
# A 5-HT2A/2C receptor agonist, 1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane, mitigates developmental neurotoxicity of ethanol to serotonergic neurons

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**ORIGINAL ARTICLE**

Full title:

**A 5-HT$_{2A/2C}$ receptor agonist, 1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane, mitigates developmental neurotoxicity of ethanol to serotonergic neurons**

First author's surname: Ishiguro

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**ABSTRACT** **Prenatal ethanol exposure causes the reduction of serotonergic (5-HTergic) neurons in the midbrain raphe nuclei. In the present study, we examined whether an activation of signaling via 5-$HT_{2A}$ and 5-$HT_{2C}$ receptors during the fetal period is able to prevent the reduction of 5-HTergic neurons induced by prenatal ethanol exposure. Pregnant Sprague-Dawley rats were given a liquid diet containing 2.5 to 5.0% (w/v) ethanol on gestational days (GDs) 10 to 20 (Et). As a pair-fed control, other pregnant rats were fed the same liquid diet except that the ethanol was replaced by isocaloric sucrose (Pf). Each Et and Pf group was subdivided into two groups; one of the groups was treated with 1 mg/kg (i.p.) of 1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane (DOI), an agonist for 5-$HT_{2A/2C}$ receptors, during GDs 13 to 19 (Et-DOI or Pf-DOI), and another was injected with saline vehicle only (Et-Sal or Pf-Sal). Their fetuses were removed by cesarean section on GD 19 or 20, and fetal brains were collected. An immunohistological examination of 5-HTergic neurons in the fetuses on embryonic day 20 using an antibody against tryptophan hydroxylase revealed that the number of 5-HTergic neurons in the midbrain raphe nuclei was significantly reduced in the Et-Sal fetuses compared to that of the Pf-Sal and Pf-DOI fetuses, whereas there were no significant differences between Et-DOI and each Pf control. Thus, we concluded that the reduction of 5-HTergic neurons that resulted in prenatal ethanol exposure could be alleviated by the enhancement of signaling via 5-$HT_{2A/2C}$ receptors during the fetal period.**

**Key Words:** 1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane, fetal ethanol, rat, 5-HTergic neuron, midbrain raphe nuclei

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## INTRODUCTION

Ethanol is one of the best-known substances capable of inducing developmental abnormalities in the central nervous system (CNS). In fact, dysfunctions of the CNS, such as intellectual disability, learning disorder, hyperactivity, and coordination defect, have been reported in children with a history of exposure to ethanol *in utero*. Now, it is widely recognized that ethanol is a main cause of intellectual disability among those of a known etiology, and the developmental abnormalities with functional defects of the CNS caused by prenatal ethanol exposure are referred to as fetal alcohol syndrome (FAS, Rosett 1980) or fetal alcohol spectrum disorders (FASD, Sokol et al. 2003; see Riley et al. 2011, for a review).

The serotonergic (5-HTergic) system is one of the places targeted by the developmental neurotoxicity of ethanol. It has been widely accepted that prenatal ethanol exposure causes a reduction of 5-HTergic neurons in the midbrain raphe nuclei (Ohta et al. 2010; Tajuddin and Druse 1999, 2001; Sari and Zhou 2004; Zhou et al. 2001, 2002, 2008). Although it is obvious that ethanol exposure during the fetal period induces a reduced number of 5-HTergic neurons in the midbrain raphe nuclei, its mechanism is still incompletely understood.

Serotonin (5-HT) is a monoamine neurotransmitter related to many behaviors, physiological functions, and psychiatric disorders, such as aggression, anxiety, stress response, sexual behavior, and depression (Oliver 2015), while it has been expected to play important roles in the immature brain (see Bonnin and Levitt 2011; Whitaker-Azumita et al. 1996, for reviews). For example, 5-HT inhibits growth corn motility and synaptogenesis (Haydon et al. 1984) and modulates responsiveness of thalamocortical axons to a axonal guidance cue (Bonnin et al. 2007), and lack of 5-HT

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altered serotonergic innervations in the suprachiasmatic nucleus, praraventricular nucreus, nucleus accumbens, and hippocampus (Migliarini et al. 2013). Activation of 5-HT receptors enhance neurite outgrowth and lead to neuronal survival (Fricker et al. 2005; Lotto et al. 1999). Thus, disrupted 5-HTergic signaling during the prenatal period has been thought to be the possible cause of abnormal brain function in adulthood (Bonnin and Levitt 2011).

Seven families of the 5-HT receptor, comprising a total of 14 subtypes, have been identified (see Hoyer et al. 2002 for review), some of which already appear in the brain from the fetal period (Hellendall et al. 1993; Hillion et al. 1993; Johnson and Heinemann 1995; Bolaños-Jiménez et al. 1997). Especially, 5-$HT_{1A}$ receptor, which was identified in the cell bodies of 5-HTergic neurons in the embryonic midbrain (Héry et al. 1999; Hillion et al. 1994), has been strongly suggested to play important roles in the development of the 5-HTergic neurons. Interestingly, it has been reported that 5-$HT_{1A}$ receptor is not only located on neurons but also on astrocytes (Azmitia et al. 1996) and it would mediate some neurotrophic effects of 5-HT (Whitaker-Azmitia et al. 1990). Previously, it was demonstrated that maternal treatment with a 5-$HT_{1A}$ receptor agonist, ipsapirone, during the peak of differentiation of 5-HTergic neurons prevented the ethanol-associated reduction of 5-HTergic neurons and astrocytes in the raphe region of the developing rat brain (Tajuddin et al. 2003; Tajuddin and Druse 1999, 2001). Also, *in vitro* studies showed that ipsapirone prevents the ethanol-associated increase of apoptosis in 5-HTergic and other neurons in the rhombencephalon (Druse et al. 2004, 2005). Thus, it is considered that a facilitated 5-HT action via 5-$HT_{1A}$ receptor could protect 5-HTergic neurons from ethanol toxicity in fetal brain.

