Effects of early postnatal ethanol exposure on the migration of rat cerebellar granule cells

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Abstract

Neuroblasts are differentiated from the external cell matrix in that they migrate out of the layer and accumulate in the internal surface of the external granular layer in order to form the external layer of the mantle. Exposure to ethanol in rats during pregnancy and early postnatal period is followed by selective loss of neuron cells in the areas where the cerebellum and hippocampus are formed. The aim of this work was to analyze the effects of alcohol on granular brain cells of rats aged 6 days old, with a survival time of 2 and 24 hours after administration of BrdU (5-Bromo-2'-desoxiuridina), a thymidine analog. Accordingly, three intraperitoneal (IP) injections of 20% ethanol (3 g kg of the body weight) were administered every five hours. Sixteen hours after the last ethanol injection, BrdU (60 $\mu g.kg^{-1}$) was administered intraperitoneally. The rats were sacrificed 2 or 24 hours after administration of BrdU. The brain system was taken apart, embedded in paraffin, and then cut into sections 5 μm thick. BrdU was subsequently detected immunohistochemically in sections of brain tissue. There was a notable decrease in the number of external granular cells in all groups that received ethanol, compared to the respective control groups. The groups that received alcohol presented a decrease in body weight compared to the respective control groups. The ethanol exposure period used in this study demonstrates a critical period in the development of the cerebellar cortex.

Keywords: cerebellar cortex, external granular layer.

1 Introduction

In order to form the cerebellar cortex, neuroblasts are differentiated from the external matrix layer. They migrate out of the layer and accumulate in the external granular layer of the internal half to form the external mantle layer (FUJITA, 1967). Then they migrate in the direction of the molecular and internal granular layers. The entire internal neuron population rises during the postnatal period in the external granular layer, between the first and eighteenth postnatal day, 95% of the cell prolifetate between the fourth and the fifteenth day (FUJITA, 1967). The external granular layer is a temporary structure that contributes to the formation of cell in the cerebellar cortex and finally disappears when the brain reaches maturity (RAMÓN and CAJAL, 1955).

Exposure to ethanol in rats during pregnancy and the early postnatal period results in selective neuronal cell loss in the realm of cerebellum and hippocampus formation (BONTHIUS and WEST, 1990; MARCUSSEN, GOODLETT, MAHONEY et al., 1994; MILLER, 1953). Changes induced by ethanol in the migration standard suggest that ethanol affects the cellular organization (ADDICKES and MOLLNER, 1986). Binge-like neonatal exposure to ethanol (EtOH) in rats, during the period of brain development comparable to that of the human third trimester, produces significant, dose-dependent Purkinje cell loss in the cerebellum and deficits in eyeblink classical conditioning (GREEN, TRAN, STEINMETZ, 2002). Silva et al. (2006) observed that the ethanol aguardente shown the deleterious effects into visual cortex structures during perinatal period.

In an auto-radiographic study, Bauer-Moffett and Altman (1977) observed a different neurogenesis pattern of granular cerebellum cells in rats treated with ethanol compared with the control group. Experimental rats showed a lower number of cells at all stages of development. The 20 to 25% loss of granular cells was followed by a decrease of approximately 10% in the premature cellular population of the germinative external layer.

The aim of this work was to analyze the effects of alcohol on granular cells of the cerebellum of 6-day-old rats, within a survival time of 2 to 24 hours after administration of BrdU (5-Bromo-2'-desoxiuridine), a thymidine analog used to label S phase of the cell cycle, and to examine the proliferation, migration, and time of origin of cells in the cerebellum.

2 Material and methods

The experiments described here were carried out in accordance with the ethical principles for animal research established by the Brazilian College of Animal Experimentation (COBEA).

