Effect of Perinatal Alcohol Exposure on Ibotenic Acid-Induced Excitotoxic Cortical Lesions in Newborn Hamsters

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ABSTRACT

Alcohol is one of the most common noxious substance to which fetuses are exposed. The aim of the study was to determine the effects of *in utero* alcohol exposure on excitotoxin-induced neuronal migration disorders. Female hamsters received alcohol (7%) for 3–5 mo or for the last 9–12 d of gestation. Alcohol diet was continued for 5 d during lactation in both groups. Drinking behavior was monitored. Peak plasma alcohol levels were 104 ± 12 mg/dL and 225 ± 6 mg/dL after 30 min for hamsters receiving an intragastric dose of 3 mL or 5 mL alcohol, respectively. At birth, pups received intrapallial injections ibotenic acid (1 ng, 100 ng, or 10 μ g). Histology and *N*-methyl-D-aspartic acid (NMDA) receptor labeling by ^3H -MK-801 in the pups cortices were studied. Short-term-alcohol-exposed pups had normal body and brain weights at birth, but their body growth was retarded postnatally. Ibotenic acid induced similar neuronal migration impairments in control and alcohol-exposed pups (nodular heterotopia in the white matter and/or deep cortical layers, subpial ectopia, and micro- or polymicrogyria). The size of lesions induced by 100 ng ibotenic acid was increased in alcohol-exposed pups; the 10 μ g dose was lethal. The density of ^3H -

MK-801 binding sites was similar in the three groups, indicating that exacerbated ibotenic acid excitotoxicity in alcohol-exposed pups did not result from increased NMDA receptor density. This study shows that alcohol exposure at levels that do not induce neuron migration disorders is sufficient to enhance the effects of the hypoxia-ischemia mimicking effects of ibotenic acid. (*Pediatr Res* 57: 287–293, 2005)

Abbreviations

ARND, alcohol-related neurodevelopmental disorders
FAS, fetal alcohol syndrome
GABA, gamma-aminobutyric acid
GFAP, glial fibrillary acidic protein
 ^3H -MK-801, tritiated dizocilpine
NeuN, neuronal nuclear antigen
NGF, nerve growth factor
NR2B, NMDA receptor subunit encoded by the GRIN-2B gene
NMDA, *N*-methyl-D-aspartic acid
PAF, platelet-activating factor

Alcohol is the main neurotoxin implicated in mental retardation in infancy. Maternal consumption of alcoholic beverages during pregnancy produces a broad spectrum of dose-dependent toxic effects in the fetus. Two main clinical variants have been described, namely, ARND and FAS (1–3).

Alcohol exposure in early gestation may cause alterations in embryogenesis such as incomplete neural tube closure, corpus callosum agenesis, and microcephalus (4,5). FAS is characterized by growth retardation, brain hypotrophy, and dysmorphol-

ogy, with or without mental retardation (6). Cognitive impairments without detectable dysmorphology may occur after mild alcohol exposure *in utero* (7). There is general agreement that there is no threshold for alcohol to have teratological effects (8). Migration disorders manifest as an arrest in neuroglial cell migration in the white matter or deep gray matter (heterotopia) (9) and loss of cortex laminar organization (10). The effects of alcohol on cerebral development vary with many factors, including plasma alcohol levels, the developmental stage of the fetus, the duration and frequency of alcohol intake, and the genetic background.

Alcohol affects glutamate NMDA and GABA-A receptor activities, reducing NMDA-evoked Ca^{2+} currents (11) and potentiating GABA-A Cl^- currents in adults (12). Neuronal migration is tightly controlled by NMDA receptors (13), and mice with NMDA receptor dysfunction exhibit severe neuronal

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migration abnormalities (14). Acute exposure to high-dose alcohol induces neuronal apoptosis in rat neonates through NMDA receptor inhibition and leads to loss of brain mass (15). Nevertheless, the neurodevelopmental damage caused by alcohol cannot be ascribed solely to effects on NMDA and GABA-A receptors. The impairment of neuronal migration, the corpus callosum agenesis, and the microcephaly observed in FAS probably result from effects on the glia (16). *In utero* alcohol exposure affects radial glia morphology (10,17), as well as GFAP expression by astrocytes (18).

