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EFFECTS OF PRENATAL ETHANOL EXPOSURE ON PHYSICAL GROWTH, SENSORY REFLEX MATURATION AND BRAIN DEVELOPMENT IN THE RAT

D. LOPEZ-TEJERO*, I. FERRER†, M. LLOBERA * AND E. HERRERA‡

**Catedra de Fisiología General, Facultad de Biología, Universidad de Barcelona, †Unidad de Neuropatología, Departamento de Anatomía Patológica, Hospital Príncipes de España. Hospitalet de LLobregat and ‡Servicio de Bioquiímica, Departamento de Investigación, Centro Ramon y Cajal, Madrid*

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Effects of prenatal ethanol exposure on physical growths, sensory reflex maturation and brain development in the rat

In the offspring of ethanol-treated rats during gestation (25% ethanol in drinking water) decreased litter size, increased postnatal mortality rate, reduced body weight and body size, delayed ear opening, eyelid opening and teeth eruption, retarded air righting reflex acquisition, impaired brain growth, reduced cortical thickness and delayed maturation of layer Vth's pyramidal neurons: (a) reduced basilar dendritic arborization and (b) decreased number of spines in the apical dendrite, were observed when compared with age-matched controls fed with a standard diet. Minimal effects were found in the offspring of fibre-treated rats during gestation (standard diet mixed with cellulose) in which the body weight was similar to that of controls, although both the calorific intake from food and the mother's weight gain during pregnancy were similar to those of the ethanol-treated group. All these abnormal parameters became normal at the end of the first month of postnatal life, indicating recovery of these developmental defects produced by prenatal ethanol consumption.

Introduction

It is well known that ethanol abuse during gestation may produce a wide range of deleterious effects on the development of the offspring in human and other mammals (Jones *et al.*, 1973; Veghelyi *et al.*, 1978; Rasmussen &

Address for Correspondence: Dr I. Ferrer, Unidad de Neuropatología, Depto. Anatomía Patológica, Hospital Príncipes de España, Hospitalet de LLobregat, Spain.

Christensen, 1980; Streissguth *et al.*, 1980; Colangelo & Jones, 1982; Wisniewski *et al.*, 1983; Sulik, Lauder & Dehart, 1984). Morphological studies in the rodent brain showed developmental abnormalities in the cerebral cortex after ethanol exposure in early life (Hammer & Scheibel, 1981; Reyes *et al.*, 1983; Stoltenburg-Didinger & Spohr, 1983; Pentney, Cotter & Abel, 1984; Schapiro, Rosman & Kemper, 1984; Fabregues *et al.*, 1985). However, long term investigations have failed to establish clear agreement as to the degree of recovery of damaged pyramidal cells of the cerebral cortex.

In this study, we have sequentially examined the effects of prenatal ethanol exposure on the development of the cerebral cortex in the rat and more especially on the maturation of the Vth layer's pyramidal cells.

We also studied other physical and functional parameters of maturation to obtain a wide perspective of the development of rats exposed to prenatal ethanol consumption.

Material and methods

Animals

Female Wistar rats weighing 150±3 g from our own colony were kept three per cage under automatically controlled humidity (65±5%), temperature (22–23°C) and 12 h light-dark cycles. They were divided into three groups and treated according to the following schedules (Testar *et al.*, 1986).

1. Ethanol-treated rats receiving *ad libitum* purina rat chow and 10, 15, 20 and 25% (v/v) ethanol in drinking water on successive weeks before pregnancy. At the end of the week 4 the animals were mated with normal males and were kept on 25% ethanol in drinking water until delivery. This dose of ethanol represented 30–35% of the whole calorific intake. The alcoholic diet was isocalorific with the control diet throughout the period studied.

2. Fibre-treated rats receiving a diet containing 50% cellulose *ad libitum*. Total calorific intake in these rats was reduced to 75% when compared to age-matched controls, but the calorific intake from food and the weight gain of the dams during pregnancy were similar to those of the ethanol-treated group.

3. Control rats receiving a standard diet *ad libitum*. Immediately after delivery the pups were removed from their mothers and individually identified. At birth the number of pups per mother was adjusted to 8–10 to obtain the optimal litter size so that each pup would have access to the same amount of milk per day (Babicky *et al.*, 1983; Yagil, Etzion & Berlyne, 1976).