Twenty 6-day postnatal offspring were selected from 12 broods of Wistar rats (*Rattus norvegicus*) and divided into two groups of 10 rats each. The experimental group received three intraperitoneal injections of 20% ethanol (3 g of ethanol/kg of body weight). The control group received similar injections of 0.9% (v/v) saline solution. The ethanol was administered three times every 5 hours, at 8:00 am, 1:00 pm and 6:00 pm. The animals were weighed before

and after treatment. Sixteen hours after the ethanol or saline solution were administered, BrdU (5-Bromo-2`-desoxiuridine) (B-5002 Sigma) (60 μg.kg^{-1}) was also administered in all the animals via intraperitoneal. Ten rats, 5 from the control group and 5 from the experimental group (groups P62hC and P62hE, respectively) were sacrificed 2 hours after BrdU administration and 10 other animals, 5 from the control group and 5 from the experimental (groups P6 24hC and P6 24hE, respectively) were sacrificed 24 hours after BrdU administration.

After the treatment period, the animals were weighed and anesthetized with sodium pentobarbital (40 mg.kg^{-1} body weight) via intraperitoneal. This was followed by perfusion with 0.9% saline solution for 15 minutes at room temperature, then perfusion with 70% ethanol solution for 15 minutes. Then the cerebellum was dissected, sectioned in two parts and left overnight in 70% ethanol at 4 °C. Sections were embedded in paraffin and sectioned (5 μm thickness) and the tissues collected on gelatinized glass slides.

In order to obtain the immunohistochemical reaction the sections were deparaffinized, incubated at 60 °C for an hour, and then in xylol (twice for a period of 10 minutes each). Sections were hydrated and treated with 2 N hydrochloric acid for 20 minutes and then with 0.1 M sodium borate.

The immunohistochemical reaction was obtained by an indirect method in which the section was incubated with a monoclonal anti-BrdU antibody (Clone BU-33) (1:500; Sigma). After washing with 0.1 M PBS a secondary antibody B-GAR (Vectastain Goat Anti-Rat IgG Biotinylated Antibody) (Vector) was added with an enzymatic marker (peroxidase) that specifically identified any prime antibody. Another washing was made with 0.1 M PBS and avidin-biotin complex (Vectastain, PK-6104, ABC Elite Kit, Vector) was added, this being an amplified system that connects to the secondary anti-immunoglobuline. Then it was washed once more with PBS and with DAB (3.3-diaminobenzidine) D-5637, Sigma), and finally the sections were counterstained with toluidine blue and safranine, dehydrated, and mounted in entellan. A negative control was used for each reaction.

A Leica microscope was used to examine the slides, coupled to a video camera and connected to a computer loaded with image-analysis software (IMAGELAB-Version 2.3). For each slide, ten lobules of the vermis cerebellar area of each section were analyzed. The external granular cell images (immunohistochemically labeled in each section) were counted in the rostro-basal, rostral media and apical areas using a 40x objective.

After counting the cells, a correction was made as described by Abercrombie (ABERCROMBIE, 1946), using the formula P = A [M/(L + M)], where P is the corrected number of nuclei, A is the observed total count, M is the thickness of the section and L is the average nuclear diameter, determined by measuring the diameter of 30 nuclei from different cerebellar lobules. Random variance analysis was used to compare the block treatments and the Mann-Whitney test was used to compare the number of granular cells in each cerebellar lobule among the different groups and the data referring to the animal's weight. In all cases, a value of $p < 0.05$ indicated significance. The statistical analyses were done using the SAS software, version 8 (SAS/STAT, 1999).

3 Results

The average number of external granular cells of the cerebellum labeled with BrdU in the control group sacrificed 2 and 24 hours after the administration of BrdU in layers I, II, IV, V, VI, VII, VIII, IX and X showed significant statistical difference, with the 24 hours group presenting a higher number of labeled cells than the 2 hours group. No significant difference was observed between the two groups in layer III ($p > 0.05$). Table 1 shows the average and the derivations of the labeled cells from group layers P62hC and P624hC.

