1983; Lauder, 1990) suggests that 5-HT plays crucial roles in the neural development (Gaspar et al., 2003; Wirth et al., 2015). In addition, disorder of 5-HT system is closely related to neuropsychiatric diseases. For example, depression is hypothesized to be caused by altered levels of 5-HT (Artigas et al., 2013; Dale et al., 2015) and commonly prescribed antidepressants such as selective serotonin reuptake inhibitors (SSRIs) target 5-HT system (Olfson and Marcus, 2009).

5-HT receptors are classified into 7 families with at least 14 different subtypes (Barnes and Sharp, 1999; Bockaert et al., 2006; Celada et al., 2013). Among these receptors, 5-HT_{1A} receptor appears in the early embryonic brain and regulates various aspects of neural development (Bonnin et al., 2006). In the matured brain, 5-HT_{1A} receptor acts as presynaptic autoreceptor in 5-HT neurons of the raphe nuclei and postsynaptic heteroreceptor in many brain regions including the cerebral cortex, hippocampus and amygdala (Artigas et al., 2013; Fiorino et al., 2014). Human studies of postmortem patients (Lopez-Figueroa et al., 2004; Szewczyk et al., 2009) and by positron emission tomography (Bhagwagar et al., 2004; Drevets et al., 2007) as well as preclinical studies using experimental animals (Haddjeri et al., 1998; Scorza et al., 2012) have demonstrated that 5-HT_{1A} receptor in the cerebral cortex is involved in action of antidepressants.

Recently, a number of studies have shown that glutamatergic system may be a novel target for treatment of major depressive disorder (MDD). α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor is likely to be involved in actions of antidepressants. AMPA receptor consists of tetramers composed of four subunits, GluR1-

GluR4. Among these subunits, only GluR2 lacks Ca^{2+} permeability, which gives diverse properties to AMPA receptors in the process of neuroplasticity (Derkach et al., 2007; Huganir and Nicoll, 2013). AMPA receptors appear early in the developing brain (Jourdi et al., 2003) and the trafficking of AMPA receptors to the synaptic membrane plays an important role during synaptogenesis and synapse maturation, as well as synaptic plasticity (Kumar et al., 2002; Fortin et al., 2012).

Previous studies suggested possible interactions between 5-HTergic and glutamatergic systems under the pathogenesis of depression. Antidepressants such as SSRI upregulate the expression of AMPA receptor subunits both *in vitro* and *in vivo* (Svenningsson et al., 2002; Barbon et al., 2006; Cai et al., 2013). However, roles of specific 5-HT receptors in the regulation of AMPA receptor expression are not well understood.

Brain-derived neurotrophic factor (BDNF) is required for neuronal development early in life and for neuronal survival and plasticity in the adult brain. It was shown that the expression of BDNF is decreased by stress whereas increased by antidepressant treatment in the hippocampus and prefrontal cortex (PFC) (Duman and Aghajanian, 2012; Duman and Voleti, 2012). In addition, BDNF upregulates mRNA expression, protein expression and membrane trafficking of AMPA receptor subunits in the hippocampus and cerebral cortex (Narisawa-Saito et al., 2002; Jourdi et al., 2003; Caldeira et al., 2007; Nakata and Nakamura, 2007; Fortin et al., 2012)

In the present study, in order to elucidate the interactions between 5-HT and glutamatergic systems during development and pathogenesis, we examined roles of 5-HT_{1A} receptor in the regulation of mRNA levels of AMPA receptor subunits (*GluR1* and *GluR2*) and brain-derived neurotrophic factor (*BDNF*) using cortical neurons both *in vitro*

and *in vivo*.

2. Materials and Method

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. Primary culture of cortical neurons

BALB/c mice (Nihon SLC, Hamamatsu, Japan) were used in the present study. The day of the vaginal plug was considered to be embryonic day (E) 0. Embryos at E18 were removed from pregnant mice under the deep anesthesia by isoflurane (Mylan, Tokyo, Japan), and quickly decapitated. The cerebral cortex was excised and meninges were carefully removed. The cerebral cortex was incubated in 0.025% trypsin-EDTA (Invitrogen, Carlsbad, CA) for 5 minutes at 37^{o}C, which was followed by incubation in DNase I (Roche Diagnostics, Mannheim, Germany) for another 5 minutes. The cells were dissociated by trituration with a Pasteur pipette. After filtration with 70-μm nylon cell strainer (BD Falcon, San Jose, CA), dissociated cells were plated on 8-well chamber slides (NUNK, Rochester, NY) at a density of 4 x 10^4 cells/cm^2 for immunocytochemistry or 24-well plates (BD Falcon) at a density of 1 x 10^5 cells/cm^2 for real-time RT-PCR. The slides and plates were coated with 0.2% polyethyleneimine (Sigma, St. Louis, MO) in advance. The cells were cultured in Minimal Essential Medium (Life Technologies, Carlsbad, CA) 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 37ºC. Eight hours after plating, the medium was replaced by Neurobasal Medium (Life Technologies) with 2% B-27 supplement (Life Technologies), 0.5 mM L-glutamine and 25 μg/ml penicillin/streptomycin. One day after plating, 5 μM cytosine-β-arabinofuranoside (Ara-C; Sigma) was added to medium for 24 hours to remove proliferating glial and neuronal progenitors.

