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RESEARCH NOTE

Ethanol-Induced Reductions in Cerebellar Growth of Infant Rats

CHRISTINA BAUER-MOFFETT AND JOSEPH ALTMAN [1]

Laboratory of Developmental Neurobiology, Department of Biological Sciences, Purdue University, West Lafayette, Indiana 47907
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Chronic ethanol consumption during gestation may adversely affect the developing human central nervous system (9, 10, 12). It is unknown, however, whether the pathologies result from circulating blood ethanol or from nutritional deficiencies and their consequences, since mothers bearing such children are generally malnourished. Morphological alterations in the developing cerebellar cortex produced by ethanol were investigated in the present study in infant rats during the period of cerebellar neurogenesis (1, 2). A vapor inhalation method was used which circumvents the damage caused to tissue when ethanol is administered by injection or the confounding factors of severe dehydration and malnutrition when mothers are forced to consume ethanol.

Purdue–Wistar rats, cross-fostered and raised eight to a litter, inhaled an air mixture calculated to contain 3.5–4% (v/v) ethanol during two 90-min daily sessions from 3 to 20 days. The vapor mixture, monitored by a BSV 640 Matheson flowmeter, was forced into 0.015 cu m transparent chambers, the volumes of which were exchanged each min. Blood ethanol concentrations averaged 268 mg/100 ml, as measured directly by gas chromatography from blood collected by cardiac puncture from unanesthetized rats at 5, 10, 15 and 20 days immediately after the second daily exposure to ethanol vapor. The animals were not unconscious at these blood levels; subjectively, however, younger animals appeared less active and older animals slightly ataxic immediately after ethanol treatment. Control rats were placed in identical chambers and similarly treated, but exposed only to air.

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Rats were weighed at 5, 10, 15, and 21 days. The two rats in each litter best approximating the median litter body weight were used for histological analyses; fresh liver, cerebellar, and remaining brain tissue weights were taken on the remaining litter-mates, killed by cardiac puncture following ether anesthesia. Although ethanol depressed body weights ($p < 0.001$) at 5 days, body weights at later ages were not significantly affected (Fig. 1). Livers from both groups of animals did not differ significantly in fresh weight at any age nor had those from ethanol-treated animals undergone any pathological alterations by 21 days of age. Lung samples from 21-day rats also appeared grossly and microscopically normal. In contrast, fresh weight data indicated developing brain tissue was highly sensitive to ethanol: Brain (minus cerebellar) tissue weights were reduced by ethanol significantly and approximately to the same extent at all ages (Fig. 1). Cerebellar weight reductions in 10, 15, and 21 day ethanol-treated animals were nearly twice that observed for remaining brain tissue (Fig. 1).

The reduction in cerebellar and in remaining brain weights of ethanol-treated animals cannot be attributed to undernutrition. The body weights of control and ethanol-treated animals generally did not differ significantly, and the work of Barnes and Altman (5) indicates that in order to produce a comparable cerebellar stunting by undernutrition the body weight had to be reduced by 42%. Further, daily injections of 10 μg thiamine hydrochloride, the minimum daily requirement for rats (7), did not increase body cerebellar, or remaining brain tissue weights of ethanol-treated animals, indicating that the observed effects cannot be attributed to thiamine deficiency, a state associated with some human neuropathologies (8, 11).

To determine whether reductions in dry weights paralleled wet weight decreases, cerebellar and remaining brain tissues from 42 control and 33 ethanol-treated animals were removed at 21 days, frozen in isopentane cooled to -50 C with solid carbon dioxide and acetone, and dried for 48 hr in a Thermovac lyophilizer cooled to -50 C and maintained at 100 Mtorr pressure. The reduction in dry weights of cerebellar and remaining brain tissue from ethanol-treated animals, 26.0 and 14.0%, respectively, approximately the wet weight reductions previously described (Fig. 1). We conclude, therefore, that the reductions in cerebellar and in remaining brain tissue weights represent alterations in development that are direct consequences of ethanol administration to developing rats.

Gross-morphologically, the rat cerebellum is nearly mature at 21 days (1–4). Therefore, an histological analysis was conducted at this age to determine which cortical layers and cell types were altered to account for the 26.0% weight reduction. The animals selected for histology were killed by cardiac perfusion with a 10% neutral buffered formalin, after which the brain was placed in Bouin's fixative 24 hr. Following several changes of