In a previous study, we showed that ibotenic acid provoked neuronal migration disorders through NMDA activation (19). The heterotopia and subpial ectopias corresponding to migration beyond the normal target to the superficial cortical layer I were somehow reminiscent of those seen in FAS (20). Alcohol exposure was known to amplify NMDA receptor expression (21,22) and activity (23). Hence, the present study was designed to determine whether *in utero* alcohol exposure could be followed by increased sensitivity to NMDA receptor-mediated neuronal migration disorders.

MATERIALS AND METHODS

Animals. Golden hamsters (100–180 g) were purchased from Janvier (Le Genest, France) and bred in the laboratory. The animals had free access to food and water or alcohol solution and were subjected to a 12 h/12 h light/dark cycle. Males and females were housed together for 3 d. Nonalcohol-exposed pups were obtained from water-drinking dams ($n = 17$). One group of dams was subjected to short-term alcohol exposure started 5 d after removal of the males (on d 5–8 of pregnancy, which lasts 17 d in hamsters) and continued until the pups were killed on postnatal d 5 (P5). The alcohol was added to the dams' drinking water in a concentration of 7% (vol/vol) ($n = 36$), 10% ($n = 5$), or 12% ($n = 3$). In another group ($n = 9$), long-term alcohol exposure was obtained by giving female hamsters 7% alcohol in their water for at least 3 mo (up to 5 mo) before mating and subsequently until the pups were killed on P5. The excitotoxic effects of ibotenic acid (1 ng to 10 μ g) were studied in newborns from unexposed control dams, short-term-exposed dams, and long-term-exposed dams. Newborn were used within 6 h after birth, a period noted P0, then the age was counted as P1 to P5.

Drinking behavior and plasma alcohol kinetics. In eight animals, liquid intake was determined for up to 14 d using a monitoring device (Columbus Instruments, Columbus, OH) that recorded the number of laps per 10-min period. Two groups were studied successively in the four-compartment device, each composed of two water-drinking and two alcohol (7%) drinking animals. The liquid consumption per day was also recorded. In the animals given water with 7% alcohol, the maximum volume ingested in 10 min was 5.2 ± 0.7 mL. Therefore, we administered 3 mL or 5 mL of 7% alcohol by gastric gavage to measure plasma alcohol kinetics in separate nonpregnant females with a mean weight of 121 ± 9 g. Ketamine (Imalgene, Merial Laboratory, Lyon, France) 5 mg/kg was used to anesthetize the animals. Blood collected from the venous sinus of the eye after 15, 30, 60, 180 min, and 24 h was centrifuged, and the plasma was frozen until alcohol assay. In addition, 36 blood samples were collected using the same procedure from freely drinking females at various times during the day; 20 samples between 0700 and 1200 h; 16 samples between 1200 and 1600 h.

Alcohol assay. Alcohol levels in blood samples from freely drinking females or females given 7% alcohol by gastric gavage were assayed using the Emit II Plus device (Dade Behring, Paris, France), in the biochemistry department of the university hospital in Rouen, which is accredited to perform alcohol assays requested by the justice department. A pregnant female was given 5 mL of 7% alcohol by gastric gavage on E17 (full term). Thirty minutes later, alcohol levels were measured concomitantly in the dam's plasma and brain and in the brains and placentas of six fetuses. For tissue ethanol assays, brain tissues were homogenized by ultrasonication in saturated NaH_2PO_4 solution. After centrifugation, the underlying liquid phase was collected,

filtered, and frozen at -80°C until alcohol assay, which was done as for the blood samples.

Energy intake estimation. Liquid and dry food intakes were measured in 4 dams given water and in 12 dams given 7% alcohol in water. Dams were allowed free access to rodent laboratory chow for pregnant animals (11.6 kJ/g) (Special Diets Services, Vigny, France). The energy content of alcohol was considered to be 20 kJ/g. Daily chow intake was weighted for 4 d in 12 females exposed to alcohol for 7 d before and in 4 control dams.