2.2. Immunocytochemistry

Cortical neurons were cultured for 3 or 14 days *in vitro* (DIV) as described above and fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (PB) for 30 minutes 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 minutes.

To examine the expression of 5-HT_{1A} receptor, the cells cultured for 3 DIV were incubated overnight at 4ºC with the rat anti-5-HT_{1A} receptor antibody (4A6, 1:1000 dilution, Wako) and the chicken anti-MAP2 antibody (1:4000 dilution, Merck Millipore, Darmstadt, Germany). Cultured neurons were then incubated with Alexa Fluor 488-conjugated goat anti-rat IgG antibody (1:500 dilution, Invitrogen) and Alexa Fluor 594-conjugated goat anti-chicken IgG antibody (1:500 dilution, Invitrogen) for 1 hour at room temperature. In addition, cells cultured for 14 DIV were incubated with the rat anti-5-HT_{1A} receptor antibody and then Alexa Fluor 488-conjugated goat anti-rat IgG antibody. After the incubation with the secondary antibody, neurons were incubated with rhodamine-phalloidin (1:100 dilution, Invitrogen) which selectively labels F-actin for 30 minutes to visualize dendritic protrusions.

To examine the localization of 5-HT_{1A} receptor and GluR1 receptor, cortical neurons

at 3 DIV and 14 DIV were incubated overnight at 4°C with the rat anti-5-HT_{1A} receptor antibody (1:1000 dilution) and rabbit anti-GluR1 antibody (#13185, 1:200 dilution, Cell Signaling Technology, U.S.A.), followed by the incubation with Alexa Fluor 488-conjugated goat anti-rat IgG antibody and Alexa Fluor 594-conjugated goat anti-rabbit IgG antibody for 1 hour at room temperature.

In confirmation of the specificity of the primary antibodies, the western blot analysis of mouse frontal cortex by anti-GluR1 antibody reveled a single band of 100 kD in the present study (data not shown). In addition, the incubation except for anti-5-HT_{1A} receptor antibody or anti-GluR1 antibody yielded no specific staining (data not shown).

X-Y plane or Z-stack images of stained neurons were taken respectively at 20x or 63x with confocal laser scanning microscopes (LSM 510 META ver.3.2, and LSM 800 with Airyscan, Carl Zeiss, Oberkochen, Germany).

2.3. Quantitative real-time PCR

2.3.1. Cortical neurons in vitro

Cells cultured for 3 or 14 DIV were treated with 5-HT_{1A} agonist ((R)-(+)-8-Hydroxy-2-(dipropylamino) tetralin hydrobromide, 8-OH-DPAT, Sigma) at concentrations of 1, 10 and 100 nM for either 3 hours or 24 hours. Total RNA was extracted with RNAiso (Takara Bio, Shiga, Japan), according to the instructions of the manufacturer. The full content of a 24-well plate, with 2 x 10^5 cells/well, was collected for each experimental condition, and was immediately frozen in liquid nitrogen and kept at −80 ◦C until RNA extraction. Chloroform was added to separate RNA into aqueous layer. After centrifugation at 15,000 rpm at 4 ◦C for 15 min, supernatant was collected, and then isopropanol and ethachinmate (Nippon Gene, Tokyo, Japan) was added to precipitate RNA. Precipitated RNA was

washed with 75% ethanol and centrifuged at 15,000 rpm at 4 ◦C for 5 min. Supernatant was discarded and RNA was dried out and dissolved into RNase-free water. Concentration of total RNA was measured using spectrophotometer (Eppendorf Bio Spectrometer). Genomic DNAs were removed and cDNAs were synthesized from 300 ng of total RNA using QuantiTect Reverse Transcription Kit (Qiagen, Hilden, Germany). The sample was stored at −30 °C until further use.

For PCR amplification, cDNA was added to the reaction mixture containing SYBR Premix Ex TaqTM II (Takara Perfect Real Time; Takara Bio) and 0.2 µM of the primers. The specific primer pairs were: GluR1: forward primer, 5'-AGCGGACAACCA-CCATCTCTG-3'; reverse primer, 5'-AAGGGTCGATTCTGGGATGTTTC-3'; GluR2: forward primer, 5'-ATGGAACATTAGACTCTGGCTCCAC-3'; reverse primer, 5'-CTGCCG-TAGTCCTCACAAACACA-3'; BDNF: forward primer, 5'-GACAAGGCAACTTGGCCTAC-3'; reverse primer, 5'-ACTGTCACACACGCTCAGCTC-3'; tryptophan hydroxylase 2 (Tph2): forward primer, 5'-GAGCAGGGTTACTTTCGTCCATC-3'; reverse primer, 5'-AAGCAGGTCGTCTTT- GGGTCA-3'; serotonin transporter (Sert): forward primer, 5'-AAGCCCCACCTTGACTCCTCC-3'; reverse primer, 5'-CTCCTTCCTCTCCTCACATATCC-3'. The endogenous control was 18S rRNA: forward primer, 5'-ACTCAACACGGGAAACCTCA-3'; reverse primer, 5'-AACCAGACA- AATCGCTCCAC-3'. PCR was carried out on Thermal Cycler Dice Real Time System (Takara TP800, Software Ver.3.00) according to the following protocol: 5 s at 95 ◦C and 30 s at 60 ◦C – 50 cycles. Ct values were calculated from the crossing point of amplification curve and threshold, and relative quantitative analysis of targeted genes was carried out using calibration curve. The expression of 18S rRNA as internal control was used for compensation and the relative expression of mRNA in the experiment groups was calculated when the

