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Embryonic exposure to ethanol disturbs regulation of mitotic spindle orientation via $GABA_A$ receptors in neural progenitors in ventricular zone of developing neocortex

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ABSTRACT

Neural progenitors in the ventricular zone of the developing neocortex divide oriented either parallel or perpendicular to the ventricular surface based on their mitotic spindle orientation. It has been shown that the cleavage plane orientation is developmentally regulated and plays a crucial role in cell fate determination of neural progenitors or the maintenance of the proliferative ventricular zone during neocortical development. We tested if fetal exposure to ethanol, the most widely used psychoactive agent and a potent teratogen that may cause malformation in the central nervous system, alters mitotic cleavage orientation of the neural progenitors at the apical surface of the ventricular zone in the developing neocortex. Fetal exposure to ethanol on E10.5 and 11.5 increased the occurrence frequency of a horizontal cleavage plane that is parallel to the ventricular surface on E 12.5. Administration of picrotoxin, a $GABA_A$ receptor antagonist, prior to ethanol administration canceled the effect of ethanol with the frequency of horizontal division similar to the control level, although picrotoxin itself did not show any effect on cleavage plane orientation. Phenobarbital, a $GABA_A$ receptor agonist, induced horizontal cleavage to an extent similar to that induced by ethanol administration. (+)MK801, an antagonist of NMDA receptor that is another major target of ethanol in neural cells, did not affect the cleavage plane of dividing progenitors. These results suggest that fetal ethanol exposure induced alterations in the cleavage plane orientation of neural progenitors in the ventricular zone of the neocortex via the enhancement of the function of $GABA_A$ receptors.

Normal development of the central nervous system (CNS) can be disturbed by alterations in the microenvironment of developing cells. Ethanol is not only the most widely used psychotropic agent, it is also a potent teratogenic substance. Ethanol consumption during pregnancy can lead to CNS malformations such as microcephaly, lissencephaly, cortical lamination defects and cortical ectopias [27]. Although the risk of fetal exposure to ethanol has attracted the attention of the public, the molecular mechanisms and the cell biological bases of the teratogenesis induced by ethanol still remain to be elucidated.

It has been shown that, in *Drosophila* neuroblasts, the regulation of mitotic spindle orientation plays a crucial role in determining the mode of cell division, symmetric or asymmetric [30]. Although in mammalian cortical neural progenitors, the importance of the orientation of mitosis in determining the modes of cell division is still controversial [30], several genes whose mutation is related to congenital malformations in the CNS are known to be implicated in the regulation of mitotic cleavage orientation. The knockdown of the abnormal spindle-like microcephaly-associated (ASPM) gene reduced the frequency of the cleavage plane of progenitors perpendicular to the ventricular surface (VS) [8]. It has been reported that mutations in the ASPM gene are the most common cause of human autosomal recessive primary microcephaly showing a small but otherwise grossly normal cerebral cortex associated with mental retardation [5]. Conditional knockout of mouse Lis1 gene in neuroepithelial progenitors induced the mitosis of the progenitors in which cleavage plane is parallel to the VS [34]. Deletion of Lis1 gene is a cause for Miller-Dieker lissencephaly [24]. In the developing cortex of the knockout mice of Nde1, a Lis1-interacting protein, the frequency of mitotic cells with cleavage plane perpendicular to the VS was decreased, which resulted in a microcephaly-like phenotype [7]. These show that the proper regulation of mitotic spindle orientation in the mitoses of neural progenitors is essential for normal development of mammalian neocortex. It can be hypothesized that ethanol perturbs the regulation of cleavage plane orientation of neural progenitors during neocortical development in the course of teratogenesis induced by embryonic exposure to ethanol. In this

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report, we verified this hypothesis and further examined if ethanol has its effects on the regulation of mitotic orientation of neural progenitors via $GABA_A$ receptors which is one of the major possible targets of ethanol in neural cells.