Excitotoxic lesions. All protocols and procedures complied with the guidelines established by the Institut National de la Santé et de la Recherche Médicale (INSERM) for laboratory animals and were performed under the supervision of a licensed expert (P.L., license number: 76.A.16). Excitotoxic lesions were induced in pups from unexposed dams (controls), short-term-exposed dams, and long-term-exposed dams. The excitotoxic lesions were induced in the somatosensory cortex as previously described (19) by injecting ibotenic acid (1 ng to 10 μ g) (Sigma Chemical Co., L'Isles d'Abeau Chesnes, France) dissolved in 2 μ L of sterile PBS, pH 7.4, into the cortex, using a semistereotactic method, under isoflurane anesthesia. Ibotenic acid is a glutamatergic agonist at NMDA and metabotropic receptors, although its toxicity in neonatal hamster cortex is specifically blunted by the NMDA receptor antagonist AP7 (19). Ibotenic acid injection was performed within 6 h after birth and required separation from the dam for no longer than 10 min. The pups were killed on P5 and their brains were prepared for histology, as previously described (19). Excitotoxic lesions were identified by light microscope examination of cresyl violet-stained 10- μ m serial sections. Lesion size was estimated as the rostrocaudal length of the lesion (number of abnormal sections multiplied by 10 μ m). Microscopy was performed by an expert unaware of the group assignment of the animals.

Heterotopic neuronal cells were identified by immunohistochemical labeling of nuclear neuronal specific antigen (NeuN, Chemicon, Cergy, France). Sections were exposed to microwaves (10 min in 0.1 M citrate buffer, pH 6.0) then rinsed in 0.1 M PBS (pH 7.4) containing gelatin (2 g/L) and Triton $\times 100$ (0.4%) (blocking buffer). The sections were incubated with monoclonal anti-NeuN mouse Ig (50 μ g/mL) in blocking buffer for 1 h at room temperature and finally subjected to indirect immunoperoxidase staining using a biotinylated horse anti-mouse IgG (1.5 mg/mL for 45 min), streptavidin peroxidase (ABC kit, Vector, Abcys, Paris, France), and diaminobenzidine plus urea-peroxide (Sigma Chemical Co.) as the substrate. Controls were performed by omitting the primary antibody.

Density of ^3H -MK-801 binding sites. NMDA receptors were labeled in frozen brain sections from P0 pups and their dams, using ^3H -MK-801 binding as previously described (14). Binding was detected and quantified using a radio-imager (Biospace, Paris, France). ^3H -MK-801 concentrations in the media were 4.7 nM and 8.8 nM for adult and neonate sections, respectively. ^3H -MK-801 binding was measured in four or more brain sections in two to six animals per group. Sections were collected between the interhemispheric junction of anterior commissure and the rostral limit of hippocampus. In neonates, the binding was measured over whole brain sections whereas in adults it was measured selectively in the cortex.

Statistical analyses. Mean $\pm$ SE values of daily liquid volume intakes were compared using the student *t* test. One-way or two-way ANOVA were used to evaluate dose-dependent effects of ibotenic acid and/or alcohol exposure on lesion size, body weights, and brain weights on P5. Between-group comparisons of ibotenic acid effects were achieved using the Newman-Keuls test. The nonparametric Kruskal-Wallis test was used to compare ^3H -MK-801 binding-site densities in P0 brains and adult brains. The rates of occurrence of each lesion type were compared in pup groups from water- and alcohol-drinking dams using the two-sided Fisher exact test.

RESULTS

Drinking behavior. Female hamsters drank the alcohol-water mixtures readily, so that addition of a flavoring agent to mask the alcohol taste was unnecessary. The net spontaneous volume intake was 50% higher in the group receiving 7% alcohol than in the group with no alcohol. With the higher alcohol concentrations (10% and 12%), the animals drank smaller volumes, so that the intake of alcohol was similar to

that seen with the 7% concentration. The daily volume intake was significantly greater with long-term exposure (3–5 mo) than with short-term exposure to 7% alcohol (Table 1). Two patterns of drinking behavior over the 24-h cycle were seen, an episodic pattern (usually two to five episodes of drinking during the night), with intervals of abstinence, and a more regular pattern of drinking toward the end of the light period and during the dark period (Fig. 1). These two patterns were observed alternately in the same individuals, with free access to water or to 7% alcohol.