expression of mRNA in the control group was set to 1.0.

2.3.2. Frontal cortex and dorsal raphe in vivo

BALB/c mice received a single oral administration of either vehicle (5% sucrose solutions) or 8-OH-DPAT (5 mg/kg) on postnatal day 1 (P1) where birthday is counted as P0. Twenty four hours after the drug treatment, mice were anesthetized by immersion in ice and quickly decapitated. The anterior 1/3 of cerebral cortex (frontal cortex) and the dorsal raphe nucleus were dissected from the brain. They were frozen immediately in liquid nitrogen and kept at −80 ◦C until RNA extraction. These brain regions were homogenized in RNAiso on ice using sonicator (Taitec, Saitama, Japan). Total RNA was diluted to 1:100 with distilled water and the concentration of total RNA was measured using spectrophotometer (Pharmacia Biotech Ultraspec 2000). For cDNA synthesis, 1 µg of total RNA was reverse-transcribed, and quantitative PCR was performed as described above.

2.4. Statistical analysis

The statistical analyses for cultures were performed by one-way ANOVA followed by *post-hoc* analysis (Fisher's protected least significant difference test) using SPSS statistics 22 (SPSS Japan Inc.). The statistical analyses for pups were performed by student t-test using Microsoft Excel 2010. Significance was set at a value of $p < 0.05$. All the data were expressed as the mean ± SEM.

3. Results

3.1. Localization of 5-HT$_{1A}$ receptor and GluR1 receptor in cortical neurons

We examined the localization of 5-HT$_{1A}$ receptor and GluR1 in cortical neurons cultured for 3 days and 14 days using specific antibodies. At 3 DIV, neurons were immunostained by the antibody against 5-HT$_{1A}$ receptor in combination with MAP2 antibody. 5-HT$_{1A}$ receptor showed spot-like localization, and was expressed in cell bodies and dendrites of all neurons (Fig. 1A). Double staining with anti-5-HT$_{1A}$ receptor and anti-GluR1 antibodies showed that GluR1 was expressed in cell bodies and dendrites of the same neurons in which 5-HT$_{1A}$ receptor was expressed (Fig. 1B). However, close examination revealed that 5-HT$_{1A}$ receptor and GluR1 were not co-localized at subcellular level (inset in Fig. 1B).

At 14 DIV, neurons were immunostained by anti-5-HT$_{1A}$ receptor antibody in combination with either rhodamine-phalloidin or anti-GluR1 antibody. All the neurons showed the spot-like immunoreactivity for 5-HT$_{1A}$ receptor in cell bodies and dendrites (Fig. 1C). In dendrites, 5-HT$_{1A}$ receptor was localized in dendritic shafts but not in dendritic protrusions where GluR1 was localized (insets in Fig. 1C, D).

3.2. 5-HT$_{1A}$ receptor agonist increased the mRNA expression of BDNF and GluR1 in cultured cortical neurons

We examined effects of 8-OH-DPAT on the mRNA expression of *BDNF, GluR1* and *GluR2* on cortical neurons *in vitro*. Cortical neurons were cultured for 3 days and 14 days, and then treated acutely with 8-OH-DPAT (1, 10 and 100 nM) or vehicle for 3 or 24 hours. Real-time RT-PCR was performed to examine time- and dose-dependent effects of 8-OH-DPAT on the mRNA levels for each gene.

At 3 DIV, the treatment with 1 nM 8-OH-DPAT for 3 hours increased the mRNA expression of *BDNF* ($p = 0.08$) and *GluR1* ($p < 0.05$) as compared with vehicle (Fig. 2A). The treatment with 1 nM and 100 nM 8-OH-DPAT for 24 hours also increased the mRNA expression of *BDNF* ($p < 0.05$) and *GluR1* ($p < 0.01$), respectively (Fig. 2B). Other treatment had no significant effect on the mRNA expression of *BDNF* and *GluR1* at 3 DIV. In addition, 8-OH-DPAT treatment at any concentrations had no significant effect on *GluR2* mRNA expression at 3 DIV (Fig. 2A, B).