Lactation in the three groups was done by normal nurses of similar body weight who had given birth 6 h before the experimental groups.

Seven to nine litters from each group were used in the present study.

Postnatal maturation and development of sensory reflexes

After spontaneous delivery the mortality of the pups was evaluated over the next days as the percentage of deaths per litter. Death of the animals was not related to infanticide or cannibalism. The body weight and the body size (crown-rump length) were measured during the first month of life. Teeth eruption, opening of the eyelids, opening of the ear and acquisition of several sensory reflexes including the surface righting reflex, the air righting reflex and the acquisition of the olfactory sense (Grawiler & Leist, 1977; Lapointe & Nosal,

1979; Adams & Buelke-Sam, 1981; Vetula-Gallo & Weirberg, 1982) were tested on the appropriate days until the age of 20 days. Results were expressed as the cumulative percentage of pups per litter attaining mature responses.

Results were statistically processed with the Student's *t*-test or with the Mann-Whitney *U*-test.

Brain weight and morphological studies of the cerebral cortex

Pups aged 1, 4, 15 and 30 days old were killed and the brains rapidly removed and weighed fresh. Immediately the brains were fixed for histological studies or according to Golgi's rapid method.

For histological studies the brains were fixed in 1% glutaraldehyde-2·5% paraformaldehyde in phosphate buffer (pH 7·4) for 2 weeks, and then embedded in paraffin. Sections 10 μm thick were stained with cresyl violet and the thickness of the sensorimotor cortex was measured with a calibrated ocular micrometer.

For Golgi studies (Ferrer *et al.*, 1984) fresh slices of the sensorimotor cortex 3 mm thick were fixed in 3% potassium-bichromate–1% osmium tetroxide (20/5; v/v) for 3–5 days; after this time the blocks were briefly washed in 0·75% silver nitrate and immersed in fresh 0·75% silver nitrate solution for 48 h. The samples were dehydrated in ethanol, 'shelled' in paraffin and sectioned at 75–100 μm.

The pyramidal cells of the Vth cortical layer were selected for quantitative morphological studies. The number of primary basilar dendrites and the number of ramification points in concentric rings around the cell body with a radial increment of 25 μm were evaluated in rats aged 4, 15 and 30 days old. The total number of dendritic spines in the proximal 300 μm segment of the apical dendrite was measured in rats 15 and 30 days old.

Results were statistically processed with the Student's *t*-test.

Results

Number of pups at birth

The size of the litters in the ethanol and in the fibre-treated groups was smaller than that in the control group. The mean value in the latter was 11·26 ($\pm$0·49) pups per litter and 8·32 ($\pm$0·48) in the ethanol-treated and 8·82 ($\pm$0·49) in the fibre treated groups ($P < 0·001$; Student's *t*-test).

Postnatal mortality

Within the first 24 h after delivery a high per cent mortality was observed in the ethanol-treated group (23·2$\pm$5·17%) when compared to the fibre-treated group (0·48$\pm$0·48%) and to controls (0·38$\pm$0·22%). This significant mortality ($P < 0·001$; Student's *t*-test) in the ethanol-treated group was related to neither the size nor the weight of the pups. After the first postnatal day the rate of mortality was irrevelant in the three groups.

Body weight and body size

As shown in Table 1, pups born of ethanol-treated mothers were smaller in size and weighed less than those delivered from fibre-treated dams and from

Table 1. Body weight, body size and brain weight during postnatal days.