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Also other subtypes of 5-HT receptors have been considered to play key roles in brain development, because they were identified in the fetal brain (Gaspar et al. 2003) and suggested to mediate trophic effects of 5-HT (Lotto et al. 1999; Persico et al. 2006). Thus, it will be interesting to see if the agonists for these 5-HT receptors can prevent ethanol toxicity in the development of 5-HTergic neurons. Among 5-HT receptors, the 5-$HT_{2A}$ receptor is the most widely distributed in the brain. The expression of 5-$HT_{2A}$ receptors in the fetal brain gradually increases from embryonic days (EDs) 11 to 21, with a dramatic increase to a peak at ED 13, and it then decreases by ED 17 (Wu et al. 1999). The predominant 5-$HT_2$ receptors in the neonatal period are 5-$HT_{2C}$ receptors, while 5-$HT_{2A}$ receptors become predominant as the animal ages (Ike et al. 1995). In the midbrain raphe nuclei of adult rats, 5-$HT_{2C}$ receptors have been identified in the majority of GABAergic neurons, even if not in 5-HTergic neurons (Serrats et al. 2005), and the GABAergic neurons input to 5-HTergic neurons of DR (Wang et al. 1992). In addition, the possibility that a 5-$HT_{2A/2C}$ receptor agonist promotes growth of cultured embryonic brainstem 5-HT cells was suggested (Whitaker-Azmita et al. 1996). Thus, it is of interest whether the enhancement of signaling via 5-$HT_{2A/2C}$ receptors during the fetal period has the potential to protect 5-HTergic neurons from the developmental toxicity of ethanol.

In the present study, we examined whether a 5-$HT_{2A/2C}$ receptor agonist, 1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane (DOI), has a preventive effect against the reduction of 5-HTergic neurons in the midbrain raphe nuclei resulting in prenatal ethanol exposure in rats. We also measured monoamines and their metabolite levels using high-precision liquid chromatography (HPLC), and determined the expression level of genes involved in the generation and differentiation of 5-HTergic neurons using

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a real-time reverse-transcriptase polymerase chain reaction (RT-PCR) in the whole brain of fetuses prenatally exposed to ethanol.

## MATERIALS AND METHODS

### Animals, diets, and treatment

All animal procedures were conducted according to the Guide for the Care and Use of Laboratory Animals, and were reviewed by the Institutional Animal Care and Use Committee of the University of Tokushima. Great care was taken to minimize the number of animals used, and their suffering.

Pregnant Sprague-Dawley rats were obtained from Japan SLC (Hamamatsu, Japan). All of the rats used for the present study were maintained under 12-hour/12-hour of light and dark cycle. The presence of a vaginal plug was referred to as gestational day (GD) 0. At first, the pregnant dams were divided into two groups; prenatal ethanol-exposed (Et) and pair-fed control (Pf) groups. The same procedure as described in our previous study (Ohta et al. 2010) was used to prepare the rats of each experimental group. Briefly, pregnant rats of the Et group were allowed free access to a liquid diet (Oriental Yeast Co., Tokyo, Japan) containing ethanol during GDs 10 to 20, corresponding to the second-trimester of pregnancy in human. The ethanol concentration of the diet was gradually raised: 2.5% (w/v) on GDs 10 to 12, 4.0% (w/v) on GDs 13 to 15, and 5.0% (w/v) on GDs 16 to 20, to avoid a diminishing maternal intake and body weight gain (Sakata-Haga et al. 2002). In our preliminary study, the blood ethanol concentration (BEC) at the end of dark-phase during this administration regimen was measured in non-pregnant female rats (n=6) by AM1 Alcohol Analyzer (Analox Instrument, London, UK). During the 1$^{st}$ to 3$^{rd}$ day, the period rats fed a diet

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containing 2.5% ethanol, the mean of BEC was 94.5 ± 8.5 mg/dl. During the 4th to 6th day, the period rats fed a diet containing 4.0% ethanol, the mean of BEC was 182.3 ± 9.8 mg/dl. During the 7th to 10th day, the period rats were fed a diet containing 5.0% ethanol, and the mean of BEC was 266.0 ± 9.8 mg/dl. Pregnant rats of the Pf group were given an equivalent amount of the same liquid diet consumed by Et dams on a daily basis, except that the ethanol was replaced by isocaloric sucrose. Calorie content of both liquid diets was 1 kcal/ml. Each day between GDs 13 and 19, approximately half of the Pf and Et dams were given an intraperitoneal injection of a DOI solution, containing 1 mg of DOI hydrochloride (Sigma-Aldrich, St. Louis, MO) in 1 ml of saline, once a day at a daily dose of 1 mg/kg/day. Other dams of each Pf and Et group received only saline vehicle at the equivalent dose. DOI has a higher affinity for both 5-$HT_{2A}$ and 5-$HT_{2C}$ (*Ki* value is 0.7 nM and 2.4 nM, respectively) receptors than for 5-$HT_{2B}$ receptor (*Ki* value is 20 nM) (Nelson et al. 1999), and it has been widely used as an agonist for 5-$HT_{2A/2C}$ receptors. DOI also has been used to examine the effect of the activation of 5-$HT_{2A/2C}$ receptors during the fetal period, because it crosses the placental barrier (Bou-Flores et al. 2000; Bras et al. 2008). The four treatment groups, Pf-Sal (*n*=7) Pf-DOI (*n*=8), Et-Sal (*n*=8), and Et-DOI (*n*=6), were established in the present study by the combination of maternal diet and drug treatment. On GD 19 or 20, pregnant dams were anesthetized and their fetuses were removed by cesarean section. Although it has been reported that there was a gender difference in effects of prenatal ethanol exposure on the development of the 5-Hergic system (Hofmann et al., 2007), only male fetuses were used for the present study. The brains from ED 19 fetuses, which were collected within 4 h after a final injection of saline or DOI to their dams, were used for the observation using quantitative real-time PCR. To avoid miscount of