To elucidate the onset of 8-OH-DPAT effects in more detail, we treated cortical neurons with 1 nM 8-OH-DPAT for 30 minutes at 3 DIV, which yielded no significant changes in the mRNA expression of *BDNF, GluR1* and *GluR2* (data not shown). Furthermore, to confirm the specific effects of 8-OH-DPAT, we treated cortical neurons with DOI, 5-$HT_{2A/2C}$ receptor agonist at 3 DIV for 3 hours. The DOI treatment showed no significant effects on the mRNA expression of *BDNF, GluR1* and *GluR2* (supplemental Fig. 1).

At 14 DIV, the treatment with 8-OH-DPAT for 3 hours and 24 hours had no significant effects on the mRNA expression of *BDNF, GluR1* and *GluR2* (Fig. 2C, D).

3.3. 5-HT_{1A} receptor agonist decreased the mRNA expression of BDNF in newborn frontal cortex in vivo

To examine *in vivo* roles of 5-HT_{1A} receptor in the mRNA expression of *BDNF, GluR1* and *GluR2* in the frontal cortex, mice received a single oral administration of either 8-OH-DPAT or vehicle at P1 and brain was removed after 24 hours. The treatment with 8-OH-DPAT decreased the mRNA expression of *BDNF* ($p < 0.05$) but had no significant effects on the mRNA expression of *GluR1* and *GluR2* in the frontal cortex (Fig. 3A),

which showed the different effects compared with those of cortical neurons *in vitro*. Next, to clarify whether these differences of effects between *in vitro* and *in vivo* were caused by interactions between frontal cortex and other brain regions, we examined effects of 8-OH-DPAT on the mRNA expression of *Tph2* and *5-HTT* in the dorsal raphe. 8-OH-DPAT treatment increased the mRNA expression of *Tph2* ($p < 0.01$) but had no significant effects on the mRNA expression of *5-HTT* (Fig. 3B).

4. Discussion

The present study examined roles of 5-HT_{1A} receptor in the mRNA expression of *BDNF*, *GluR1* and *GluR2* of mouse cortical neurons *in vitro* and *in vivo*. In embryonic cortical neurons cultured for 3 days, 5-HT_{1A} receptor activation upregulated the mRNA expression of *BDNF* and *GluR1*, but had no effects in neurons cultured for 14 days. In contrast, 5-HT_{1A} receptor activation in newborn mice *in vivo* downregulated mRNA expression of *BDNF* but had no effect on the mRNA expression of *GluR1* in the frontal cortex. These results demonstrate that 5-HT_{1A} receptor may regulate the mRNA expression of *BDNF* and *GluR1* in mouse cortical neurons *in vitro*, which may be modulated indirectly by other brain regions such as the dorsal raphe *in vivo*.

4.1. The types of cultured neurons

In the present study, we cultured E18 cortical neurons for 3 days and 14 days. In the mouse cerebral cortex at E18, most neuronal progenitors have completed the final cell division and begin differentiation into layer II-VI neurons (Caviness, 1982). Thus, most neurons in the present study are supposed to include layer II-VI neurons. A previous study

reported that in the culture of embryonic mouse cortical neurons, the expression of synaptic proteins begin to increase at 5-10 DIV together with dendrite development. Subsequently at about 15-25 DIV, the expression of synaptic proteins reaches the highest levels (Lesuisse and Martin, 2002). These results suggest that 3 DIV culture and 14 DIV culture in the present study may correspond to the periods of dendrite elongation and synapse maturation, respectively.

4.2. Roles of 5-HT$_{1A}$ receptor in the mRNA expression of AMPA receptor subunits and BDNF in vitro

In E18 cortical neurons cultured for 3 days, 8-OH-DPAT treatment upregulated the mRNA expression of *GluR1*, but not *GluR2*. Different effects on the expression of AMPA receptor subunits may be due to the developmental pattern of expression. A previous study reported that the expression of *GluR1* mRNA rapidly increases during late embryonic days, while *GluR2* mRNA expression gradually increases during postnatal days (Jourdi et al., 2003). Therefore, we treated embryonic cortical neurons at 3 DIV with 8-OH-DPAT during the period of dynamic changes of *GluR1* mRNA expression, but not *GluR2* mRNA.

In the present study, the activation of 5-HT$_{1A}$ receptor upregulated the subunit-specific transcription of *GluR1* mRNA, which may suggest the increase of calcium-permeable AMPA (CP-AMPA) receptor. CP-AMPA receptor lacks GluR2 subunit and mainly consists of GluR1 subunit. It has been shown that excitatory glutamatergic synapses express CP-AMPA receptors during early postnatal development, and these receptors are supposed to play an important role in synapse maturation and synaptic plasticity in developing networks (Kumar et al., 2002; Fortin et al., 2012). Therefore, it is likely that

in vitro activation of 5-HT_{1A} receptor promotes the expression of CP-AMPA receptor in an early stage of cortical neurons, which might contribute to the synapse maturation and plasticity. Further studies are needed to examine this possibility.

