A selective loss of small-diameter myelinated optic nerve axons in rats prenatally exposed to ethanol

Kazuhiko Sawada¹, Hiromi Sakata-Haga¹, Suguru Komatsu¹, Kyoko Ohta¹, Young-Gil Jeong², and Yoshihiro Fukui¹
¹Department of Anatomy and Developmental Neurobiology, University of Tokushima School of Medicine, Tokushima, Japan, and ²Department of Anatomy, College of Medicine, Kongyang University, Nonsan, Chungnam, South Korea.

ABSTRACT Pregnant rats were fed an ethanol-containing liquid diet between gestational days 10 and 21. The optic nerves of their litters at 49 days of age were examined using quantitative stereological procedures. Cross-sectional areas of the optic nerve in ethanol-exposed rats were significantly smaller than those in controls. This was reflected in the reduced number of myelinated fibers, but not of non-myelinated fibers. The size distribution histogram indicated a decreased number of small axonal-diameter myelinated fibers in ethanol-exposed rats. The results suggested optic nerve hypoplasia in ethanol-exposed rats characterized by a selective loss of small-diameter myelinated fibers.

Key words: fetal alcohol syndrome, optic nerve, stereology, ethanol

INTRODUCTION Ethanol consumption during pregnancy has teratogenic effects on fetuses and causes a cluster of symptoms termed "fetal alcohol syndrome (FAS)" (Clarens et al., 1978; Jones and Smith, 1973). FAS children and its model animals show morphological abnormalities in the central nervous system (CNS), i.e. leptomeningeal heterotopias on the surface of cerebral cortex (Clarens et al., 1978; Wisniewski et al., 1983; Komatsu et al., 2001), dysgenesis of cerebral cortex (Sakata et al., 2002) and fusion of cerebellar folia (Sakata et al., 2001). Visual impairment is one of the common ophthalmic abnormalities in FAS, and involves optic nerve hypoplasia characterized by a reduction in cross-sectional area, a subnormal number of axons, and delayed myelination of axons (Phillips et al., 1991; Pinazo-Duran et al., 1993, 1997; Hellström et al., 1999; Harris et al., 2000).

Correspondence: Kazuhiko Sawada, Ph.D., Department of Anatomy and Developmental Neurobiology, University of Tokushima School of Medicine, 3-18-15 Kuramato, Tokushima 770-8503, Japan. E-mail: sawada@basic.med.tokushima-u.ac.jp

Received March 14, 2002; revised and accepted May 27, 2002.

MATERIALS AND METHODS Pregnant Sprague-Dawley rats purchased from Japan SLC were given a liquid diet (Oriental Yeast Co., Japan) containing either 5% (w/v) ethanol (n = 6) or with isocaloric sucrose (n = 5) (pair-fed) instead of ethanol from 10:00 a.m. gestational day (GD) 10 to 10:00 a.m. GD 21. The amount of liquid diet given pair-fed dams was determined on the basis of the consumption by ethanol-exposed dams during the previous 24 hours. On GD 21, all dams were given a commercial diet (NMF; Oriental Yeast Co.) and tap water ad libitum, allowed to give birth, and culled randomly to 8 pups. The pups were reared by their own dams, and weaned at 21 days of age.

Nine ethanol-exposed rats and eight pair-fed controls at 49 days of age were deeply anesthetized with an intraperitoneal injection of sodium pentobarbital (25 µg/10 g body weight), and were perfused with 0.9% NaCl followed by 3% glutaraldehyde in 0.1 M phosphate buffer (PB) (pH 7.4). Optic nerves situated between the posterior pole of the eyeball and the optic chiasma were dissected out, cut at approximately 2 mm in length, and fixed with the same fixative overnight. Following a rinse with 0.1 M PB, the tissues were postfixed with 0.5%
osmium tetroxide for an hour, dehydrated and embedded in Epon 812.

All animal procedures were performed according to the Guide for the Care and Use of Laboratory Animals, and were reviewed by the Institutional Animal Care and Use Committee of University of Tokushima. All efforts were made to mini-
mize the number of animals used and their suffering.