Plasma alcohol levels. Of 36 plasma alcohol assays done in samples from animals drinking 7% ethanol, 21 were below the detection limit (10 mg/dL), 13 showed low values (10–40 mg/dL), and 2 showed values >120 mg/dL. In animals given 3 mL or 5 mL of 7% alcohol by gastric gavage, the maximum concentration was observed after 30 min. In the group given 5 mL, plasma alcohol levels were around 200 mg/dL for 60 min and were still >100 mg/dL after 3 h. In the two animals given 3 mL of 7% alcohol, plasma alcohol levels expressed as the proportion of the volume of alcohol administered were lower at all time points than in the group given 5 mL of 7% alcohol. Plasma alcohol levels in the group given 3 mL were within the range of values observed in some spontaneously drinking animals, the other having probably not drank for several hours when blood sampling were done. Alcohol was undetectable in plasma 24 h after gavage (Table 2).

Thirty minutes after gavage, the plasma alcohol level in the pregnant female was 139 mg/dL; tissue levels were 692 ± 9 μ g/g in the dam's brain, 625 ± 32 μ g/g in the fetal brains, and 422 ± 36 μ g/g in the placentas, respectively.

Effect of alcohol exposure on energy intake and body weight at the end of gestation. We monitored the total energy intake in animals given short-term 7% alcohol and in controls. The short-term-exposed animals reduced their dry food consumption in proportion to the energy provided by the alcohol, so that their total energy intake was not statistically different from that in controls (Table 3). No difference was found in energy intake between pregnant and nonpregnant females (not shown). Alcohol intake affected neither the body weight increase in dams during the last 9–12 d of gestation nor the size of the litters (Table 3).

Effects of alcohol exposure on pup growth and brain weight. Pup birth weight was not affected by short-term alcohol exposure *in utero*. Conversely, a two-way ANOVA study shows that alcohol exposure and ibotenic acid (100 ng) injections affected postnatal growth very significantly [$F(1,50) = 32$; $p < 0.001$]. The posthoc Newman-Keuls test revealed that among pups given intracortical PBS on P0, those exposed to

Figure 1. Drinking behavior profile over two 24-h cycles in a female given 7% alcohol. (A) Episodic intake; (B) more regular moderate intake. The gray zone indicates the 12-h dark period.

Table 2. Plasma alcohol levels (mg/dL) in female hamsters given 7% alcohol by intragastric gavage (3 mL, 210 mg; or 5 mL, 350 mg)

Alcohol dose	15 min	30 min	60 min	180 min	24 h
210 mg (n = 2)	—	92–116	48–92	10–36	n.d.
350 mg (n = 4)	205 $\pm$ 38	225 $\pm$ 6	191 $\pm$ 23	134 $\pm$ 20	n.d.

n.d., Not detectable.

Table 3. Energy intake (kJ/d/100 g body weight), body weight, and litter size in dams given water or short-term 7% alcohol

Energy source	Water	Alcohol
Chow	82.5 $\pm$ 4.9	53.7 $\pm$ 4.0
Alcohol	—	21.1 $\pm$ 2.2
Total	82.5 $\pm$ 4.9 (n = 4)	74.8 $\pm$ 4.6 (n = 12)
Initial body weight	124 $\pm$ 9	123 $\pm$ 13
Final body weight	148 $\pm$ 8	147 $\pm$ 11
Litter size	6.2 $\pm$ 0.8 (n = 12)	5.9 $\pm$ 0.9 (n = 6)

alcohol had significantly lower body weights on P5 ($p < 0.001$), compared with pups not exposed to alcohol (Table 4). Intracortical ibotenic acid injection (100 ng) was also associated with growth retardation on P5 in litters from unexposed dams [$F(1,50) = 35$; $p < 0.001$] (Table 4). A significant interaction between alcohol exposure and ibotenic acid was

Table 1. Fluid intake (mL/d/100 g body weight) and alcohol consumption (g) in female hamsters given water, short-term alcohol, or long-term alcohol

	Water	Short-term alcohol 7%	Short-term alcohol 10%	Short-term alcohol 12%	Long-term alcohol 7%
Fluid intake (n)	11.9 $\pm$ 1.5 (10)	17.8 $\pm$ 1.1* (30)	14.5 $\pm$ 2.1 (5)	11.8 $\pm$ 0.6 (3)	23.0 $\pm$ 1.6**, [†] (8)
Alcohol intake (g)	—	1.00	1.16	1.14	1.29

n, number of females.