In contrast to the effects in short term culture, the treatment of more matured cortical neurons cultured for 14 days with 8-OH-DPAT had no significant effects on the mRNA levels of AMPA receptors. We examined immunocytochemically the localization of 5-HT_{1A} receptor and GluR1 subunit in cortical neurons that were cultured for 3 days and 14 days. 5-HT_{1A} receptor and GluR1 showed similar localization pattern demonstrating the both were expressed in the same neurons but not co-localized at sub-cellular level at 3 DIV and 14 DIV. These results indicated that the differences in the effects of 8-OH-DPAT between young and more matured neurons are not due to the localization of 5-HT_{1A} receptor and GluR1. It is likely that 5-HT_{1A} receptor modulates the expression of AMPA receptors depending on developmental stages of cortical neurons. Different roles of 5-HT_{1A} receptor during development were shown in the regulation of dendrite development. We have shown that 5-HT_{1A} receptor activation has no effects in the dendrite elongation of embryonic rat cortical neurons at 4 DIV (Ohtani, 2014) but inhibits maturation of dendritic spines at 14 DIV (Yoshida et al., 2011). Because 5-HT_{1A} receptor can couple to variety of effectors such as Gi/adenylate cyclase/protein kinase A signaling pathway (Wirth et al., 2015), it is possible that signaling mechanisms downstream to 5-HT_{1A} receptor may change depending on the developmental stages.

In addition to *GluR1* mRNA, 5-HT_{1A} receptor activation upregulated the mRNA expression of *BDNF*. It was reported that BDNF increases the expression of AMPA receptor subunits both in mRNA and protein levels of cultured hippocampal neuron at 7 DIV, but this effect disappears at 14 DIV (Caldeira et al., 2007). It was also shown that

BDNF regulates GluR1 expression in a subunit-specific manner. In cultured hippocampal neuron, BDNF enhances synaptic strength via the trafficking to membrane of newly translated GluR1 subunits as CP-AMPA receptors (Fortin et al., 2012). Taken together with the present study, it is probable that BDNF is involved in the regulation of GluR1 expression in cortical neuron *in vitro*.

In cultured cortical neurons at 3 DIV, 8-OH-DPAT treatment showed dose-dependent effects on the mRNA expression of *BDNF* and *GluR1*. 8-OH-DPAT has not only full agonist activity for 5-HT_{1A} receptor (EC_{50} = 9.6 nM、K_i = 0.65 nM) but also partial agonist activity for 5-HT_7 receptor (EC_{50} = 1000 nM、K_i = 39 nM) (Sprouse et al., 2004). Though it was reported that 5-HT_7 receptor antagonist increased the mRNA expression of BDNF, exact roles of 5-HT_7 receptor in the expression of BDNF remain unclear (Fumagalli et al., 2012; Homberg et al., 2014). Therefore, it is possible that 5-HT_7 receptor may be involved in the effects of 8-OH-DPAT treatment at higher dose.

4.3. Roles of 5-HT_{1A} receptor in the mRNA expression in the frontal cortex and dorsal raphe in vivo

The oral administration of 8-OH-DPAT to newborn mice downregulated the mRNA expression of *BDNF* but had no effects on the mRNA expression of AMPA receptor subunits in the frontal cortex. These results indicated that 5-HT_{1A} receptor activation induced different changes between *in vitro* and *in vivo*. It is possible that *in vivo* effects on the frontal cortex may be mediated indirectly through other brain regions including the dorsal raphe. 5-HT neurons in the dorsal raphe express 5-HT_{1A} receptor and project to various brain regions such as the cerebral cortex (Adell et al., 2002; Celada et al., 2013;

Fiorino et al., 2014). To clarify indirect effects of 8-OH-DPAT on cortical neurons via the dorsal raphe, we examined the mRNA levels of *Tph2* and *SERT* in the dorsal raphe which encode 5-HT synthesizing enzyme and 5-HT transporter, respectively. 8-OH-DPAT treatment increased the mRNA expression of *Tph2* but not *SERT*. These results suggest that 5-HT synthesis may be upregulated to increase 5-HT release in the frontal cortex. Then, the increased release of 5-HT may induce the decrease of mRNA expressions of *BDNF*, considering a pervious report that the increase of 5-HT concentration during an early stage of postnatal development downregulates mRNA levels of *BDNF* in the prefrontal cortex (Calabrese et al., 2013). It is possible that 5-HT receptors other than 5-HT_{1A} receptor in cortical neurons mediate the downregulation of BDNF level (Homberg et al., 2014). Considering that 5-HT_{2A} receptor has opposite effects to 5-HT_{1A} receptor in various neuronal functions (Azmitia, 2001; Yoshida et al., 2011), we treated cultured embryonic cortical neurons with DOI, 5-$HT_{2A/2C}$ receptor agonist at 3 DIV, which had no significant effects on the mRNA expression of BDNF (supplemental Fig. 1). These results suggest that 5-$HT_{2A/2C}$ receptors in cortical neurons are not involved in the effects of 5-HT_{1A} receptor activation observed *in vivo*.