By analyzing the experimental groups P62hE and P624hE, significant differences between the two groups were observed in layers I, II, III, IV, V, VII, X, the 24 hours group showing an average number of labeled cells higher than the 2 hours group. A significant difference was observed between the two groups in layers VI and IX ($p > 0.05$). Table 1 shows the average and standard deviations.

By analyzing the average number of total granular cells per cerebellum of the P62hE and P62hC groups, significant differences were observed between the 2 hours experimental and control ($p < 0.05$) groups, the group treated with ethanol presenting a lower number of cells (8.32 ± 1.93) than the one treated with saline solution.

In the 24 hours experimental group and the 24 hours control group, a significant difference was observed in the average number of external granular cell per cerebellum between them ($p < 0.05$); the control group (19.48 ± 3.88) presented a higher number of cells than the one treated with alcohol.

By comparing the average number of cells per layer of cerebellum in the 2 hours control and 2 hours experimental groups, it was observed that in most layers the group treated with ethanol suffered reduction in the average number of cells compared with the group treated with saline solution, except layer IX, where significant differences were observed between the cell number of the control and experimental groups. Table 1 shows the average and standard deviation of the average number of cells from the group layers mentioned above (Figure 1).

Comparing the control group versus the experimental group with a survival time of 24 hours after BrdU (24hC x 24hE) administration, it was observed that in all layers the control group had a higher average of cells than its respective experimental group (Figure 1).

The variation in rat weight with 6 days of postnatal life treated with ethanol was -0.435 ± 0.2795 g, that is, lower weight gain than in the group treated with saline solution (1.981 ± 1.046 g, ($p < 0.01$)) (Figure 2).

4 Conclusion

In our experiment, in the rats aged 6 days old and sacrificed 2 hours after administration of BrdU, a significant decrease was noticed in the number of external granular cells (with the exception of layer IX, where this reduction was not significant) in the group that received intraperitoneal ethanol, compared with the group that received saline solution. Between 24hE and 24hC, the experimental group suffered a significant reduction in the number of cells in all layers of cerebellum in relation to its respective control group. Similar results were found by Fiuza and Moraes (2005), who observed a significant reduction in the number of external

Table 1. Average number of granular cells in each cerebellar lobule of the experimental (E) and control (C) groups of 6-day postnatal rats (5 rats per group) sacrificed 2 or 24 hours after BrdU administration. (Mann Whitney test).

Cerebellar lobules	Treatments				Statistical analysis P	
	2 hours after BrdU administration		24 hours after BrdU administration			
	E	C	E	C	C x E	2hE x 24hE
I	7.22 ± 1.53	13.40 ± 1.54	10.78 ± 1.45	19.56 ± 4.89	<0.01	<0.01
II	7.60 ± 1.80	12.38 ± 1.83	11.70 ± 2.10	19.14 ± 3.63	<0.01	<0.05
III	7.84 ± 0.99	13.70 ± 2.42	12.68 ± 2.06	18.42 ± 4.88	*	<0.01
IV	8.42 ± 1.89	13.52 ± 1.88	13.60 ± 2.66	20.56 ± 4.05	<0.01	<0.01
V	7.62 ± 1.80	13.74 ± 2.59	11.90 ± 2.79	17.96 ± 3.88	*	<0.05
VI	8.32 ± 1.26	11.68 ± 1.21	10.80 ± 2.85	18.52 ± 2.82	<0.01	>0.05
VII	8.66 ± 1.36	13.44 ± 1.88	13.30 ± 3.71	20.96 ± 3.22	*	<0.05
VIII	8.76 ± 1.52	13.24 ± 2.02	14.46 ± 2.42	20.46 ± 5.33	*	<0.01
IX	11.58 ± 2.54	14.28 ± 1.33	13.60 ± 3.09	19.76 ± 5.01	#	>0.05
X	7.20 ± 1.31	11.64 ± 1.70	11.92 ± 2.96	19.42 ± 3.09	<0.01	<0.01

The values are the mean ± SD; *p < 0.05 for the 24 hours groups and p < 0.01 for the 2 hours groups; #p < 0.05 for the 24 hours groups and p > 0.05 for the 2 hours groups; and 2hE = experimental group sacrificed 2 hours after BrdU administration. 24hE = experimental group sacrificed 24 hours after BrdU administration.