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5-HTergic neurons by direct effect of DOI on TPH expression and also to understand a change of monoamines status consequent to the DOI treatment, the brains used for the immunohistological detection of 5-HTergic neurons and quantification of monoamines and their metabolite contents were obtained from ED 20 fetuses, which were collected at least 24 h after a final injection of saline or DOI.

**Immunohistochemistry for 5-HTergic neurons in midbrain raphe nuclei**

For an immunohistological observation of 5-HTergic neurons, seven fetuses from three of Pf-Sal dams, seven fetuses from four of Pf-DOI dams, ten fetuses from four of Et-Sal dams, and seven fetuses from two of Et-DOI dams were used. The fetal brains were removed and immersed into 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) just after removal from the skull. The brains were embedded in paraffin and serially sliced along the frontal plane at 5-μm thickness. The sections were irradiated with microwaves for 5 min in 10 mM citrate buffer (pH 6.0), and then incubated with mouse anti-tryptophan hydroxylase (TPH) antibody (1:1000, Oncogene Research Products, San Diego, CA, USA) for 24 h at 4°C. Next, the sections were incubated with horseradish peroxidase-conjugated anti-mouse IgG antibody (1:400, MBL, Nagoya, Japan) for 2 h at 37°C. The immunoreactivity was then visualized with a solution containing 0.035 g 3,3-diaminobenzidine tetrahydrochloride (Nakarai, Kyoto, Japan), and 2.5 g nickel ammonium sulfate hexahydrate (Nakarai, Kyoto, Japan) in 100 ml of 0.1 M acetate buffer for 5 min at 37°C in the presence of 0.003% $H_2O_2$.

TPH-immunoreactive (TPH-ir) cells were counted in the dorsal raphe (DR) and the median raphe (MR) nuclei. At first, the most rostral section containing both DR and MR was identified as the first section, and every 5 sections (a total of five sections) were



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picked and supplied for a TPH-ir cell counting procedure (Ohta et al. 2010). TPH is a rate-limiting enzyme for 5-HT biosynthesis and is widely used as a marker of 5-HTergic neurons. The monoclonal antibody used in the present study has been used to describe the distribution of TPH-containing neuron and to classified 5-HTergic neurons if the neuron was immunoreactive for TPH (Zhang et al. 2006; Zhang and Hammond 2009). Thus, TPH-ir neurons were considered as 5-HTergic neurons.

**Quantitative measurement of monoamines and their metabolites in whole brains**

For a quantification of monoamines, sixteen fetuses from two of Pf-Sal dams, eleven fetuses from two of Pf-DOI dams, eleven fetuses from two of Et-Sal dams, and eight fetuses from two of Et-DOI dams were used. Brains were quickly weighed after removal from the skull, frozen in liquid nitrogen, and stored at -30°C until use. Each brain was homogenized with 1 ml of 0.2 M perchloric acid (Wako, Osaka, Japan) containing 100 μM EDTA, and then 1 μl of isoproterenol (ISO) solution (100 ng/μl) was added to the homogenate as an internal standard. The homogenates were incubated for 30 min on ice and centrifuged for 15 min at 20,000 ×g at 0°C. Each supernatant was corrected, pH was adjusted to 3.0 with 1 M sodium acetate, and filtered with a 0.45 μm pore-size filter (Millipore, USA). Each 10 μl of the aliquot was injected into the HPLC system (HTEC-500, Eicom, Kyoto, Japan) with an electrochemical detector (ECD-300, Eicom, Kyoto, Japan) and a reverse-phase column (EICOMPAK SC-50DS, ϕ3 mm×150 mm, Eicom, Kyoto, Japan). As a mobile phase, a solution composed of 0.1 M sodium acetate-citric acid buffer with 190 mg/l sodium 1-octanesulfonate, 5 mg/l EDTA-2Na and 15% (vol/vol) methanol (pH 3.5), was used, and the flow rate was maintained at 0.5 ml/min. The detector response was plotted and measured using an

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HPLC chromatogram analysis software Power Chrom (ver. 2.3.1, eDAQ Japan, Nagoya, Japan). A standard solution, containing 5-HT and its metabolite, 5-HIAA; dopamine (DA) and its metabolite, 3,4-dihydroxyphenylacetic acid (DOPAC); and ISO at each concentration of 10 pg/μl was prepared, and 10 μl of the standard solution was applied to obtain a chromatogram before sample analysis. The amount of each monoamine was calculated from the integrated chromatographic peak area and expressed as ng/g wet tissue. As an index of DA and 5-HT turnover, the DOPAC/DA and 5-HIAA/5-HT ratios were also calculated.

