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REGULAR PAPER

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Prenatal exposure to ethanol induces leptomeningeal heterotopia in the cerebral cortex of the rat fetus

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Abstract Pregnant rats were fed an ethanol-containing liquid diet between gestational day (GD) 10 and GD 21. Leptomeningeal heterotopias were observed in the cerebral cortex of ethanol-exposed fetuses. They appeared on the brain surface of the lateral cortical region near the rhinal fissure, and were found more numerously in the rostral than the caudal region. These abnormalities contained certain neuronal perikarya, microtubule-associated protein (MAP) 1b-positive neuronal processes, and Rat-401-positive radial glial fibers. Immunostaining for Rat-401 revealed that the heterotopias protruded through breaches in the glia limitans. In adult rats exposed to ethanol prenatally, the heterotopias persisted in the lateral cortical region. We conclude that prenatal exposure to ethanol might induce defects in the glia limitans, resulting in the genesis of leptomeningeal heterotopias. These abnormalities may be related to mental retardation or the cognitive deficits associated with human fetal alcohol syndrome (FAS).

Keywords Fetal alcohol syndrome · Glia limitans · Leptomeningeal heterotopia · Rat-401 · Microtubule-associated protein 1b

Introduction

Heavy ethanol consumption during pregnancy has teratogenic effects on fetuses and causes a cluster of symptoms termed the fetal alcohol syndrome (FAS) [2, 8]. One of the major signs of FAS is dysfunction of the central nervous system (CNS), i.e., aberrant neuronal and glial migration, altered formation of axonal and dendritic projections, and cortical dysgenesis [5, 6, 16]. Neuropathological examinations show that leptomeningeal heterotopias appear on the brain surface of the cerebral cortex in FAS children [3, 23]. The CNS dysfunction in FAS is involved in behavioral abnormalities such as mental retardation and cognitive deficits [2, 16].

Prenatal exposure to ethanol alters the morphology of the radial glia [5, 10] and delays the onset of expression of glial fibrillary acidic protein (GFAP) [10, 21]. In primary cultures, ethanol decreases DNA and RNA syntheses, GFAP expression, and levels of plasma membrane glycoproteins [6, 20]. Radial glial cells serve as guides to the migration of neurons in the developing CNS [14]. Several abnormalities in the radial glia, such as a microcavitation, involvement in rosettes, and disruption of attachment to the meningeal cells, are caused by prenatal treatment with ionizing irradiation [17, 19] or methylazoxymethanol [25], and are considered to be responsible for the genesis of neuroglial heterotopias.

In the present study, we successfully produced leptomeningeal heterotopias in the rat cerebral cortex after prenatal exposure to ethanol, and examined immunohistochemically the features of the glial elements in relation to the genesis of these heterotopias.

Materials and methods

Pregnant Sprague-Dawley rats supplied by Japan SLC were divided into three dietary groups on gestational day (GD) 10. Rats in the first group received a liquid diet (Oriental Yeast Co., Japan) containing 5% (w/v) ethanol ad libitum from 10:00 on GD 10 to 10:00 on GD 21 (n=6). Blood samples from the rats were collected on GD 21 and the ethanol concentrations were measured using a blood ethanol enzymatic assay kit (Sigma Chemical Co., USA). In the ethanol-exposed dams, the mean blood ethanol concentration was 147.5±41.7 mg/100 ml (mean ± SD). In the second group, rats were given the same liquid diet with the ethanol replaced by isocaloric sucrose, in an amount equivalent to that consumed by the ethanol group during the previous 24 h (n=5) (pair-fed). The third group of rats was given a commercial diet (NMF, Oriental Yeast Co., Japan) and tap water ad libitum (n=2). They served as intact controls for the effects of malnutrition imposed on the pair-fed rats due to their restricted diet.