In addition to the indirect effects via changes of 5-HT release from the dorsal raphe discussed above, there may be another indirect mechanisms. Because it was reported that 8-OH-DPAT treatment of neonatal mice affects respiratory function (Corcoran et al., 2014), we can not exclude the possibility that oral administration of 8-OH-DPAT changed BDNF mRNA expression in the frontal cortex through general effects on respiratory activity.

Considering these results, we must be careful for the interpretation of the effects of 8-OH-DPAT *in vivo* and *in vitro*. As a treatment of human patients such as neuropsychiatric

disorders, we must consider effects by oral administration. In contrast, *in vitro* experiments may be useful to examine signaling pathways affected through 5-HT receptors because of the availability of direct experimental manipulations.

In conclusion, the present study showed that activation of 5-HT_{1A} receptor modulates mRNA expression of AMPA receptor subunit and BDNF in cortical neurons. The effects are different between *in vitro* and *in vivo* treatments, which may represent direct and indirect effects, respectively.

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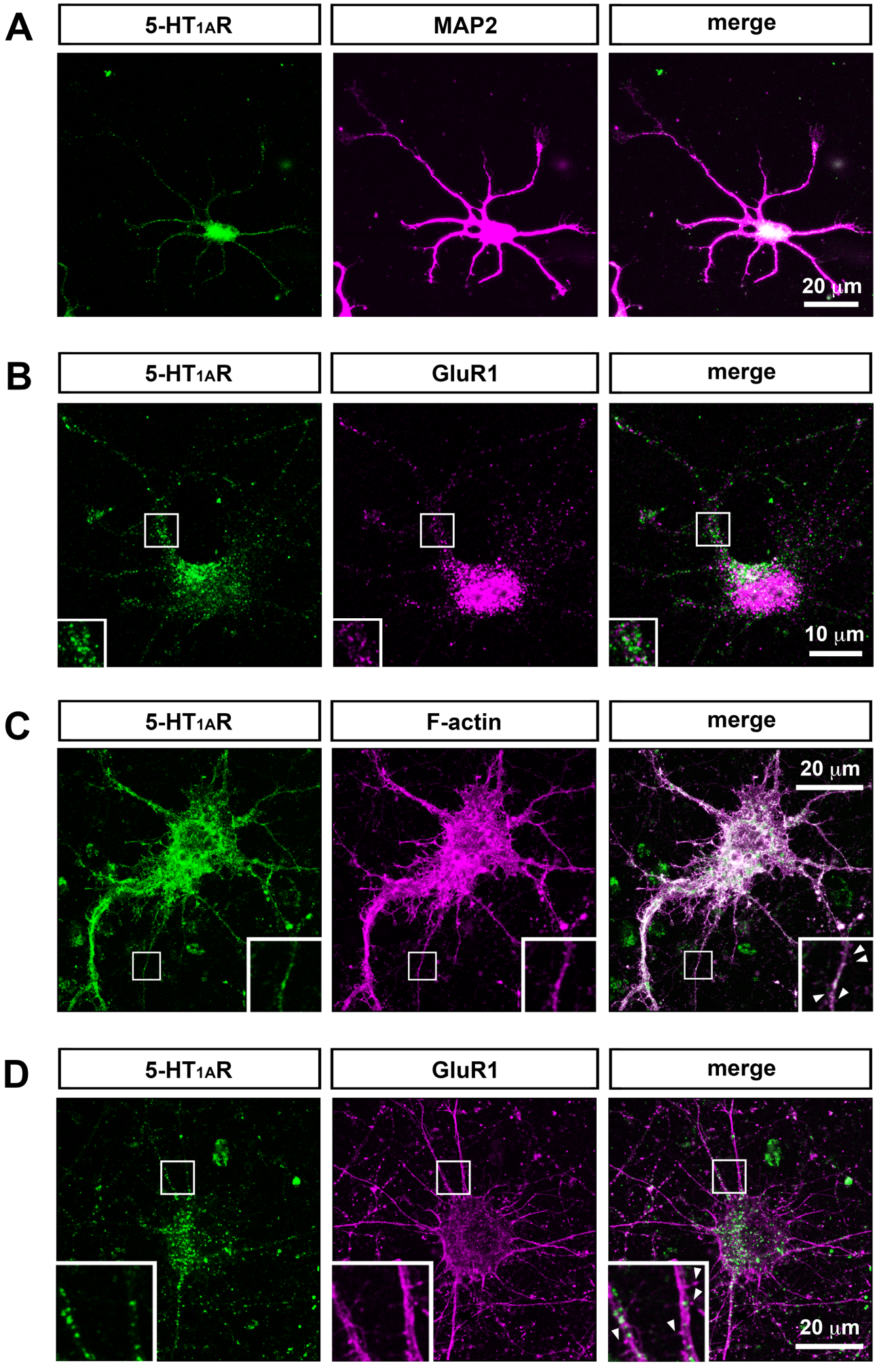
Figure legends

Fig. 1. Expression of 5-HT_{1A} receptor and AMPA receptor subunit GluR1 in cortical neurons cultured for 3 days (A, B) and 14 days (C, D). (A) Neurons at 3 DIV were stained by anti-5-HT_{1A} receptor antibody (green) and anti-MAP2 antibody (magenta). (B) Neurons at 3 DIV were stained by anti-5-HT_{1A} receptor antibody (green) and anti-GluR1 antibody (magenta). (C) Neurons at 14 DIV were stained by anti-5-HT_{1A} receptor antibody (green) and rhodamine-phalloidin (magenta). (D) Neurons at 14 DIV were stained by anti-5-HT_{1A} receptor antibody (green) and anti-GluR1 antibody (magenta). Arrowheads in C and D show dendritic protrusions where GluR1 but not 5-HT1A receptor was localized. Higher magnification of each figure is shown in inset. Scale bars: 20 μm (A, C, D), 10 μm (B).