**Quantitative real-time PCR**

Total RNA was isolated from frozen brains of 18, 11, 8 and 13 fetuses from two dams each in the Pf-Sal, Pf-DOI, Et-Sal and Et-DOI groups according to the manufacturer's instructions with Isogen (Nippon Gene Inc., Tokyo, Japan) and quantitated by a spectrophotometer (Smartspec 3000, Bio-Rad Laboratories, Hercules, CA, USA; 260/280 nm ratio). The integrity of the RNA was checked by agarose gel electrophoresis. First-strand cDNA was synthesized from 1 μg of total RNA using oligo (dT) and Super Script III First-strand Synthesis System (Invitrogen Life Tech., CA, USA). Real-time PCR was performed on an ABI 7500 instrument and its associated software (Applied Biosystems, Foster City, CA, USA). PCR reactions were performed in 20 μl solutions containing 2 μl of cDNA, 200 nM primer pair, and 1× SYBR green PCR master mix in 96-well plates. The primer sets for the PCR reactions are listed in Table 1. The PCR cycling conditions were 95°C for 3 min, followed by 40 cycles at 95°C for 30 s, and the appropriate annealing temperature for the primer set under study was for 30 s and 72°C for 40 s (see Table 1 for annealing temperatures). For each reaction, a standard curve

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was obtained by analyzing a dilution series of pooled cDNA samples. To correct for sample-to-sample variation, glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) was used as an internal standard for RT-PCR. The expression of each target gene was quantified as a relative expression against the *Gapdh* expression. PCR product purity was confirmed by dissociation curve analysis for each gene at the end of the PCR reaction.

**Statistical analysis**

Statistical analyses were conducted using SPSS 16.1J (IBM Japan, Tokyo, Japan). Data of the cell counts, body weight, brain weight, monoamine content, and RNA expression were analyzed using a two-way analysis of variance (ANOVA) with factors Food and Treatment. When an interaction reached significance, a simple main effect test was used to confirm a simple main effect of each factor, while an interaction was not significant, Tukey test (for data sets with equal variances) or Dunnett's T3 test (for data sets with unequal variance) was performed as Post-hoc analysis. Homogeneity variances were analyzed with Levene's test. Results are expressed as mean ± SD. Statistical significance was considered at $p < 0.05$.

## RESULTS

**Body weight and brain weight of fetuses**

Body weight, brain weight, and brain to body weight ratio of fetuses at ED 20 were shown in Table 2. In the body weight of pups, no interaction was detected between Food and Treatment, and a significant main effect of Food was significant. Post-hoc analysis revealed that there were significant differences between PF-sal and Et-sal or Et-DOI.

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Thus, it was suggested that prenatal ethanol exposure induced growth deficiency of fetuses regardless of DOI treatment. On the other hand, a significant Food and Treatment interaction was detected in the brain weight of pups. A simple main effect of Food was significant in the saline-treated group (Pf-Sal and Et-Sal) and also that of Treatment was significant in the non ethanol-exposed group (Pf-Sal and Pf-DOI). Thus, it was suggested that prenatal ethanol exposure inhibited brain growth, and the inhibitory effect of ethanol on fetal brain growth was not prevented by DOI. In the ratio of brain weight to body weight, the main effect of Food was significant, whereas the main effect of Treatment was not, and no interaction was detected between Food and Treatment. Post-hoc analysis revealed that there were significant differences between PF-Sal and Et-Sal or Et-DOI. Thus, it was suggested that prenatal ethanol exposure altered the ration of brain weight to body weight regardless of DOI treatment.

**Number of TPH-ir cells in raphe nuclei**

In all four experimental groups, TPH-ir cells were mainly distributed in the dorsal and the median raphes (DR and MR, respectively) of the midbrain (Figs. 1A and B). Fig. 2 shows the relative numbers of these cell in each group (based on that in Pf-Sal). In both the DR and MR, a significant interaction of Food and Treatment was detected. A simple main effect of Food was confirmed in only the saline-treated group and that of Treatment was significant in the ethanol-exposed group in both nuclei. Thus, the present study suggested that prenatal ethanol exposure induced a significant decrease in the number of THP-ir cells in both DR and MR, while the inhibitory effect of ethanol on the number of 5-HTergic neurons was attenuated by co-treatment with DOI (Figs. 1 and 2).

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**Monoamine contents and their turnover in fetal brains**

Contents of DA, 5-HT, and their metabolites in whole fetal brain were shown in Table 3. For the contents of 5-HT or 5-HIAA in the whole fetal brains, the main effect of Food was significant and no interaction was detected between Food and Treatment. Post-hoc analysis revealed that there were significant differences between Pf-Sal and Et-Sal or Et-DOI and also between Pf-DOI and Et-Sal or Et-DOI. Thus, it was suggested that prenatal ethanol exposure induced a reduction of 5-HT content in the whole fetal brain regardless of DOI treatment. On the other hand, significant differences of 5-HIAA content were detected between Pf-Sal and Et-Sal, but not between Pf-Sal and Et-DOI. There results suggested that prenatal ethanol exposure without DOI treatment reduced 5-HIAA content, but DOI treatment mitigated the ethanol-induced reduction of 5-HIAA content in the fetal whole brain. The turnover ratio of 5-HT was not altered by any factors of Food or Treatment. For the contents of DA in the whole fetal brains, the main effect of Food was significant and no interaction was detected between Food and Treatment. However, a significant difference was never detected between any couple of two experimental groups. Significant interactions between Food and Treatment were shown in the content of DOPAC and the turnover ratio of DA. For both DOPAC content and the turnover ration of DA, a simple main effect of Treatment was significant in the pair-fed group, but not in ethanol-exposed group. Thus it was suggested maternal DOI treatment facilitated turnover of DA in the fetal brain, but the prenatal ethanol-exposure interrupted the DOI efficacy.