Sampling and subsequent stereological procedures have been described in a previous report (Fukui et al., 1991; Sawada et al., 2001), and were briefly summarized as follows. Transverse semithin sections (about 0.5 μm) and adjacent ultrathin sections (about 70 nm) were prepared by an LKB ultramicrotome. The semithin sections were stained with toluidine blue, photographed, and the cross-sectional area of each optic nerve was measured by an MC-300 image analyzer (FLOVEL, Tokyo). The adjacent ultrathin sections were stained with uranyl acetate and lead citrate, examined with a JOEL transmission electron microscope, and photographed. At least 20 microphotographs selected by a systematic random sampling procedure (Weibel, 1972) were obtained from a single section of the optic nerve. The thickness of the myelin sheaths of myelinated fibers and the mean axonal diameter of both myelinated and non-myelinated fibers were measured by an MC-300 image analyzer. Densities of myelinated and non-myelinated fibers were separately calculated by counting their numbers within the frame, and these values together with the cross-sectional area of each optic nerve (as determined above) were used to estimate the total number of

Fig. 1 Light micrograph of coronal section of the optic nerve in a 7-week-old rat prenatally exposed to ethanol. Bar = 100 μm.

Fig. 2 Electron micrograph of coronal section of the optic nerve in a 7-week-old rat prenatally exposed to ethanol. m: myelinated fiber, n: non-myelinated fiber, Bar = 1 μm.
Table 1 Cross-sectional area and estimated number of myelinated and non-myelinated fibers of the optic nerve in ethanol-exposed rats.

<table>
<thead>
<tr>
<th></th>
<th>Ethanol (n = 9)</th>
<th>Pair-fed (n = 8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cross-sectional area ($\mu m^2$)</td>
<td>$234048 \pm 15121^*$</td>
<td>$256163 \pm 21285$</td>
</tr>
<tr>
<td>Total number of optic nerve fibers</td>
<td>$134544 \pm 15914^*$</td>
<td>$153927 \pm 10636$</td>
</tr>
<tr>
<td>Number of myelinated fibers</td>
<td>$127950 \pm 16374^*$</td>
<td>$145734 \pm 10995$</td>
</tr>
<tr>
<td>Number of non-myelinated fibers</td>
<td>$6597 \pm 852$</td>
<td>$8193 \pm 2786$</td>
</tr>
</tbody>
</table>

Data are given as mean ± SD.
*: P < 0.05 vs pair-fed controls (Student's t-test)

Table 2 Mean diameter of myelinated and non-myelinated fibers of the optic nerve in ethanol-exposed rats.

<table>
<thead>
<tr>
<th></th>
<th>Ethanol (n = 9)</th>
<th>Pair-fed (n = 8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diameter of myelinated fibers ($\mu m$)</td>
<td>$1.16 \pm 0.06$</td>
<td>$1.10 \pm 0.05$</td>
</tr>
<tr>
<td>Axonal diameter ($\mu m$)</td>
<td>$0.82 \pm 0.05^*$</td>
<td>$0.77 \pm 0.04$</td>
</tr>
<tr>
<td>Myelin sheath thickness ($\mu m$)</td>
<td>$0.17 \pm 0.02$</td>
<td>$0.17 \pm 0.01$</td>
</tr>
<tr>
<td>Diameter of non-myelinated fibers ($\mu m$)</td>
<td>$0.48 \pm 0.07$</td>
<td>$0.44 \pm 0.08$</td>
</tr>
</tbody>
</table>

Data are given as mean ± SD.
*: P < 0.05 vs pair-fed controls (Student's t-test)

both fibers. All measurements were allowed within an oblong frame (4.5 x 2.5 $\mu m$) of each microphotograph according to the 'forbidden' line rule (Gundersen, 1977). Data were statistically analyzed by Student's t-test following one-way analysis of variance (ANOVA).

RESULTS

After birth, there was no difference in body weight between ethanol-exposed and pair-fed control rats (1 day of age: ethanol-exposed rats, 6.4 ± 0.6 g; pair-fed controls, 6.3 ± 0.4 g; 7 weeks of age: ethanol-exposed rats, 231.9 ± 43.7 g; pair-fed controls, 238.0 ± 53.8 g), indicating that ethanol-exposed rats did not experience undernutrition during postnatal development.

Figs. 1 and 2 show light and electron micrographs of transverse sections of the optic nerve in ethanol-exposed rats. Such micrographs were used to estimate the cross-sectional area of optic nerves, the total number and mean diameter of both myelinated and non-myelinated fibers, and the thickness of the myelin sheath.