Fig. 2. Effects of 5-HT_{1A} receptor agonist 8-OH-DPAT on the mRNA expression of *BDNF* and AMPA receptor subunits, *GluR1* and *GluR2*, in cortical neurons *in vitro*. Neurons were cultured for 3 days (A, B) and 14 days (C, D), and were treated with 8-OH-DPAT (1, 10 and 100 nM) or vehicle acutely for 3 hours (A, C) or 24 hours (B, D). *$p < 0.05$; **$p < 0.01$.

Fig. 3. Effects of 5-HT_{1A} receptor agonist 8-OH-DPAT on the mRNA expression of *BDNF* and AMPA receptor subunits, *GluR1* and *GluR2*, in the frontal cortex (A), and *Tph2* and *5-HTT* in the dorsal raphe (B) in newborn mice *in vivo*. *$p < 0.05$; **$p < 0.01$.

Fig.1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GluR1
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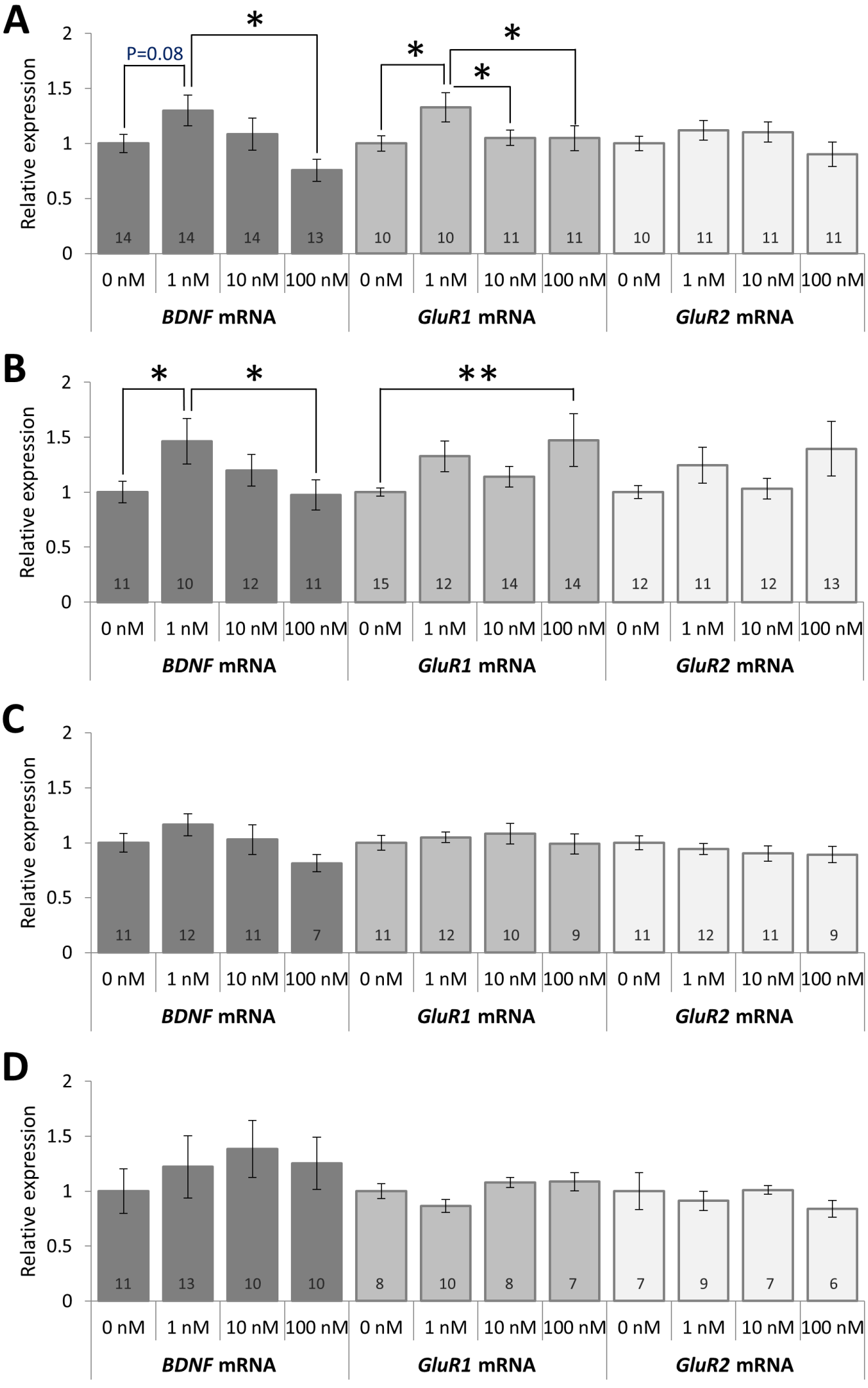
A
B
C
D
Relative expression
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1.5
1
0.5
0
P=0.08
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11 10 12 11 15 12 14 14 12 11 12 13
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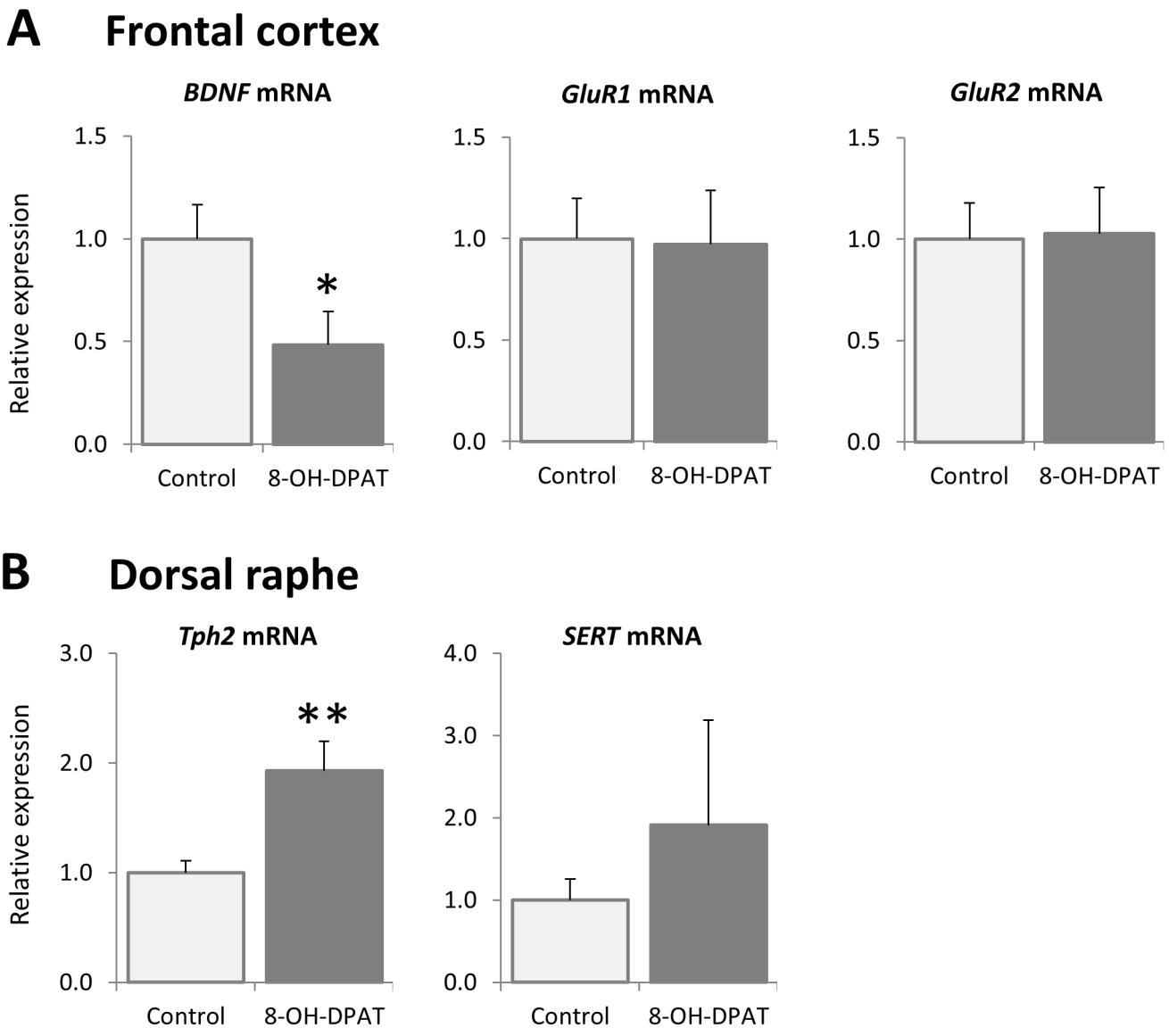
0 nM
1 nM
10 nM
100 nM
BDNF mRNA
GluR1 mRNA
GluR2 mRNA


A
Frontal cortex
BDNF mRNA
GluR1 mRNA
GluR2 mRNA
Relative expression
1.5
1.0
0.5
0.0
*
Control
8-OH-DPAT
B
Dorsal raphe
Tph2 mRNA
SERT mRNA
3.0
2.0
1.0
0.0
4.0
3.0
2.0
1.0
0.0
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