Days	*0*	*1*	*4*	*15*	*30*
Body weight (g)					
C	5·60 ± 0·09	6·38 ± 0·22	10·47 ± 0·32	27·22 ± 1·12	91·66 ± 2·23
E	5·09 ± 0·10†	5·81 ± 0·19‖	9·15 ± 0·46*	27·23 ± 1·33	91·75 ± 2·70
F	5·45 ± 0·16	6·30 ± 0·28	9·89 ± 0·59	29·45 ± 0·97	98·14 ± 2·19
Body size (cm)					
C	4·87 ± 0·09	5·05 ± 0·06	6·09 ± 0·09	8·66 ± 0·17	—
E	4·66 ± 0·04	4·65 ± 0·04‡¶	5·55 ± 0·06‡‖	8·06 ± 0·20*¶	—
F	4·86 ± 0·09	5·39 ± 0·10‡	6·07 ± 0·12	9·21 ± 0·14*	—
Brain weight (mg)					
C	245·24 ± 2·69	274·17 ± 5·93	465·89 ± 5·02	1254·67 ± 29·69	1618·11 ± 26·65
E	229·86 ± 3·33†	248·46 ± 5·37†§	415·20 ± 8·42‡	1207·56 ± 23·50	1561·61 ± 23·64
F	231·35 ± 5·28*	266·08 ± 4·80	435·92 ± 12·61*	1219·21 ± 31·79	1589·68 ± 22·14

C, offspring of control; E, ethanol treated; F, fibre treated- mothers.

All values are the mean ± SEM of 7–9 different litters. Comparisons have been made between $\frac{E}{F}$ versus C (*,†,‡) and E versus F (§,‖,¶). *, §, $P < 0.05$; †, ‖, $P < 0.01$; ‡, ¶, $P < 0.001$.

controls. Significant differences in body weight ($P < 0.05$; Student *t*-test), were observed until the 4th postnatal day, and in body size until the fifteenth day ($P < 0.001$ on days 1 and 4; $P < 0.05$ on day 15; Student's *t*-test).

Other parameters of physical maturation and the development of sensory reflexes

Teeth eruption, ear opening and opening of the eyelids were significantly retarded in pups from the ethanol-treated group when compared with age-matched controls ($P < 0.05$ for the teeth eruption and ear opening; $P < 0.01$ for the eyelid opening; Mann-Whitney *U*-test). Teeth eruption was also retarded in pups from the fibre-treated group when compared to controls ($P < 0.05$). No significant differences between the three groups were found when the acquisition of the olfactory sense and the surface righting reflex were tested. However, a significant delay in the development of the air-righting reflex was found in pups from ethanol-treated dams when compared with age-matched controls ($P < 0.01$; Mann-Whitney *U*-test) (Figure 1).

Brain weight

As shown in Table 1, the weight of the brain was less in the ethanol-treated group compared to controls until the 4th postnatal day ($P < 0.001$; Student's *t*-test). A lesser reduction in brain weight was also observed in pups from fibre-treated dams relative to controls ($P < 0.05$) at the age of 4 days.

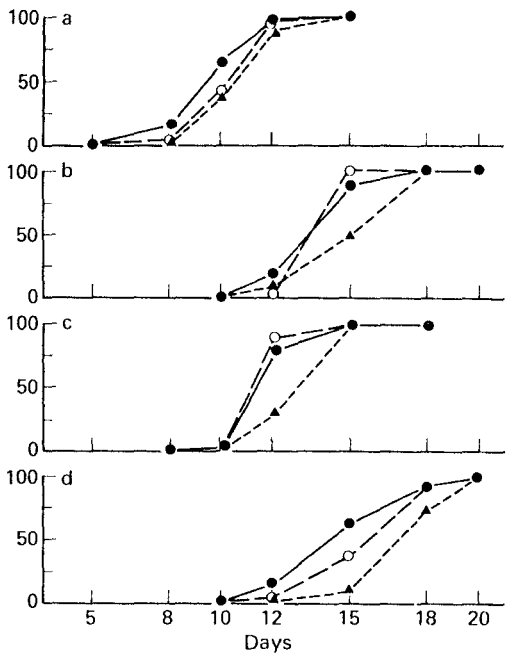

Figure 1. Accumulative percentage of pups per litter attaining mature acquisition for teeth eruption, ear and eyelid opening; and positive response for air righting reflex, during postnatal days. **a,** teeth eruption; **b,** eyelid opening; **c,** ear opening; **d,** air righting reflex. (●) Offspring from control mothers (C); (▲) offspring from ethanol-treated mothers (E); (○) offspring from fibre-treated mothers (F). Comparisons are versus C, with Mann-Whitney *U*-test (* = $P < 0.05$; † = $P < 0.01$); teeth eruption, E versus C(*) and F versus C(*); eyelid opening, E versus C(†); ear opening, E versus C(*); air righting reflex, E versus C(†).