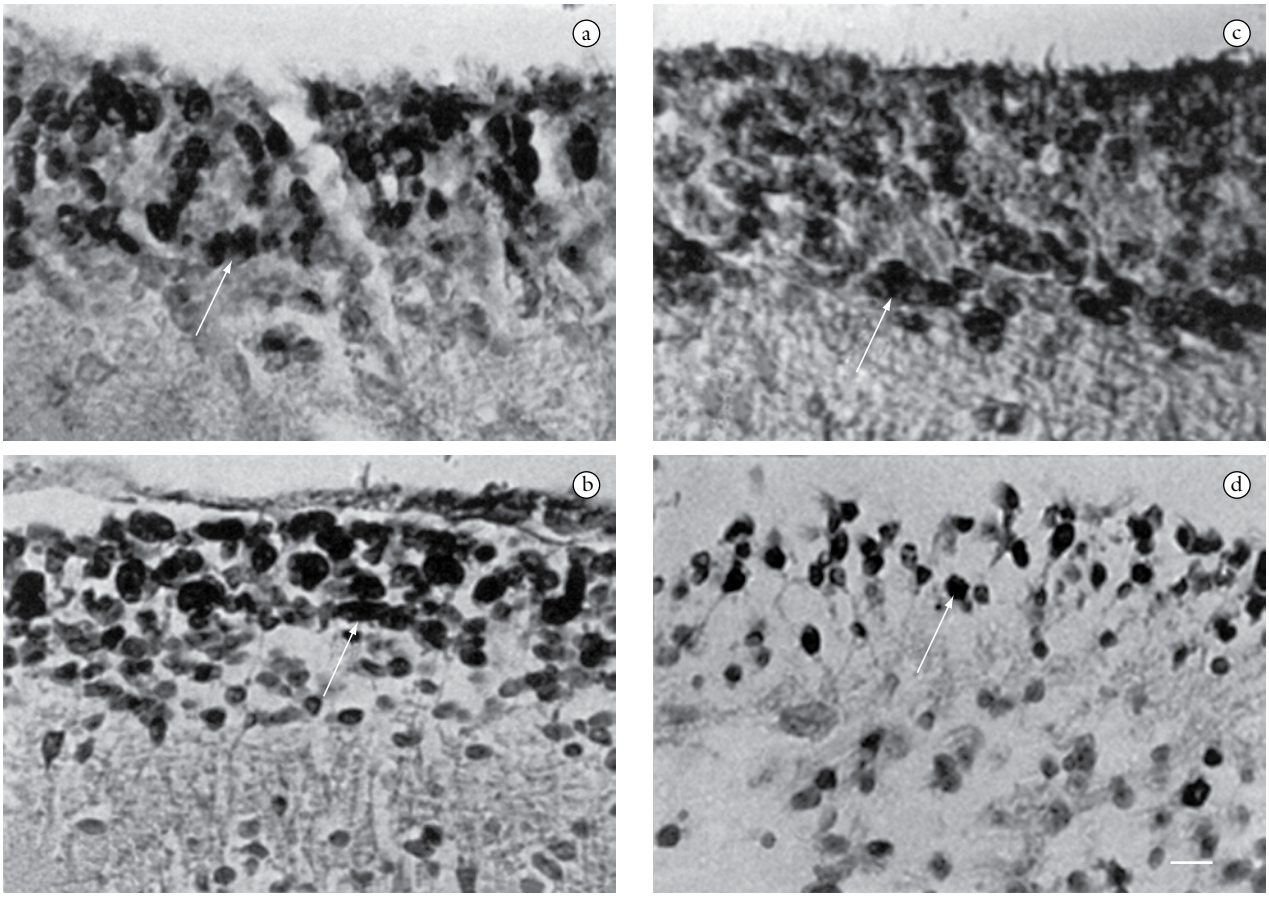

Figure 1. Photomicrographs of the cerebellar cortex area in 6-day postnatal rats showing granular cells labeled with BrdU (arrows). a) Control rat sacrificed 2 hours after BrdU administration (P62hC); b) Ethanol-treated rat sacrificed 2 hours after BrdU administration (P62hE); c) Control rat sacrificed 24 hours after BrdU administration (P624hC); and d) Ethanol-treated rat sacrificed 24 hours after BrdU administration (P624hE). The groups treated with ethanol (b and d) presenting a lower number of cells than the one treated with saline solution (a and c). Bar = 25 μm.

granular cells with BrdU in the layer of cerebellum of rats aged 12 days old treated with ethanol, in relation to its respective control group. Borges and Lewis (1983) described the total number of granular cells from the cerebellar cortex as being significantly reduced after treatment with ethanol, resulting in decrease of granular cells and Purkinje cells between 9 and 14%, in 3 lobules selected from the cerebellum. According to Borges and Lewis (1983), the ethanol

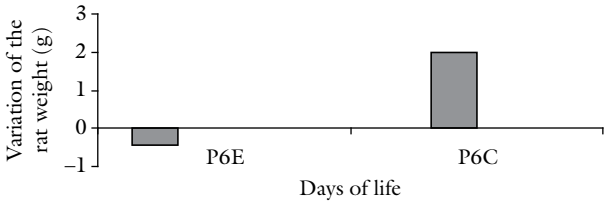

Figure 2. The variation in rat weight with 6 days of postnatal life treated with ethanol (P6E) and the group treated with saline solution (P6C).

can produce reduction in the total number of granular cells in the development of the cerebellum in two ways. The first is by increasing the level of cellular death among the granular cells, the second by changing the dynamics of migration from the external layer. Liesi (1997) observed that ethanol represses the migration of neurons.

Between the 2hE and 2hC groups, the ethanol reduced the external granular cells from the layers of cerebellum in a different manner. The most affected layers were I, III, IV, V, and the least affected were VI, VIII, IX. This different pattern of cellular loss in different layers of cerebellum, in the vermis, can be explained by the fact that the layers become mature in different periods.