**Expression of genes related to generation and differentiation of 5-HTergic neurons**

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Changes of gene expression in whole fetal brain on GD 19 were shown in Fig. 3. For expression of *Phox2b* and *Lmx1b*, a significant interaction between Food and Treatment was detected. Whereas a simple main effect of Food was confirmed in both the saline- or DOI-treated groups, a simple main effect of Treatment was significant only the ethanol-exposed group. For relative expression of *Lmx1b*, a simple main effect of Food was significant in the saline-treated group, and also that of Treatment was significant only the ethanol-exposed group. It was suggested that prenatal ethanol-exposure suppressed relative expressions of *Phox2b* and *Lmx1b*, however, the ethanol-induced reductions of each *Phox2b* and *Lmx1b* expression were completely prevented by co-treatment of dams with DOI. For expression of *Fgf8*, the main effect of Food was significant and no interaction was detected between Food and Treatment. However, a significant difference was never detected between any couple of two experimental groups in post-hoc analysis. For expression of *Nkx2.2*, the main effect of Treatment was significant and no interaction was detected between Food and Treatment. Post-hoc analysis revealed that there were significant differences between Et-DOI and PF-Sal or Et-Sal. Thus, it was suggested that DOI treatment reduced relative expression of *Nkx2.2,* regardless ethanol exposure *in utero*.

## DISCUSSION

In the present study, we found that a 5-$HT_{2A/2C}$ receptor agonist, DOI, had the ability to prevent a reduction in 5-HTergic neurons in the DR and MR induced by prenatal ethanol treatment. Previously, Tajuddin and colleagues reported that maternal treatment with a 5-$HT_{1A}$ receptor agonist, ipsapirone, during the peak of differentiation of

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5-HTergic neurons prevented the ethanol-associated reduction of 5-HTergic neurons in the midbrain raphe nuclei (Tajuddin and Druse 1999, 2001; Tajuddin et al. 2003). The present study suggested that also an agonist of 5-$HT_{2A/2C}$ receptors also protect midbrain 5-HTergic neurons from developmental neurotoxicity of ethanol.

A mechanism of the protective effect of DOI against the developmental toxicity of ethanol might be more complicated than that of 5-$HT_{1A}$ receptor agonists, because 5-$HT_{2A}$ and 5-$HT_{2C}$ receptors have never been identified on 5-HTergic neurons in the immature mesencephalon, and the distribution of these receptors during brain development remains incompletely defined, unlike that of 5-$HT_{1A}$ receptor. In addition, prenatal ethanol exposure has no apparent effect on expression of mRNA for 5-$HT_{2A}$ or 5-$HT_{2C}$ receptors in rats (Kim et al. 1997; Hofmann et al. 2007), whereas ethanol exposure *in utero* impairs the development of 5-$HT_{1A}$ receptor-expressing neurons in the parietal and frontal cortex. However, 5-HT signaling via 5-$HT_{2A}$ and 5-$HT_{2C}$ receptors must be important for normal brain development, as evidenced by the fact that both receptors are expressed in immature brain with developmental stage-related changes (Ike et al. 1995; Wu et al. 1999) and would mediate trophic effects of 5-HT (Persico et al. 2006).

It was reported that mRNA of both 5-HT2A and 5-HT2C receptors are expressed in the DR and periaqueductal gray, that is adjacent to the DR, in adult rats (Wright et al. 1995). Subsequently, it was demonstrated that 5-$HT_{2C}$ receptors were mainly distributed in GABAergic neurons in the midbrain raphe nuclei, even if they were not in 5-HTergic cells (Serrats et al. 2005). 5-HTergic neurons of the DR receive input from GABAergic neurons via $GABA_A$ receptors (Wang et al. 1992), and an activation of 5-$HT_{2A}$ and 5-$HT_{2C}$ receptors by DOI administration increases inhibitory input from GABAergic

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neurons to 5-HTergic neurons of the DR (Liu et al. 2000). It was reported that GABA acted as a neurotrophic factor against immature monoamine neurons, including 5-HTergic neurons, and regulated their survival in an *in vitro* study using primary dissociated cells from rat fetuses on ED 14 (Liu et al. 1997). Therefore, the protective effect of DOI against the reducing of 5-HTergic cells by prenatal ethanol exposure might be exerted through activation of GABA neurons in the raphe nuclei.

In quantitative measurement of their monoamines, it was revealed that prenatal ethanol administration induced decreases in the content of both 5-HT and 5-HIAA in the whole brain. DOI supplementation tended to mitigate the reduction of 5-HT content, however this DOI effect was not statistically significant. On the other hand, DOI treatment improved 5-HIAA content in the ethanol-exposed fetus. Thus, it was suggested that DOI treatment was effective to protect developing 5-HTergic system from ethanol toxicity, while it might be not enough.