Embryonic stages were calculated with the day of the vaginal plug detection as embryonic day 0 (E0). On E10.5 and E11.5, timed-pregnant ICR mice (SLC Japan, Hamamatsu, Shizuoka, Japan) received two intraperitoneal (i.p.) injections of either 25% (v/v) ethanol in saline (2 g/kg body weight), 8 mg/ml phenobarbital in saline (80 mg/kg; Sigma, St. Louis, MO, USA), 0.5 mg/ml picrotoxin in saline (5 mg/kg; Sigma) or 0.05 mg/ml (+)MK801 hydrogen maleate in saline (0.5 mg/kg; Sigma) a day 4 h apart. Control pregnant mice received two i.p. injections of saline at the same time. 'Picrotoxin + ethanol'-treated mice received two i.p. injections of 2 g/kg ethanol a day 4 h apart as well as an i.p. injection of 5 mg/kg picrotoxin 30 min prior to each ethanol injection. Pregnant mice were killed by cervical dislocation on E12.5. The doses of the drugs administered to pregnant mice were chosen according to the previous studies so as not to induce a high incidence rate of embryo resorption and death [2,10,32]. The observed incidence rate of embryo resorption and death in our experiment was 2.3% (control), 15.4% (2 g/kg ethanol), 0% (80 mg/kg phenobarbital), 4.2% (5 mg/kg picrotoxin), 14.8% (5 mg/kg picrotoxin + 2 g/kg ethanol) and 6.5% (0.5 mg/kg MK801), respectively. After i.p. injection of pregnant mice with ethanol at 3.97 g/kg body weight, the blood ethanol concentration (BEC) reached over 400 mg/dl within 30 min after injection and started to decline at an approximate rate of 50 mg/dl/h [10]. Accordingly, it is expected that a single injection of 2 g/kg ethanol increases BEC to reach over 200 mg/dl, and ethanol remains in the blood for about 4 h in our protocol (embryos were exposed to ethanol 8–9 h totally a day). Healthy men given 1.6–2.0 g/kg ethanol exhibited a peak BEC of 232 ± 21 mg/dl [1]. When a man weighing 60 kg drinks approximately 1–1.25 bottles of 750 ml wine containing 13% alcohol, she or he ingests approximately 1.6–2.0 g/kg of ethanol. The concentration of ethanol that embryos are exposed to and the time of exposure in our experiments are within the possible range in human cases of prenatal exposure to ethanol. 5 mg/kg picrotoxin injection did not cause any apparent maternal convulsive symptoms. All animal manipulations were performed in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23) revised 1996, and the guidelines for animal experiments at the University of Tokushima Graduate School.

The heads of E12.5 embryos were cut and immersion-perfused in Bouin's fixative. The tissues were dehydrated in a graded ethanol series and embedded in paraffin. Serial coronal sections, 5-μm thick, were cut from the block samples and stained with hematoxylin. With each embryo, every 10th section between the sections positioned at the anterior and the posterior ends of the lateral ventricle was examined. All anaphase and telophase cells with determinable orientation of cleavage planes were identified at the apical surface of the ventricular zone of almost the entire neocortex (Fig. 1a) [13] and photographed using a Zeiss AxioImager.A1 AX10 equipped with a Nikon digital camera DXM1200F and an oil-immersion objective, ACHROPLAN 100X. The angle of the cleavage plane was measured on a printed photograph to one decimal place by drawing a line between the dividing chromatids and a reference line parallel to the VS (Fig. 1b and c). Results were statistically analyzed using Instat Ver. 3 (Graphpad Software, La Jolla, CA, USA) [31]. To compare the means of the samples, an unpaired two-tailed Mann–Whitney *U* test was applied. In all comparisons, $P < 0.05$ was considered to indicate statistical significance.