In our work, it was observed that ethanol changed the weight of the 6 day old rats significantly if compared with the respective control groups. The rats from the experimental group showed a reduction of weight. From these data it can be affirmed that alcohol produces malnutrition in animals, a fact widely described in the literature. Bauer-Moffett and Altman (1977) reported that the body weight of animals treated with ethanol tended to be slightly lower than in the control group. They noticed that the animals aged 5 days and treated with ethanol weighed 8% less than the control animals. Smith et al. (1986) and Banuelos-Pineda et al. (1995) also observed significant reductions between the body weight of rats treated with alcohol in comparison with the control group.

According to Smith et al. (1986), the affects of cerebellum malnutrition resulted in persistent delay of the external granular layer, delay in the migration and differentiation of granular cells, and delay in the growing of apical dendrites from the Purkinje cells. But Barnes and Altman (1973) pointed out that to produce a considerable change in the cerebellum through malnutrition, the body weight has to be reduced by 42%. Tavares and Barbosa (1982) described that alcoholic deterioration of the cerebellum was related to malnutrition rather than an isolated neurotoxic effect of ethanol.

These results suggest that ethanol causes damage when exposure occurs during the generation period and neuronal migration. The exposure period of ethanol used in this research represents a critical period of cerebellar cortex development. The quantitative neuron changes of the external granular layer can explain the delay of the migration of granular cells observed in the development of the cerebellum when exposed to ethanol in the postnatal period, and can be useful to a better understating of induced disorders or the development of CNS.