Quantitative real-time PCR revealed the expression status of genes involved serotonergic differentiation on ED 19. In rats, the peak of 5-HTergic differentiation takes place at EDs 15 to16, and shows an assembled distribution of 5-HTergic neurons with adult by ED 19. Hence, differentiation into 5-HTergic neurons is due to be completed by ED 19 in rats. Thus, these gene expressions in rats at ED 19 may show the results of the altered differentiation into 5-HTergic neurons. Et-Sal rats displayed significantly lower levels of *Phox2b* mRNA expression than both Pf-Sal and Et-DOI. *Phox2b* is a molecule that suppresses the premitotic cell differentiation into 5-HTergic neurons and expression of *Phxo2b* is suppressed in differentiating 5-HTergic neurons (Pattyn et al. 2003). Thus, the changes in relative expression of *Phox2b* seen in our study might reflect the changes in the number of differentiated 5-HTergic neurons

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depending on foods and treatments. Also, *Lmx1b*, which was expressed in postmitotic 5-HTergic neurons (Hendricks et al. 1999, 2003), showed parallel changes with the number of 5-HTergic neurons. As expected, relative expression of *Lmx1b* in Et-Sal rats was significantly lower than that of Pf-Sal or Et-DOI. Expression of *Fgf8*, which reported a reduced expression after prenatal ethanol exposure, was reduced in fetal brain by prenatal ethanol exposure. On the other hand, expression of *Shh*, which also reported prenatal ethanol-related change, was not affected in the present study. However, a time series comparison from earlier stage of development and observation of 5-HTergic cell specific changes would be required to understand the correlation of these genes in the ethanol-induced reduction of 5-HTergic neurons and its prevention by DOI.

In the present study, we suggested that enhancement of signaling via 5-$HT_{2A}$ and/or 5-$HT_{2c}$ receptors could possibly prevent the reduction of 5-HTergic neurons induced by prenatal ethanol exposure. The protective effects of the 5-$HT_{2A\ /2C}$ agonist on the ethanol-induced reduction of 5-HTergic neurons would be exerted through direct and/or indirect actions on 5HTergic neurons, such as GABAergic neurons and/or astrocytes. To further understand these mechanisms, it is also important to know in detail the roles of each 5-$HT_{2A}$ or 5-$HT_{2C}$ receptor in brain development, particularly in development of the 5-HTergic system.

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## DISCLOSURES

The authors have declared no conflict of interest.



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**Fig. 1** A: Sketch of a frontal plane containing the dorsal raphe (DR) and the median raphe (MR). B: Distribution of TPH-ir cells in the midbrain raphe nuclei on a frontal section of rat fetus on GD 20 (A scale bar = 400 μm). The DR and MR of rat fetus on prenatal day 20 were identified by reference to an atlas for prenatal rat brain (Foster 1998). C and D: Comparison of TPH-ir cell distribution in the DR and MR among experimental groups, respectively (Scale bars = 100 μm). In both the DR and MR, Et-DOI fetuses showed a pronounced reduction in TPH-ir cells compared to all other groups. Aq, aqueduct; mlf, medial longitudinal fasciculus; xscp, decussation of the superior cerebellar peduncle.

**Fig. 2** Comparison of TPH-ir cell numbers in DR and MR among experimental groups. Values are represented as mean + SD of percentage of relative expression in Pf-Sal. $^{\bullet\bullet}p < 0.01$ for a significant simple main effect of Food. $^{\blacktriangle}p < 0.05$ or $^{\blacktriangle\blacktriangle}p < 0.01$ for a significant simple main effect of Treatment.

The number of TPH-ir cells in both the DR and MR showed a significant Food and Treatment interaction [$F_{(1, 27)} = 5.54$, $p < 0.05$ and $F_{(1, 27)} = 5.31$, $p < 0.05$, respectively]. A simple main effect of Food in the saline-treated group (Pf-sal and Et-sal) was significant in both DR and MR [$F_{(1, 27)} = 19.83$, $p < 0.01$ and $F_{(1, 27)} = 27.01$, $p < 0.01$, respectively]. Also, a simple main effects of Treatment was significant in the ethanol-exposed group (Et-Sal and Et-DOI) in both DR and MR [$F_{(1, 27)} = 6.17$, $p < 0.05$ and $F_{(1, 27)} = 10.17$, $p < 0.01$, respectively]. Thus, it was suggested that DOI treatment could protect ethanol-induced reduction of 5-HTergic neurons in both DR and MR.

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**Fig. 3** Gene expression of *Shh*, *Fgf8*, transcription factors involved in serotonergic neuron development (*Phox2b*, *Nkx2.2*, *Gata2*, *Lmx1b* and *Pet1*) in whole fetal brains. Values are represented as mean + SD of percentage of relative expression in Pf-Sal. $^{\bullet}p < 0.05$ or $^{\bullet\bullet}p < 0.01$ for a significant simple main effect of Treatment. $^{\blacktriangle}p < 0.05$ or $^{\blacktriangle\blacktriangle}p < 0.01$ for a significant simple main effect of Treatment. $^{*}p < 0.05$ compared to Pf-Sal. $^{\#}p < 0.05$ compared to Et-Sal.

For expression of *Phox2b* and *Lmx1b*, a significant interaction between Food and Treatment was detected [$F_{(1, 46)} = 16.95$, $p < 0.01$ and $F_{(1, 46)} = 6.53$, $p < 0.05$, respectively]. For *Phox2b* expression, a simple main effect of Food in both saline treated groups (Pf-sal and Et-sal) and DOI treated groups (Pf-DOI and Et-DOI) was significant [$F_{(1, 46)} = 11.40$, $p < 0.01$ and $F_{(1, 46)} = 5.90$, $p < 0.05$, respectively] , and a simple main effects of Treatment was significant in ethanol-exposed groups (Et-Sal and Et-DOI) was significant [$F_{(1, 46)} = 17.29$, $p < 0.01$ ]. For *Lmx1b* expression, a simple main effect of Food in saline treated groups (Pf-sal and Et-sal) was significant [$F_{(1, 46)} = 7.01$, $p < 0.05$] , and a simple main effects of Treatment was significant in ethanol-exposed groups (Et-Sal and Et-DOI) was significant [$F_{(1, 46)} = 5.22$, $p < 0.05$ ]. For expression of *Fgf8*, the main effect of Food was significant [$F_{(1, 46)} = 6.48$, $p < 0.05$], whereas that of Treatment was not, and interaction of Food and Treatment was also not significant. Post-hoc analysis showed no significant differences between any two groups. On the other hand, expression of *Nkx2.2* was significantly affected by Treatment [$F_{(1, 46)} = 9.98$, $p < 0.01$], but not by Food, and interaction of Food and Treatment was also not significant. Post-hoc

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analysis detected significant differences between Et-DOI and Pf-Sal or Et-Sal ($p < 0.05$ and $p < 0.05$, respectively). For expressions of *Shh*, *Gata2*, and *Pet1*, the main effects of each Food and Treatment were not significant, and interactions of Food and Treatment were also not significant.