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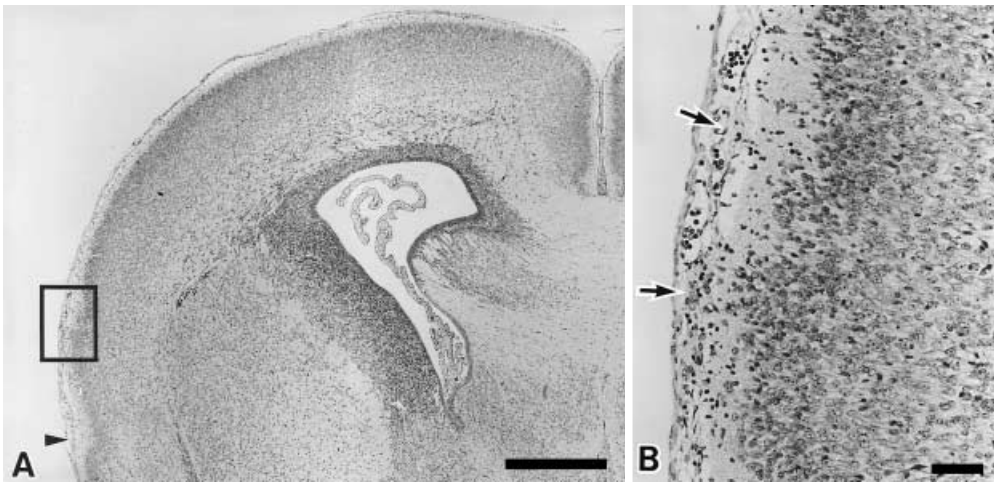

Fig. 1 A, B Frontal sections of the cerebral cortex of an ethanol-exposed fetus. **A** The laminar structure of the cerebral cortex is disrupted in the lateral cortical region. The *boxed area* is shown at higher magnification in **B**. An *arrowhead* indicates the rhinal fissure. **B** Leptomeningeal heterotopias protrude from the cerebral cortex (*arrows*). *Bars* **A** 500 μm, **B** 50 μm

Pregnant rats were killed on GD 21 under deep ether anesthesia. Fetuses were removed. Some pregnant rats were allowed to give birth and the offspring were killed at 7 weeks of age under sodium pentobarbital anesthesia. The brains were removed from the skull and were fixed in Bouin's fluid without acetic acid or 4% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4). They were weighed, dehydrated, embedded in paraffin, and serially sectioned in a frontal plane at 5 μm.

For histological observations, sections were stained with hematoxylin and eosin or cresyl violet. For immunohistochemical examinations, antibodies to microtubule-associated protein (MAP) 1b and Rat-401 (nestin) were used. MAP1b is a neuron-specific marker, and is expressed in the CNS during neonatal period [13, 15]. The Rat-401 is an intermediate filament protein which is found in radial glial cells [10]. Sections were irradiated with microwaves for 5 min in 10 mM citrate buffer (pH 6.0). Sections were then incubated with the anti-MAP1b antibody (1:20,000, Sigma) or anti-Rat-401 antibody (1:1,000, Developmental Studies Hybridoma Bank, the University of Iowa) overnight at 4°C. Immunoreactions were visualized using an immunoperoxidase method employing avidin-biotin-peroxidase complex (ABC elite kit, Vector).

Results

There was no difference in the brain weight between pair-fed (205±2 mg; n=15) (mean ± SD) and intact fetuses (207±2 mg; n=15) on GD 21. The weight in ethanol-exposed fetuses was 191±2 mg (n=21) on GD 21, significantly lower than in the pair-fed or intact fetuses (P<0.05, Duncan's multiple range test).

Laminations of the cortical plate were disrupted in the lateral region of the cerebral cortex near the rhinal fissure in ethanol-fed fetuses (Fig. 1A). Heterotopic cell masses protruded from the cerebral cortex on the brain surface in 66.7% (14/21) of the ethanol-exposed fetuses (Fig. 1B). No brain malformations were observed in the pair-fed controls.

Figure 2 shows the regions where each heterotopia appeared at various levels in the frontal sections of the forebrain. The heterotopias were bilaterally localized in the lateral cortical region throughout the entire cortex. They were more frequent at the rostral than the caudal level.