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References

ABERCROMBIE, M. Estimation of nuclear population from microtome sections. *Anatomical Record*., 1946, vol. 94, p. 239-247.

ADDICKES, ED. and MOLLNER, TJ. Ethanol-induced cytoskeletal dysgenesis with dietary protein manipulations. *Alcohol Alcohol*., 1986, vol. 21, no. 4, p. 347-355.

BAÑUELOS-PINEDA, J., CARMONA-CALERO, E., PERIS-SANCHIS, R. et al. Alcohol intake effects on the dorsal vagal complex of the rat: A cellular morphometric study. *Acta Anatomica*., 1995, vol. 153, no. 2, p. 145-150.

BARNES, D. and ALTMAN, J. Effects of different schedules of early undernutrition on the preweaning growth of the rat cerebellum. *Exp. Neurol*., 1973, vol. 38, no. 3, p. 406-419.

BAUER-MOFFETT, C. and ALTMAN, J. The effect of ethanol chronically administered to preweaning rats on cerebellar development: A morphological study. *Brain Research*., 1977, vol. 119, no. 2, p. 249-268.

BONTHIUS, DJ. and WEST, JR. Alcohol-induced neuronal loss in developing rats: Increased brain damage with binge exposure. *Alcohol Clin. Exp. Res*., 1990, vol. 14, no. 1, p. 107-118.

BORGES, S. and LEWIS, PD. Effects of ethanol on postnatal cell acquisition in the rat cerebellum. *Brain Res*., 1983, vol. 271, no. 2, p. 388-391.

FIUZA, TS. and MORAIS, JOR. Immunohistochemical evaluation of the postnatal effects of acute exposure to ethanol on the kinetics of granule-cell migration in rat cerebellum. *Braz. J. Morphol. Sci.,* 2005, vol. 22, no. 1, p. 19-24.

FUJITA, S. Quantitative analysis of cell proliferation and differentiation in the cortex of the postnatal mouse cerebellum. *J. Cell Biol.* 1967, vol. 32, no. 2, p. 277-287.

GREEN, JT., TRAN, T., STEINMETZ, JE. et al. Neonatal ethanol produces cerebellar deep nuclear cell loss and correlated disruption of eyeblink conditioning in adult rats. *Brain Res*., 2002, vol. 956, no. 2, p. 302-311.

LIESI, P. Ethanol exposed central neurons fail to migrate and undergo apoptosis. *J. Neurosci. Res*., 1997, vol. 48, no. 5, p. 439-448.

MARCUSSEN, BL., GOODLETT, CR., MAHONEY, JC. et al. Developing rat Purkinje cell are more vulnerable to alcohol-induced depletion during differentiation than during neurogenesis. *Alcohol*., 1994, vol. 11, no. 2, p. 147-156.

MILLER, MW. Migration of cortical neurons is altered by gestational exposure to ethanol. *Alcohol Clin. Exp. Res*., 1993, vol. 17, no. 2, p. 304-314.

RAMÓN, Y. and CAJAL, S. Histologie du système nerveux de l'homme et des vertébrés. Consejo Superior de Investigaciones Cientificas. *Madrid.,* 1955, vol. 2, p. 245-248.

SAS/STAT. User's Guide Version. 8 ed. North Caroline, USA, 1999.

SILVA, BPF., MELO-JÚNIOR, MR., ARAUJO-FILHO, JLS. et al. Ethanol exposition in the perinatal to aguardente into rats cerebral cortex. *Rev. Para. Med.,* 2006, vol. 20, no. 1, p. 7-14.

SMITH, DE., FOUNDAS, A. and CANALE, J. Effect of perinatally administered ethanol on the development of the cerebellar granule cell. *Exp. Neurol*., 1986, vol. 92, no. 3, p. 491-501.

TAVARES, MA. and BARBOSA, MMP. Alcohol-induced granulle cell loss in the cerebellar cortex of the adult rat. *Exp. Neurol*., 1982, vol. 78, no. 3, p. 574-582.

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