* $p < 0.01$; ** $p < 0.001$ compared with water; [†] $p < 0.02$ compared with short-term alcohol (7%) exposure.

Table 4. Effect of short-term 7% alcohol exposure in dams on P0 and P5 body weights (g) and on P5 brain weights (mg) of pups given intracortical PBS or Ibo (100 ng) at birth

	Water		Short-term alcohol 7%	
	PBS	Ibo	PBS	Ibo
Birth weight (g)	2.45 ± 0.9 (n = 13)	2.16 ± 0.08 (n = 10)	2.33 ± 0.06 (n = 15)	2.19 ± 0.51 (n = 19)
Body weight on P5 (g)	7.33 ± 0.10 (n = 13)	5.39 ± 0.13* (n = 10)	5.44 ± 0.35* (n = 13)	5.01 ± 0.12 (n = 17)
Brain weight on P5 (g)	206 ± 6 (n = 12)	194 ± 6 (n = 10)	219 ± 4 (n = 13)	205 ± 5 (n = 17)

* $p < 0.001$ vs PBS-injected pups born to dams given water.

observed [$F(1,50) = 14$; $p < 0.001$]. The posthoc Newman-Keuls comparison of ibotenic acid- or PBS-injected pups in alcohol exposed animals did not show an additive effect (Table 4).

Alcohol exposure did not result in reduced brain weight in animals injected with PBS. Conversely, the two-way ANOVA study indicated that brain weight was decreased in animals injected with 100 ng ibotenic acid [$F(1,48) = 5.5$; $p < 0.05$]. The effect did not reached statistical significance in either water or short-term-alcohol-exposed groups according to posthoc Newman-Keuls analysis. The brain weights were not diminished in proportion to body weight decreases in single experimental groups (Table 4). Long-term alcohol exposure of female hamsters caused dramatic alterations in fertility and maternal behavior. The consequence was that many pups from these females died before P5. Thus, we did not perform PBS injections in this group. No study on body weight and brain weight was performed on these animals.

Effects of alcohol exposure on ibotenic acid-induced brain lesions in pups at birth. Intrapallial PBS injection in P0 control hamsters caused minimal histologic alterations (20 μ m) in one of the seven injected pups. In pups from dams exposed to alcohol (7%), PBS injection on P0 caused lesions with a similar rate of occurrence (2/14 pups) and size ($30 \pm 10 \mu$ m) as in the controls.

Intrapallial ibotenic acid injection on P0 to pups from unexposed dams induced cytoarchitectural disorganization related to neuronal migration abnormalities, as previously described (19) (Fig. 2). Immunohistochemical labeling of NeuN or GFAP antigens in sections with deep heterotopia or subpial ectopia showed that cells in abnormal locations exhibited NeuN staining, although with variable intensity (Fig. 2, I and J). No typical GFAP-positive astrocytes were detected in heterotopic foci. Ibotenic acid-induced lesion types were similar in brains from pups born to unexposed and exposed (7% alcohol) dams. On P5, postmigratory neurons were found in the white matter or the deep cortical layers (heterotopia, Fig. 2, A–C) and in the superficial molecular layer in a subpial position (ectopia, Fig. 2, E and F). Abnormal gyri developed after ibotenic acid injection either when migrating neurons stopped in a deep heterotopic location (Fig. 2C) or when postmigratory neurons in layers V and VIa were destroyed (Fig. 2H). Cell loss was often noted near heterotopic foci and near gyri (Fig. 2, B, D, I, and J). Lesions reminiscent of polymicrogyria in humans were seen in association with hemisphere atrophy (Fig. 2, G and H).