Cortical thickness

No differences were observed at birth, but a reduced cortical thickness was found in the ethanol-treated group when compared with the fibre treated group and controls at the age of 4 days ($P < 0.001$; Student's *t*-test). Smaller differences ($P < 0.05$) were found at the age of 15 days, but no difference was noted at the end of the first month (Table 2).

Basilar dendritic arborization of the Vth layer's pyramidal neurons

No differences were observed between fibre-treated and control animals at any of the ages studied. However, as depicted in table III, a significant reduction ($P < 0.001$; Student *t*-test) in the number of primary basilar dendrites and in the number of dendritic ramification points at 25 and 50 μm

Table 2. Cortical thickness in microns at different postnatal ages

Days	*Controls*	*Ethanol*	*Fibre-treated*
1	610·4 ± 8·7	607·6 ± 6·4	608·5 ± 9·5
4	826·5 ± 12·5	647·9 ± 2·4†	815·3 ± 10·3
15	1605·5 ± 22·6	1525·7 ± 18·0*	1636·8 ± 38·5
30	1690·6 ± 18·4	1685·7 ± 15·8	1693·5 ± 23·6

Result expressed $n = 28$; *$P < 0.05$; †$P < 0.001$; as mean ± SD. Student's *t*-test.

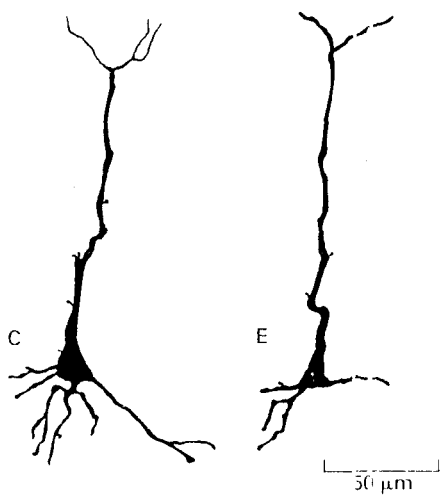

Figure 2. Camera lucida drawings of Golgi impregnated Vth layer pyramidal neurons in 4-day old rats produced by mothers treated with ethanol during gestation (E), and controls (C).

from the soma were observed in ethanol-treated pups when compared to age-matched controls at the age of 4 days (Figure 2). These differences were no longer found at 15 and 30 days.

Dendritic spine numbers in the apical dendrite of Vth layer's pyramidal neurons

As shown in Table 4, 10% reduction in the number of dendritic spines was observed in ethanol-treated rats on postnatal day 15, when compared to age-matched controls ($P < 0{\cdot}001$; Student's t-test).

However, a significant increase was found in the number of dendritic spines in fibre-treated rats when compared to age-matched controls at the end of week 2 ($P < 0{\cdot}01$). These differences were no longer observed at the end of the 1st postnatal month.

Discussion

Our general results on the physical growth and maturation of the sensory reflexes have confirmed that under these experimental conditions, prenatal ethanol consumption may produce developmental defects in the offspring that include decrease in litter size, increased early postnatal mortality, reduced body weight and body size, as well as delay in teeth eruption, in the opening of the eyelids and in ear opening (Osborne, Caul & Fernandez, 1980; Abel, 1981; Cogan, Cohen & Sparkman, 1983; Fernandez *et al.*, 1983; Abel, Jacobson & Sherwin, 1983). Delayed air-righting reflex maturation has also been observed in the offspring of ethanol-treated mothers. Sequential studies of brain development showed a delay in the brain weight gain, reduced cortical