Cross-sectional areas of the optic nerves in ethanol-exposed rats were significantly smaller than those in pair-fed controls (Table 1). In ethanol-exposed rats, the total number of the

![Fig. 3](image_url) Histogram for the distribution of axonal diameter of myelinated fibers of the optic nerve in 7-week-old ethanol-exposed rats (filled bars) and pair-fed rats (open bars). *: P < 0.05, **: P < 0.005 (Student's t-test).
optic nerve fibers (myelinated plus non-myelinated fibers) was significantly fewer than that in pair-fed controls (Table 1). This was reflected in a significantly lower number of myelinated fibers in ethanol-exposed rats. There was no difference in the number of non-myelinated fibers between either group of rats (Table 1).

Table 2 shows the mean diameter of both myelinated and non-myelinated fibers of the optic nerve. The mean diameter of non-myelinated fibers was not different between the two groups of rats. In contrast, the mean diameter of myelinated fibers was greater in ethanol-exposed rats than that in pair-fed controls. This increase was accompanied by an increasing diameter in the axons of myelinated fibers, but without any thickening of the myelin sheath (Table 2). The size distribution histogram of myelinated axons in ethanol-exposed rats showed a significantly lower number of small-diameter myelinated fibers (0.2 to 0.6 μm), indicating that the mean axonal diameter of myelinated fibers in ethanol-exposed rats was relatively increased by a loss of small-diameter axons (Fig. 3).

**DISCUSSION**

In the present study, cross-sectional areas of the optic nerves in ethanol-exposed rats were significantly smaller than those in pair-fed controls. This was reflected in the diminished number of myelinated fibers in the optic nerve. Similar results were observed in mice given a single intraperitoneal injection of ethanol on embryonic day 12 or 13 (Parson et al., 1995; Parson and Sojitra, 1995). A loss of retinal ganglion cells was observed in FAS children (Zeki et al., 1992) or chicks exposed to ethanol in ovo (Chmielewski et al., 1997). As retinal ganglion cells in rats are generated between embryonic days 14 to 17 (Reese and Colello, 1992), decreases in cross-sectional area and myelinated fiber numbers in the optic nerves of ethanol-exposed rats might indicate a loss of retinal ganglion cells.

The size distribution histogram in ethanol-exposed rats indicated a selective loss of small-diameter (0.2 to 0.6 μm) myelinated axons. The small diameter of the optic nerve axons originates from the small size of retinal ganglion cells (Perry, 1979), indicating that small retinal ganglion cells might be vulnerable to ethanol during their neurogenetic stage. Small retinal ganglion cells are known to project to the superior colliculus (Perry, 1979) and the suprachiasmatic nucleus (SCN) (Moore et al., 1995). Therefore, prenatal ethanol exposure might impair the development of retinocollicular and retinohypothalamic projections in the rat. The superior colliculus plays a role in visual locating functions such as orientation to visual stimuli, the direction of eye movements, avoidance reactions and locomotor exploration (Sefton and Lancaster, 1986). The SCN controls circadian and diurnal rhythms (Moore, 1983). However, functional alterations in the superior colliculus and the SCN have not been reported in FAS children or its model animals. Further study is required to examine in more detail the visual locating function and biological rhythmicity in ethanol-exposed rats.

In conclusion, prenatal ethanol exposure caused morphological abnormalities in the central nervous system (CNS), i.e. leptomeningeal heterotopias on the surface of cerebral cortex (Komatsu et al., 2001), dysgenesis of cerebral cortex (Sakata et al., 2002) and fusion of cerebellar folia (Sakata et al., 2001). In the present study, the small diameter of myelinated optic nerve axons was selectively reduced in rats prenatally exposed to ethanol, indicating impaired development of retinocollicular and retinohypothalamic projections. These findings might be implicated in CNS dysfunctions seen in FAS patients such as cognitive deficits, delayed motor development and visual impairments.

**ACKNOWLEDGMENTS**

This work was supported in part by Scientific Research Grants (09307018 and 12770532) from the Ministry of Education, Culture, Sports, Science and Technology, Japan. We wish to thank Mrs. S. Nitta for her expert technical assistance.

**REFERENCES**


Jones KL, Smith DW (1973) Recognition of the fetal alcohol...