The rates of occurrence of neuronal heterotopia, subpial ectopia, and microgyria were evaluated separately (Table 5). Two or three of these abnormalities were present in most

Figure 2. Cresyl violet staining (A, C, E, G) and NeuN immunohistology (B, D, F, H, I, J) of pup brain sections 5 d after intrapallial ibotenic acid (Ibo) injection. (A–F, I, J): pups from dams given water; G and H: pups from dams given short-term 7% alcohol. A, B: nodular neuronal heterotopia after 1 ng Ibo. C: nodular heterotopia with microgyria after 10 μ g Ibo. D: microgyria and subpial ectopia in the molecular layer after 1 ng Ibo. E: subpial ectopia after 100 ng Ibo. F: Subpial ectopia after 1 ng Ibo. G and H: polymicrogyria after 100 ng Ibo. I and J: enlargements of B and D. *Indicates nodular heterotopia; μ , microgyria; l, neuronal loss; arrows, subpial ectopia. Bar = 200 μ m in A–F, 360 μ m in G and H, and 50 μ m in I and J.

individuals, so that the sum of the occurrence rates exceeded 100% in all groups. Subpial ectopia was absent in pups born to unexposed dams and injected with 10 μ g of ibotenic acid

Table 5. Effect of intrapallial Ibo injection on P0 on the cortical cytoarchitecture alterations seen on P5 in pups from dams given water, short-term 7% alcohol, or long-term 7% alcohol

Alcohol (7%)	Ibo (N/n)	Nodular heterotopia	Subpial ectopia	Microgyria or polymicrogyria
0	1 ng (18/5)	56%	56%	66%
0	100 ng (14/6)	86%	86%	100%‡
0	10 µg (8/2)	100%	0*,†	100%
Short-term	1 ng (9/2)	56%	56%	33%
Short-term	100 ng (29/9)	72%	58%	90%§
Short-term	10 µg (14/3)		lethal (100%)	
Long-term	100 ng (7/3)	100%	71%	86%

(N/n) number of brains examined per group/number of litters.

* Significantly different from 1 ng ($p < 0.01$) Ibo in pups from dams given water.† Significantly different from 100 ng ($p < 0.001$) Ibo in pups from dams given water.‡ Significantly different from 1 ng ($p < 0.05$) Ibo in pups from dams given water.§ Significantly different from 1 ng ($p < 0.05$) Ibo in pups from dams given short-term 7% alcohol.

(Table 5). In contrast, the rate of neuronal heterotopia increased with the ibotenic acid dose in pups from unexposed and exposed dams, although the difference was not significant between 1 ng and 10 µg ibotenic acid in the unexposed groups ($p = 0.06$ by the Fisher two-sided test). In pups from dams chronically exposed to alcohol, deep heterotopia was consistently seen after injection of 100 ng ibotenic acid. The rate of microgyria increased significantly in relation to the ibotenic acid dose in both the unexposed and the exposed groups (Table 5). No effect of alcohol was observed on lesion patterns induced by 1 ng or 100 ng ibotenic acid, whereas the 10-µg dose was lethal in all the short-term-alcohol-exposed pups.

Conversely, alcohol affected lesion size (Fig. 3). In pups from unexposed dams, lesion size was dependent on the ibotenic acid dose [$F(2,44) = 5.1$; $p < 0.001$ by one-way ANOVA]. Injection of PBS seldom produced very small lesions, size <40 µm. Because all the pups from short-term-alcohol-exposed dams died after intrapallial injection of the 10 µg ibotenic acid dose ($n = 14$ from three different litters), a two-way ANOVA was done from the 0 to 100 ng ibotenic acid doses in unexposed and short-term-ethanol-exposed groups. The test revealed an interaction between the two parameters [$F(2,92) = 3.2$; $p = 0.046$]. The posthoc Newman-Keuls analysis revealed that short-term alcohol significantly enhanced the size of ibotenic acid-induced lesions at the 100 ng dose only (Fig. 3A). Lesion size in the water group injected with 10 µg ibotenic acid was 750 ± 78 µm ($n = 8$). The duration of dam exposure to alcohol significantly affected the effect of 100 ng ibotenic acid [$F(2,55) = 8.8$; $p < 0.001$ according to one-way ANOVA]. The posthoc Newman-Keuls analysis indicated that long-term exposure to alcohol was responsible for enhanced lesion size compared with unexposed and short-term-exposed groups (Fig. 3B).