Table 3: Number of basilar dendritic ramification points

		Control	*Ethanol*	*Fibre treated*
	B	3·80 ± 0·08	2·80 ± 0·09*	3·70 ± 0·09
4 days	25 μm	2·70 ± 0·07	1·90 ± 0·60*	2·60 ± 0·06
	50 μm	1·85 ± 0·15	0·54 ± 0·06*	1·75 ± 0·18
	B	3·86 ± 0·15	3·87 ± 0·17	3·87 ± 0·18
	25 μm	4·97 ± 0·27	4·45 ± 0·27	4·89 ± 0·29
	50 μm	3·95 ± 0·28	4·15 ± 0·23	4·08 ± 0·31
15 days	75 μm	1·06 ± 0·15	1·00 ± 0·14	1·08 ± 0·16
	100 μm	0·06 ± 0·03	0·11 ± 0·05	0·10 ± 0·06
	B	3·28 ± 0·12	3·59 ± 0·12	3·48 ± 0·14
	25 μm	5·02 ± 0·21	4·88 ± 0·21	4·92 ± 0·22
30 days	50 μm	4·24 ± 0·14	4·32 ± 0·12	4·30 ± 0·14
	75 μm	1·63 ± 0·11	1·61 ± 0·15	1·64 ± 0·10
	100 μm	0·20 ± 0·05	0·30 ± 0·07	0·32 ± 0·06

Results are expressed as mean ± standard deviation (B, primary basilar, and at 25–100 μm concentric rings from the soma) of Vth layer pyramidal neurons in the 4, 15 and 30-day-old rats. ($n = 100$ for each group; $P < 0·001$; *, Student's *t*-test).

thickness and impaired maturation of the Vth layer pyramidal cells, including the spread of the dendritic arborization and the number of dendritic spines; all these parameters, returned within normal values at the end of the 1st month of postnatal life. These results are in accordance with those reported by Pentney *et al.* (1984) in which no significant differences were observed in pyramidal cells of the cerebral cortex of rats aged 8–10 months (ethanol-treated versus control) in which ethanol was given only during gestation. On the contrary, 16% loss of dendritic spines in cortical pyramidal cells was observed by Reyes *et al.* (1983) in rats aged 30 days in which ethanol was given during gestation and lactation.

These discrepancies may be explained by the fact that ethanol consumption during lactation may produce additional negative effects on the development of the rat's cerebral cortex. A similar conclusion was obtained

Table 4. Dendritic spine counts in the apical dendrites of Vth layer pyramidal neurons

Days	*Control*	*Ethanol*	*Fibre-treated*
15	73·26 ± 0·84	66·55 ± 0·84*	77·15 ± 1·15†
30	156·42 ± 1·20	155·33 ± 1·28	155·84 ± 0·85

Results expressed as mean ± SD. $n = 88$; *$P < 0·001$; †$P < 0·01$; Student's *t*-test; *, ethanol versus control; †, fibre-treated versus control.

by Lancaster *et al.* (1984) for the myelination in the offspring of ethanol-treated rats, when *in utero* versus lactational exposure were compared.

Although the results obtained in rodents ought not be applied without reserve to human infants affected with the foetal alcohol syndrome (Pentney *et al.*, 1984), the recovery of certain morphological and behavioural parameters in the rat (Volk, 1977; Chan & Abel, 1982; Øisund, Fjorden & Mørland, 1978; Vetula Gallo & Weinberg, 1982; Stoltenburg-Didinger & Spohr, 1983; Ludeña *et al.*, 1983; Pentney *et al.*, 1984; Lancaster *et al.*, 1984; present results) may have valuable implications in human pathology, since some degree of recovery has also been observed in infants born from alcoholic mothers (Hanson, Jones & Smith, 1976; Santolaya *et al.*, 1978; Staisey & Fried, 1983).

As a complementary control we used a group which received a fibre diet during gestation. Some delay in the development of certain parameters (slight decrease in the brain weight, retarded teeth eruption, opening of the eyelids and air righting reflex acquisition) was observed in the offspring of this group. However, in contrast to ethanol-treated pups, the body weight in this group was similar to controls. Although the calorific intake by food in fibre-treated rats was similar to that of the ethanol-treated group and the weight gain of the mothers during pregnancy was similar to the latter, additional facts, i.e. inhibition of the placental uptake and transport of essential nutrients following ethanol exposure (Grace & Lin, 1981; Fisher *et al.*, 1981; Patwardhan *et al.*, 1981, Jones, Leichter & Lee, 1981; Henderson *et al.*, 1982) and placental transfer of ethanol (Dilts, 1970; Idänpään-Heikkilä *et al.*, 1971; Kaufman & Woollan, 1981; Mann *et al.*, 1975) may largely contribute to the misdevelopment of offspring from alcoholic mothers.

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