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**Table 1** List of primer pairs used in quantitative real-time PCR

| Genes | | Sequence 5'→3' | Amplicon size (bp) | Annealing temperature (°C) |
|---|---|---|---|---|
| *Gapdh* | Forward<br>Reverse | AGACAGCCGCATCTTCTTGT<br>TGATGGCAACAATGTCCACT | 142 | 58 |
| *Shh* | Forward<br>Reverse | AGGCTGGATTCGACTGGGTCTA<br>AACTTGGTGCCACCCTGCTC | 142 | 64 |
| *Fgf8* | Forward<br>Reverse | CATCAACGCCATGGCAGAA<br>CAGCACGATCTCCGTGAACA | 187 | 64 |
| *Phox2b* | Forward<br>Reverse | TACGCCGCAGTTCCATACAAACTC<br>TCTTTGAGCTGCGCGCTTGTGAAG | 104 | 56 |
| *Nkx2.2* | Forward<br>Reverse | CATGTCGCTGACCAACACAAAG<br>TCGCTGCTGTCGTAGAAAGGA | 210 | 56 |
| *Gata2* | Forward<br>Reverse | GTGGAACGTACTCTTGGCTCCTG<br>TCCAAACAAACACTGTCCGTGAA | 196 | 64 |
| *Lmx1b* | Forward<br>Reverse | TCAGTGTGCGTGTGGTCCAG<br>TCTGCTGCTCTTGCTGTTGC | 93 | 64 |
| *Pet1* | Forward<br>Reverse | CCCTGCTGATCAACATGTACCTACC<br>CAGCTCCAGTAGAAACTGCCACAA | 143 | 60 |

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**Table 2** Body weight, brain weight, and brain to body weight ratio of fetuses

| | Pf-Sal ($n$ = 16) | Pf-DOI ($n$ = 11) | Et-Sal ($n$ = 11) | Et-DOI ($n$ = 8) |
|---|---|---|---|---|
| Body weight (g) | 3.987 ± 0.413 | 3.671 ± 0.166 | 3.440 ± 0.222** | 3.409 ± 0.196** |
| Brain weight (g) | 0.181 ± 0.007••,▲ | 0.175 ± 0.008▲ | 0.170 ± 0.004•• | 0.172 ± 0.007 |
| Brain/Body weight ratio (%) | 4.59 ± 0.39 | 4.76 ± 0.12 | 4.96 ± 0.27* | 5.06 ± 0.29* |

Values are reported as mean ± SD. *$p < 0.05$ or **$p < 0.01$ compared to Pf-Sal. ••$p < 0.01$ for a significant simple main effect of Food between the saline-treated groups. ▲$p < 0.05$ for a significant simple main effect of Treatment between the pair-fed controls. In the body weight of pups, no interaction was detected between Food and Treatment, and a significant main effect of Food [$F_{(1, 42)}$, = 20.72, $p < 0.01$], but not of Treatment, was detected. Post-hoc analysis revealed there were significant differences between Pf-Sal and Et-Sal ($p < 0.01$) or Et-DOI ($p < 0.01$), respectively. In the brain weight of pups, a significant Food and Treatment interaction was detected. A simple main effect of Food in the saline-treated group (Pf-Sal and Et-Sal) [$F_{(1, 42)}$, = 19.12, $p < 0.01$] and that of Treatment in the pair-fed control (Pf-Sal and Pf-DOI) [$F_{(1, 42)}$, = 7.06, $p < 0.05$], was significant. In the brain/body weight ratio, no interaction was detected between Food and Treatment, and a significant main effect of Food [$F_{(1, 42)}$, = 14.04, $p < 0.01$], but not of Treatment, was detected. Post-hoc analysis revealed there were significant differences between Pf-Sal and Et-Sal ($p < 0.05$) or Et-DOI ($p < 0.05$), respectively.

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**Table 3** Monoamine levels and their turnover ratios in whole brain of rat fetuses

| | | Pf-Sal ($n$ = 16) | Pf-DOI ($n$ = 11) | Et-Sal ($n$ = 11) | Et-DOI ($n$ = 8) |
|---|---|---|---|---|---|
| Contents of monoamine (ng/g wet tissue) | 5-HT | 193.4 ± 17.9 | 194.5 ± 20.2 | 152.0 ± 22.1$^{**, \#\#}$ | 168.5 ± 23.2$^{*, \#}$ |
| | 5-HIAA | 313.7 ± 62.3 | 339.5 ± 86.3 | 252.7 ± 43.7$^{*}$ | 290.1 ± 75.5 |
| | DA | 139.0 ± 23.9 | 154.1 ± 17.8 | 134.3 ± 22.4 | 131.5 ± 18.0 |
| | DOPAC | 23.4 ± 13.8$^{▲}$ | 37.6 ± 15.2$^{▲}$ | 30.8 ± 17.9 | 23.4 ± 13.8 |
| Turnover ratio | 5-HIAA/5-HT | 1.61 ± 0.19 | 1.73 ± 0.33 | 1.67 ± 0.19 | 1.70 ± 0.26 |
| | DOPAC/DA | 0.16 ± 0.07$^{▲}$ | 0.24 ± 0.08$^{▲}$ | 0.22 ± 0.10 | 0.17 ± 0.08 |