Effects of alcohol exposure on ^3H -MK-801 binding-site density in P0 and adult hamster brains. NMDA-receptor labeling by ^3H -MK-801 revealed higher binding-site densities in adults than in neonates, although incubation was performed

Figure 3. Effect of intrapallial ibotenic acid (Ibo) injected to newborn (P0) hamsters (A), and effect of short-term (Alc. short) or long-term alcohol (Alc. long) on the size of lesions in newborn hamsters injected with 100 ng ibotenic acid (B). Brain lesions were evaluated on P5. Open bars: pups from unexposed dams; gray bars: pups from dams with short-term exposure to 7% alcohol; and black bar: pups from dams with long-term exposure to alcohol. Symbols indicate statistically significant differences between groups evaluated using the Newman-Keuls test. N/n over histograms indicate the number of brain examined per group and the number of litters of origin of these pups, respectively. * $p < 0.05$ and † $p < 0.001$ vs effect of 100 ng Ibo in pups from unexposed dams; ‡ $p < 0.001$ vs effect of 1 ng Ibo in pups from short-term-exposed dams; § $p < 0.05$ vs effect of 100 ng Ibo in pups from short-term-exposed dams. C, Controls.

with a lower radioligand concentration. Compared with controls from unexposed dams, ^3H -MK-801 binding-site density was 205% ($p < 0.05$) in brain sections from short-term-exposed dams and 167% in those from long-term-exposed dams (Fig. 4A). Conversely, ^3H -MK-801 binding-site densities were not significantly increased in pups from the dams exposed for short term or long term to alcohol (Fig. 4B).

Figure 4. Effect of short-term (*Alc. short*) or long-term (*Alc. long*) prepartum alcohol exposure of female hamsters on ³H-MK-801 binding-site density (median, Q1, Q3) in cerebral cortex and telencephalon frozen sections from the dams (A) and P0 pups (B). **p* < 0.05 according to the Kruskal-Wallis nonparametric test vs values in control females not exposed to alcohol and in their pups. C, Controls.

DISCUSSION

Neuronal migration is not yet completed in hamsters at birth. Therefore, exposure to excitotoxic insults on P0 impairs neuronal migration in the cortex in hamsters. Deep heterotopias are believed to result from premature cell migration arrest, whereas subpial ectopia result from migration beyond the normal target (19). In our study, these effects were strengthened when pups were antenatally exposed to alcohol. Many studies reported the effect of alcohol on the expression of the cell adhesion molecule N-CAM by neurons and consequences on neuron migration (24–27). Heterotopic clusters of neurons have been described in rats fed ethanol-containing diet resulting from altered cell cycle, *i.e.* decreased proliferating rate, prolonged sojourn of postmitotic neurons in the proliferation area, and delayed migration by several days (9,28). In mice, alcohol exposure induced a premature radial glia transformation and disorganized cortical neuronal pattern (10,17). In addition, neurons modify extracellular environment by degrading laminin (24). The latter effect is partly rescued by the NMDA receptor antagonist MK-801, indicating the involvement of glutamate NMDA receptor in neuronal migration. The LIS-1 gene associated with severely impaired neuronal migration and lissencephaly encodes the PAF acetyl-hydrolase that degrades PAF. This mediator plays a key role in neuronal migration since both over-activation of its receptors and disturbance of its synthesis results in neuronal migration disruption (14,29). This effect is concomitant to impaired NMDA receptor function (14). Altogether, these data indicate that alcohol interferes with many molecular events that direct neuronal migration, and in particular with NMDA receptor activity.

Most of the acquired cerebral lesions have multifactorial origin needing at least one acute insult and a favorable genetic and epigenetic background. In the present model, we found that alcohol significantly exacerbated NMDA receptor-mediated migration disorders after mild exposure, although alcohol 7% used alone during partial or full gestation period (and before) was not sufficient to mimic fetal alcohol-induced malformations observed in FAS at these doses and timing of exposition.

Moreover, brain weight was preserved when postnatal growth was impaired.