MAP1b-positive neuronal processes were seen in, and extended irregularly below, the heterotopias (Fig. 3B). The Rat-401 immunoreactivity was found in radial glial fibers extending straight and perpendicularly through the

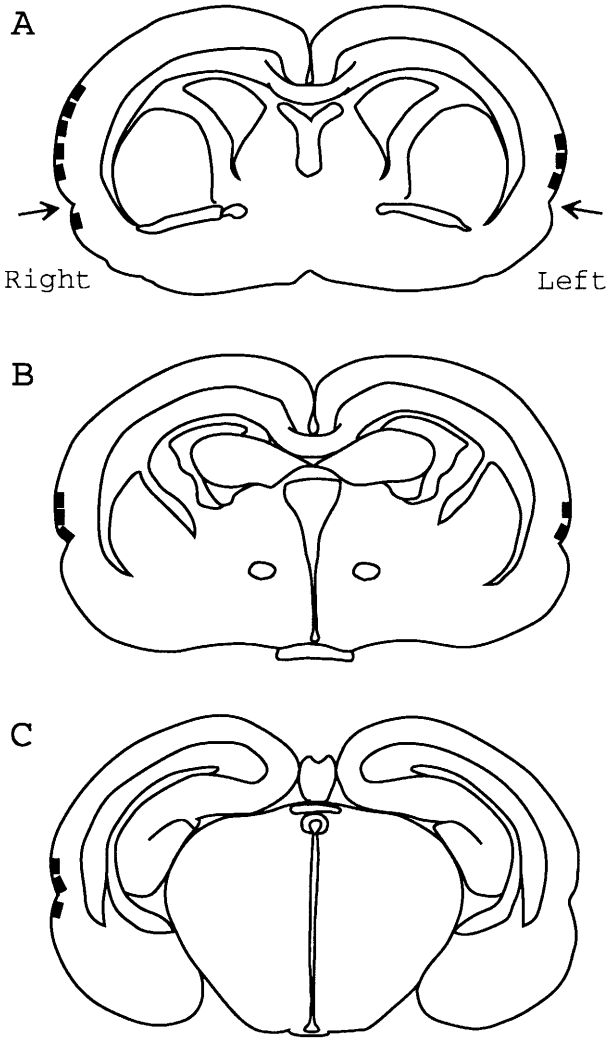

Fig. 2 Schematic drawings of frontal sections of the forebrain, from rostral (**A**) to caudal (**C**), representing the distribution of leptomeningeal heterotopias in an ethanol-exposed fetus. The leptomeningeal heterotopias are shown as *closed squares*. *Arrows* indicate the rhinal fissure