Neurogenesis starts to occur around at E10 in the rodent telencephalon [11]. It is expected that the change that occurs in the properties of neural progenitors in the early phase of neurogenesis can possibly cause the long-lasting (and severe) effect in the

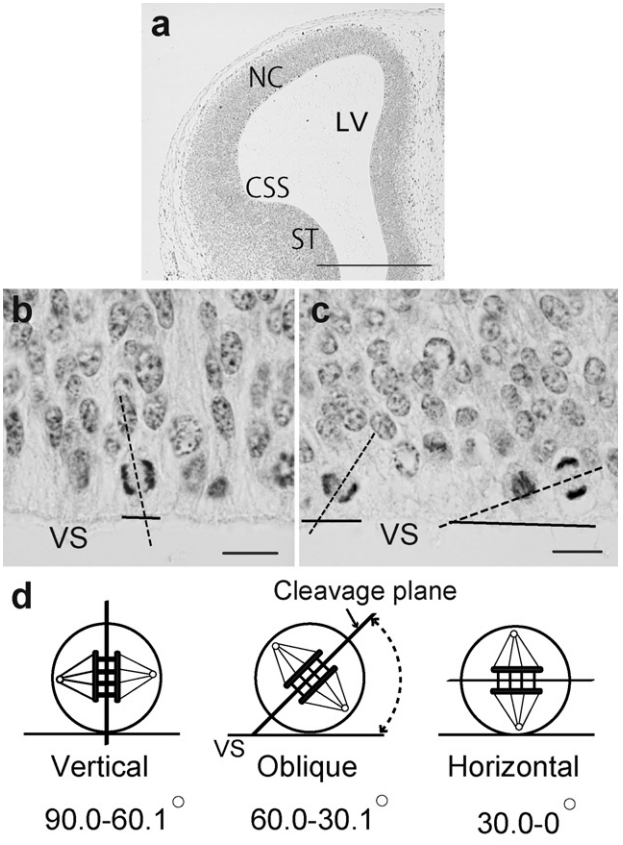

Fig. 1. Analysis of cleavage plane orientation of neural progenitors at apical surface of the ventricular zone (VZ) of the neocortex. (a) Micrograph showing neocortical hemisphere and lateral ventricle (LV) of an E12.5 embryo in the hematoxylin-stained section. The ventricular zone in the neocortex excluding the medial part of cortex, striatum and amygdala was examined in this study [13]. Bar, 0.5 mm. NC, neocortex; CSS, corticostriatal sulcus; ST, striatum; LV, lateral ventricle. (b and c) Micrographs showing mitotic cells at the apical surface of the VZ of neocortex. Bold lines indicate the reference lines parallel to the ventricular surface (VS). Dashed lines indicate the orientation of the dividing chromatids of the cells in anaphase. Bars in the bottom right corners, 10 μm. (d) Angle between the cleavage plane of the dividing neural progenitors and the VS (double-headed dashed arrow) was measured in the cleavage plane orientation analysis. Dividing progenitors were classified into three groups according to the angle of cleavage plane to the VS: Vertical (90–60.1°), Oblique (60.0–30.1°), and Horizontal (30.0–0°).

course of neocortical development. To test if fetal ethanol exposure affects the regulation of mitotic spindle orientation of dividing progenitors at the apical surface of the ventricular zone (VZ) in the early phase of neurogenesis, we administered ethanol to pregnant mice on E10.5 and E11.5 and measured the angles of the cell cleavage plane of the dividing progenitors against the ventricular surface in the control and ethanol-exposed neocortices on E12.5 (Fig. 1a, b and c). We classified three alternative orientations of the cleavage planes with reference to VS, including vertical (90.0–60.1°), oblique (60.0–30.1°) and horizontal (30.0–0°) (Fig. 1d) according to the previous studies [6,23]. This analysis revealed that fetal exposure to ethanol on E10.5 and 11.5 altered the orientation of the cleavage plane on E12.5. In the ethanol-exposed embryos, the frequency of the dividing progenitors with a cleavage plane vertical to VS decreased from 67.8% to 54.9% ($P < 0.02$), while the progenitors with a horizontal cleavage plane significantly increased from 15.2% to 24.3% ($P < 0.02$) (Fig. 2). The proportion of cells with an oblique cleavage plane increased from 17.0% to 20.8%, but statistical significance was not attained ($P = 0.0649$). Detailed analysis of the cleavage plane angle showed the proportion of the cells with a cleavage angle of 0–40.0° was larger in the EtOH-exposed neocortex compared to the control (Supplementary figure), while there