Values are reported as mean ± SD. $^{*}p < 0.05$ or $^{**}p < 0.01$ compared to Pf-Sal. $^{\#}p < 0.05$ or $^{\#\#}p < 0.01$ compared to Pf-DOI. $^{▲}p < 0.05$ for a significant simple main effect of Treatment in the pair-fed group (Pf-Sal and Pf-DOI). For the contents of 5-HT or 5-HIAA in the whole fetal brains, no interaction was detected between Food and Treatment, and the main effect of Food was significant [$F_{(1, 42)}$, = 29.43, $p < 0.01$ and $F_{(1, 42)}$, = 7.24, $p < 0.05$, respectively], whereas the main effect of Treatment was not significant. Post-hoc analysis revealed there were significant differences of 5-HT content between Pf-Sal and Et-Sal ($p < 0.01$) or Et-DOI ($p < 0.05$), respectively, and between Pf-DOI and Et-Sal ($p < 0.01$) or Et-DOI ($p < 0.05$), respectively. Meanwhile. a significant difference of 5-HIAA content was detected only between Pf-Sal and Et-Sal ($p < 0.05$). For the turn over ratio of 5-HT, the main effects of Food or Treatment were not significant, and no interaction was detected between Food and Treatment. For the contents of DA, no interaction was detected between Food and Treatment, and the main effect of Food was significant [$F_{(1, 42)}$, = 4.46, $p < 0.05$], whereas the main effect of Treatment was not significant. However, there were no significant differences between any two groups. On the other hand, significant interactions between Food and Treatment were shown in the content of DOPAC and the turnover ratio of DA [$F_{(1, 42)}$, = 5.49, $p < 0.05$, or $F_{(1, 42)}$, = 6.83, $p < 0.05$, respectively]. A simple main effect of Treatment was significant in the pair-fed group [$F_{(1, 42)}$, = 5.72, $p < 0.05$, or $F_{(1, 42)}$, = 6.50, $p < 0.05$, respectively].

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Fig. 1.

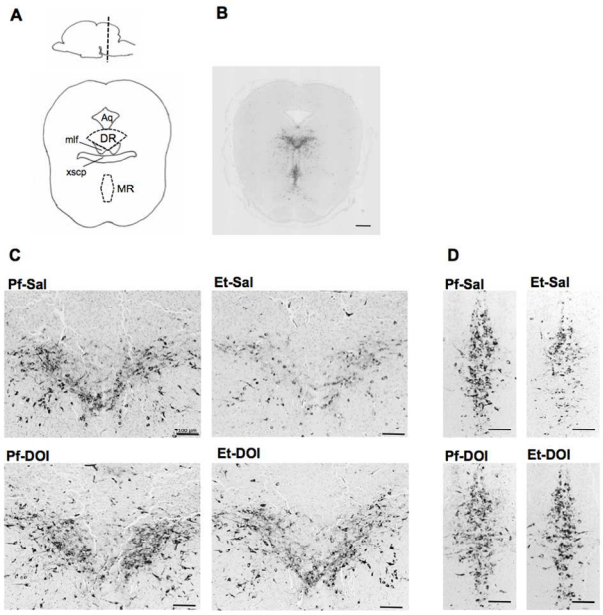


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Fig. 2

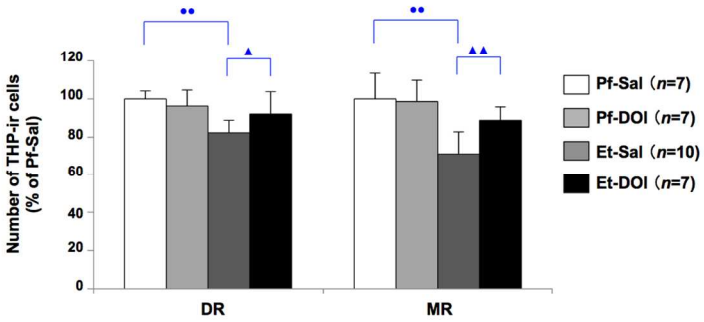


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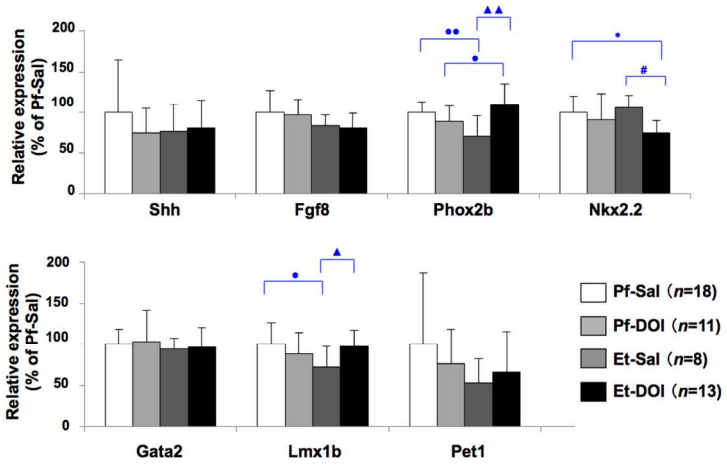
Fig. 3

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