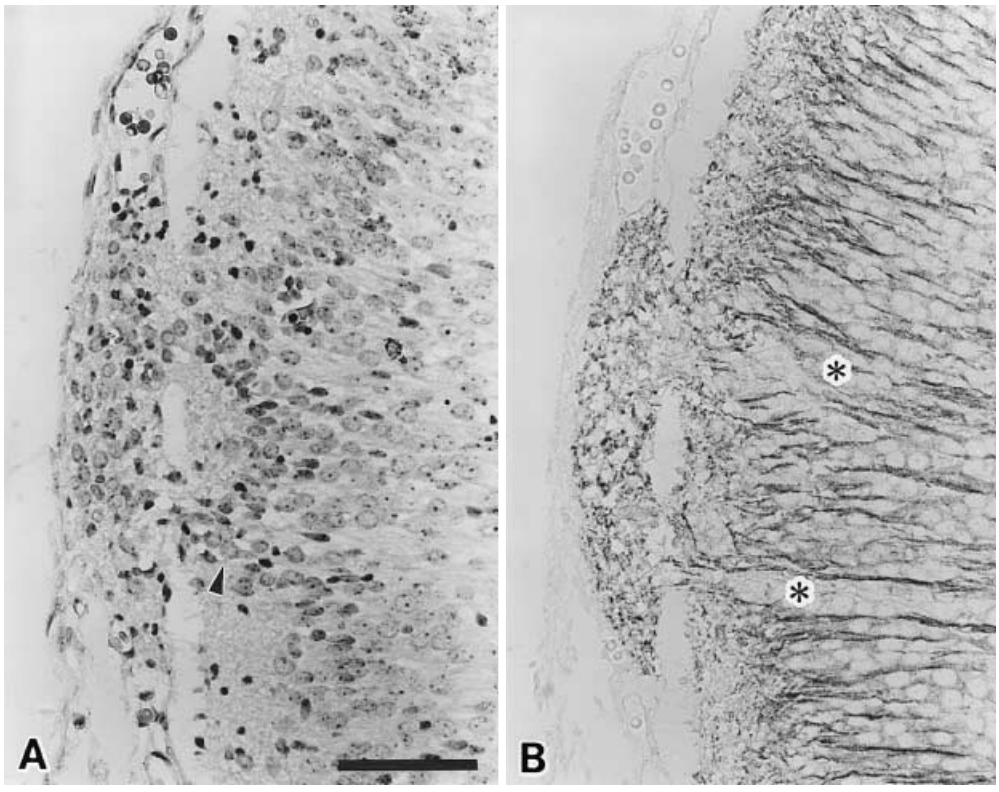

Fig. 3 A, B Consecutive sections of lateral cerebral cortex in an ethanol-exposed fetus. **A** A leptomeningeal heterotopia protrudes from the cerebral cortex; hematoxylin and eosin staining. **B** MAP1b-positive neuronal processes are observed in, and extended irregularly below, the heterotopias (*asterisks*). *Bar* 50 μm

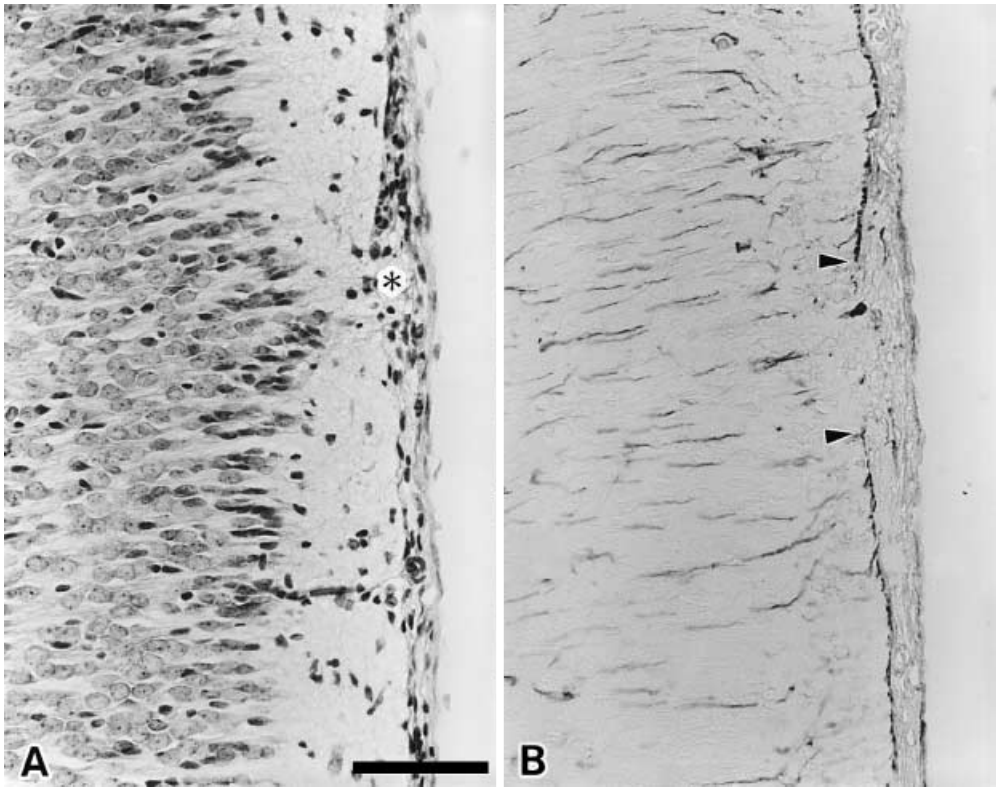

Fig. 4 A, B Consecutive frontal sections of lateral cerebral cortex in an ethanol-exposed fetus. **A** A leptomeningeal heterotopia protrudes from the cerebral cortex (*asterisk*); hematoxylin and eosin staining. **B** A breach in the Rat-401-positive glia limitans is observed in the region where the heterotopia protrudes (*arrowheads*). Rat-401-positive radial glial fibers are also found in the heterotopia. *Bar* 50 μm

cortex, and in the glia limitans as horizontal-oriented linear profiles at the brain surface (Fig. 4B). A breach in the Rat-401-positive glia limitans was observed in the region where the heterotopias protruded (Fig. 4B).