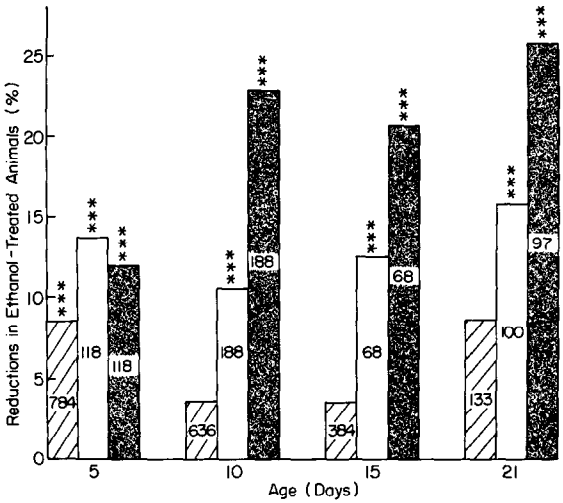

FIG. 1. Effects of ethanol inhalation on body, brain (minus cerebellum), and cerebellar wet weights at 21 days. Each bar represents the percentage reduction between mean ethanol-treated and control weights: ruled bars, body; open bars, brain (minus cerebellum); closed bars, cerebellum. Total numbers of control and ethanol-treated animals (used in approximately equal numbers) are given within each bar. *** $P <$ 0.001 (Student's t test).

formalin, the brains were blocked, dehydrated in a graded series of alcohol, and embedded in Tissue Prep (Fisher). Homologous, midsagittal, 6 μm, hematoxylin and eosin stained sections from 15 control and 15 ethanol-treated animals were enlarged 65× with a Zeiss micro-macro-projector; the boundaries of the cerebellar layers were then traced, and their areas measured with a planimeter. In the ethanol-treated animals the Medullary, granular, and molecular layers were reduced 34.0, 23.8, and 27.4%, respectively. Reductions in the latter two areas suggested reduced numbers of postnatally formed microneurons. But the areal reduction observed in the medullary layer, comprised of Purkinje cell axons, climbing and mossy fibers, suggested that ethanol affected prenatally as well as postnatally forming elements. The cerebellar weight reduction already apparent at 5 days of age (Fig. 1) also implied prenatally formed neurons were being affected by ethanol, since at this time very few microneurons have begun to differentiate in the rat cerebellum (1–4). A general effect on both prenatally and postnatally formed neurons was verified in a quantitative assessment of the numbers of Purkinje cells and granule cells in the sampled sections. For each cerebellum on which areal measurements were performed, all Purkinje cells in three serial sections having nucleoli and granule cells within a grid area of 0.024 mm^2, five samples/per lobule, were counted at 675×. These results (Fig. 2) sug-

gested that the numbers of postnatally forming granule cells may depend on the number of Purkinje cells surviving the toxic effects of ethanol. Although basket and stellate cell densities were not measured, the reduction in the molecular area of ethanol-treated animals, averaging 27.4%, suggested their cell populations were also affected by the number of surviving Purkinje cells.

The ethanol-induced reduction of cerebellar neurons thus differs from that produced by infantile undernutrition which affects selectively the postnatally acquired cells (6). This consideration might suggest that the differential vulnerability of the cerebellar cortex is due to factors other than late maturation. However, a regional analysis of cell losses in different lobules of the vermis showed that the temporal order in the maturation of these lobules had a discernible effect. According to a previous autoradiographic study (2), lobules 1 to 4 in the anterior lobe and 9 and 10 in the posterior lobe are among the earlier maturing regions of the vermis, lobules 6 and 7 are the latest. The earliest maturing lobules, particularly those of the anterior lobe, showed greater reduction in Purkinje cells attributable to ethanol treatment than the late-maturing dorsal lobules (Fig. 2). The corresponding regional reduction in granule cells might be an indirect transneuronal effect, since granule cells (by the way of their axons, the parallel fibers) synapse extensively with Purkinje cells. But if the developing Purkinje cells are the primary targets of the toxic effects of ethanol, the greater resistance of the late-maturing Purkinje

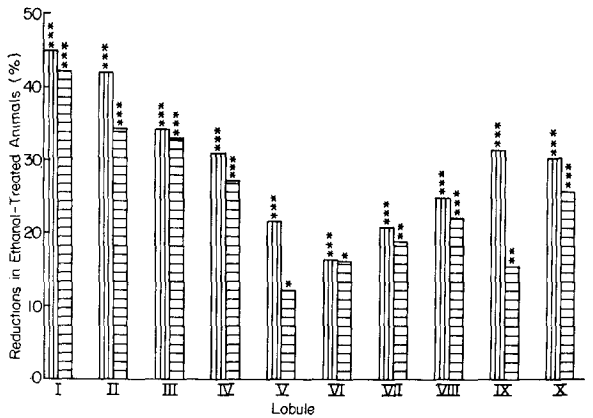

FIG. 2. Effects of ethanol inhalation on the total numbers of Purkinje and granule cells in each of the ten cerebellar lobules at 21 days. Each bar represents the percentage reduction between mean ethanol-treated and control total cell numbers: vertical rules, Purkinje cells; horizontal rules, granule cells. Fifteen control and 15 ethanol-treated animals were used. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$ (Student's t test).

cells must be due to a complex age-dependent interaction between alcohol levels in the blood and vulnerable periods in Purkinje cell development.

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