The plasma alcohol levels in animals given free access to a water-alcohol mixture, together with the level of alcohol in brain and the plasma kinetics in animals given alcohol by gastric gavage, indicate that fetal alcohol exposure was mild. When the dams drank episodically, their fetuses were exposed to alcohol peaks (100–200 mg/dL) for a few hours each day, during which brain alcohol concentrations reached 63 mg/dL (near 15 mM). These values were far below the toxicity thresholds associated with NMDA receptor-mediated apoptosis (>200 mg/dL for several hours) (15) or NR2B protein phosphorylation (>300 mg/dL) after acute alcohol administration (30). When the dams had a pattern of regular drinking, the fetuses were exposed to lower concentrations that appeared to undergo faster detoxification. As the dams drank little or not at all for about 12 h per 24-h cycle (Fig. 1), the fetuses had daily alcohol-free periods. All dams alternated between episodic and regular drinking, suggesting that our model may mimic the human behavior of regular alcohol intake with sporadic binge drinking. Our finding that exposed neonates had no brain damage or brain weight decrease yet showed increased sensitivity to ibotenic acid, suggests that our model may mimic conditions of alcohol exposure associated with ARND in humans (7). The absence of spontaneous dysmorphology, microcephaly, or low birth weight in newborns indicates that short-term alcohol exposure used was inappropriate to promote a FAS. Alcohol exposure modified tissue responses to high, but not to low doses of ibotenic acid, suggesting that different doses of ibotenic acid may recruit different effectors with different sensitivity to alcohol. Similar dose-dependent effects of ibotenic acid have been reported previously in P5 mice (31).

Chronic ethanol has been reported to enhance NMDA-receptor protein expression in adult rat cortex (21), as well as in the hippocampus of young rats (P14 and P28) exposed to alcohol *in utero* (22). We noted similar effects in the cortices of dams exposed to alcohol for 17 d but found no increase in ³H-MK-801 binding in the cortex of their pups on P0 (the age selected for the ibotenic acid injury). The difference of effects was not due to distinct bioavailability of alcohol, inasmuch as we observed the same brain concentration in the dam and its fetuses. A previous study showed that *in utero* and postnatal alcohol exposure in rats did not affect ³H-MK-801 affinity (22), suggesting that the absence of alterations in ³H-MK-801 binding-site density in our hamster model was indicative of an absence of alteration in NMDA receptor density.

Ibotenic acid (100 ng) produced larger brain lesions in pups from alcohol-exposed dams than in those from unexposed dams. The possibility that NMDA receptor activity was enhanced despite the absence of an increase in NMDA receptor density has previously been reported *in vitro* (23). Alternatively, the potentiating effect of alcohol exposure on ibotenic acid toxicity in newborns may result from alcohol effects on their dams. The enhancement of ibotenic acid toxicity in pups from chronically alcohol drinking females indicates that chronic alcohol modifies the physiology of pregnancy that in turn result in enhanced vulnerability of pups brains to exogenous injury (*i.e.*, hypoxia-ischemia). The decreased postnatal

growth in pups from short-term-alcohol-exposed dams was perhaps related to alcohol-induced changes in maternal behavior or milk production. Although alcohol exposure did not modify the energy intake of the dams, the protein intake was diminished by 35%. Protein deficiency could in part be responsible for the effects of alcohol exposure observed inasmuch as it has been shown to have effects on brain maturation in newborn rats (32). Alcohol exposure *in utero* or during lactation also affects folic acid intestinal absorption and reduces its concentration in milk (33). Reduced folic acid intake would be expected to alter NMDA-mediated neurotransmission *via* homocysteine accumulation (34). Potentiation of the effects of NMDA activation after *in utero* alcohol exposure may also be a consequence of fetal hypoxia and impaired antioxidant defenses (35).

In conclusion, alcohol exposure *in utero* was associated with exacerbated toxicity of ibotenic acid in newborn hamsters, indicating that alcohol exposure during development was deleterious even in the absence of brain malformation. Alcohol is one of the most widely abused substances in pregnant women. This study suggests that even modest alcohol levels may increase the vulnerability of the fetal cortex to the large variety of agents capable of triggering an excitotoxic cascade (36). The absence of effect of such exposure on NMDA receptor density lets unsolved the question of the biochemical targets of alcohol in the dam/fetus-neonate couple.

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