The leptomeningeal neuronal heterotopias persisted in the lateral cortical region even in adult rats exposed to ethanol prenatally (Fig. 5).

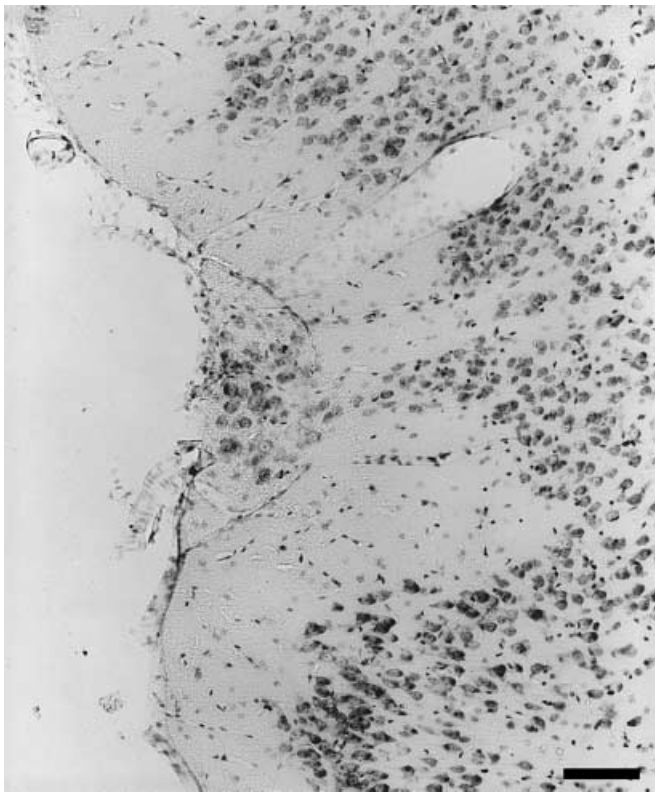

Fig. 5 A frontal section of the lateral cerebral cortex of a 7-week-old rat exposed to ethanol prenatally. A leptomeningeal heterotopia protrudes from the cerebral cortex. *Bar* 100 μm

Discussion

FAS children exhibit cerebral cortical dysplasia, including leptomeningeal heterotopias [3, 23]. In the present study, ectopic cell masses containing certain neuronal perikarya, MAP1b-positive neuronal processes and Rat-401-positive radial glial fibers protruded from the lateral regions of the cerebral cortex in ethanol-exposed rats. The evidence indicates that over-migration of neurons and glial cells results in leptomeningeal heterotopias.

The glia limitans of the brain surface plays a role in the arrest of neural migration in the developing brain [11]. Defects in the glia limitans caused by various insults result in the over-migration of neurons into extracortical areas [9, 22]. An immunohistochemical study with anti-Rat-401 antibody revealed that the leptomeningeal heterotopias protruded on the brain surface through breaches in the glia limitans in ethanol-exposed fetuses. These results suggest that defects in the glia limitans may be responsible for the genesis of leptomeningeal heterotopias.

Several researchers have reported that ethanol exposure alters normal development and delays the onset of GFAP expression in radial glial cells [10, 21]. The glia limitans is generated in a process through which the end-feet of radial glial fibers contact the meningeal cells [18]. In the present study, ethanol was administered to pregnant rats from GD 10 to GD 21. In this period, the genesis of the glia limitans may be retarded by the interference in the development of radial glia due to ethanol. Cortical neurons are generated with specific neurogenetic gradients: a transverse one from lateral to medial, and a longitudinal one from rostral to caudal [1]. Therefore, in ethanol-exposed fetuses, over-migration of cortical neurons may occur through breaches in the glia limitans in the rostrolateral regions of the cerebral cortex.

The present study demonstrated that leptomeningeal heterotopias persisted in the brain of adult rats exposed to ethanol prenatally. Leptomeningeal heterotopias have been observed in human neurological disorders such as dyslexia [4] and Fukuyama congenital muscular dystrophy [24]. These patients exhibit abnormal behavior, including mental retardation and cognitive deficits [7, 12]. Therefore, leptomeningeal heterotopias may be involved in the expression of abnormal behavior in FAS.

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