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IMMUNOHISTOCHEMICAL EVALUATION OF THE POSTNATAL EFFECTS OF ACUTE EXPOSURE TO ETHANOL ON THE KINETICS OF GRANULE-CELL MIGRATION IN RAT CEREBELLUM*

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ABSTRACT

Exposure of the developing central nervous system (CNS) to ethanol leads to impaired cellular migration. In the cerebellar cortex, cell proliferation occurs in the early postnatal period. Granular cells generated in the external granular layer (EGL) migrate to their final destination at the internal granular layer. In this work, we examined the ethanol-induced alterations in cerebellar granular cells during their formation in 12-day postnatal (P12) Wistar rats (*Rattus norvegicus*). Three intraperitoneal (i.p.) injections of 20% ethanol (3 g/kg of body weight) were administered to each rat at 5 h intervals folowed by 5'-bromo-2- deoxyuridine (BrdU, 60 mg/kg, i.p.) 16 h after the last injection. The rats were sacrificed 2 h or 24 h after the administration of BrdU and the brain was removed and embedded in paraffin. BrdU was subsequently detected immunohistochemically in sections of brain tissue. There was a decrease in the number of external granular cells and in the number of cell layers in the cerebellar EGL in all of the groups that received ethanol when compared to their respective controls. There was also a decrease in these parameters in the 2 h and 24 h survival period after BrdU administration. These results indicate that exposure to ethanol during granule cell generation and neuronal migration in the cerebellum is harmful, and that a study of the quantitative alterations in EGL neurons of the developing rat cerebellum exposed to ethanol in the postnatal period can provide a better understanding of ethanol-induced or related disturbances in the CNS.

Key words: Cerebellum, ethanol, granule-cell

INTRODUCTION

The cerebellum contains two separate germinative zones: the primary germinative zone, which covers the 4th ventricle roof, and the external granular layer, which overlays the cerebellar cortex. The progenitors cells that originate from each germinative layer migrate in opposite directions and differentiate in the area between the two germinative zones [26].

The teratogenic effects of prenatal exposure of the CNS to ethanol are well documented [16]. The deep neuroanatomical changes caused by ethanol, including microcephaly, have been related to interference in many developmental processes in the CNS, including neuronal proliferation, migration, differentiation [8,18,24], and glial maturation and gliosis [10,20]. The postnatal exposure of rats to ethanol results in specific damage to the CNS. In humans, such exposure can give rise to behavioral disturbances and learning deficiencies in children born to alcoholic mothers. Pre- and postnatal exposure to a large amount of ethanol during a critical developmental period results in memory and learning deficits and the loss of hipocampal neuronal cells [7,11]. Neuropathological changes can occur after early postnatal exposure to ethanol [3,6,22]. However, in these studies of newborn rats, the effects on the nervous system were found to result from the high level of ethanol in the blood.

In this work, we examined the effects of ethanol on cerebellar granular cells during their formation in 12-day postnatal rats (P12) (*Rattus norvegicus*).

MATERIAL AND METHODS

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

Twenty 12-day postnatal offsprings were selected from 12 broods of Wistar rats (*Rattus norvegicus*) and were 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) at 5-h intervals. The control group received similar injections of 0.9% (w/v) saline solution. The plasma volume in both was calculated based on the body weight.

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Sixteen hours after the last injection of ethanol or saline solution, BrdU (5'-bromo-2 deoxyuridine, Sigma, 60 µg/kg body weight, i.p.) was administered to both groups of rats. Ten rats, 5 from the control group and 5 from the experimental group, were sacrificed 2 h after BrdU administration, while the remaining animals in each group were sacrificed 24 h after the injection of BrdU. After anesthesia with sodium pentobarbital (40 mg/kg body weight, i.p.), the heart was exposed and the rats perfused with 0.9% saline solution via the left ventricle for 15 min at room temperature, followed by perfusion with 70% ethanol for 15 min. The cerebellum was then dissected, sectioned at the cerebellar vermis, left overnight in 70% ethanol at 4ºC, and finally embedded in paraffin. The paraffin blocks were sectioned (5 µm thick) and the tissues collected on glass slides precoated with gelatin. The sections were deparaffinized, hydrated in a graded ethanol series, and treated with 2 N hydrochloric acid at 60 ºC and then with 0.1 M sodium borate (20 min each). The sections were subsequently incubated with a monoclonal anti-BrdU antibody (1:500, Sigma) and washed with 0.1 M PBS (phosphate buffered saline) before incubation with a goat anti-rat IgG biotinylated antibody secondary antibody (Vector). After washing with 0.1 M PBS, avidin-biotin complex (Vectastain, PK-6104, ABC Elite Kit, Vector) was added and the sections again washed with PBS, prior to the addition of DAB (3,3-diaminobenzidine, Sigma). Finally, the sections were counterstained with toluidine blue and safranine, dehydrated, and mounted in entellan. A negative control was used for each reaction.

The slides were examined with a Leica microscope coupled to a video camera and connected to a computer loaded with image-analysis software (IMAGELAB - Version 2.3). Ten lobules of the vermis cerebellar area of each section were analyzed. The immunohistochemically-labeled cells in each section were counted in the rostro-basal, rostral medial and apical areas (125 µm long each) (Fig. 1) using a 40X objective. After counting the cells, a correction was made as described by Abercrombie [1], 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. In all cases, a value of $P < 0.05$ indicated significance. The statistical analyses were done using the SAS software, version 8 [23].

RESULTS

The average numbers of external granular cells labeled with BrdU per unit area of the cerebellar lobule in the 2-h and 24-h control groups (2 hC and 24 hC, respectively) and layers I, II, V and VIII were significantly different, with the 24 hC group having a greater number of labeled cells than the 2 hC group (Table 1). A comparison of the average number of external granular cells per layer in the cerebellum of groups 2-h and 24-h experimental alcohol-treated groups (2 hE and 24 hE) revealed significant differences between the two groups ($P < 0.05$) in all ten layers of the cerebellum: the 2 hE group had a smaller number of granular cells in all layers compared to the 24 hE group (Table 1).

Table 1. Average number of granular cells in each cerebellar lobule of the experimental (E) and control (C) groups of 12-day postnatal rats (5 rats per group) sacrificed 2 h and 24 h after BrdU administration.

Cerebellar lobules	Treatment					
	2 hours		24 hours		P	
	Experimental	Control	Experimental	Control	CxE	2 hE x 24 hE
I	5.68 ± 1.08	10.94 ± 0.71	7.38 ±0.34	13.16 ± 1.67	< 0.01	< 0.05
II	5.26 ± 1.30	10.84 ± 1.18	8.38 ± 1.23	12.10 ± 1.72	< 0.01	< 0.05
III	5.48 ± 0.92	11.38 ± 2.07	7.28 ± 0.53	12.22 ± 2.27	< 0.01	< 0.05
IV	6.24 ±0.95	11.36 ± 1.33	8.16 ± 1.34	13.30 ± 2.56	< 0.01	< 0.05
V	5.68 ± 0.61	10.16 ± 1.31	8.08 ± 2.25	12.44 ± 0.87	*	< 0.05
VI	6.84 ± 0.75	11.04 ± 1.72	8.50 ± 1.17	12.26 ± 0.43	< 0.01	< 0.05
VII	6.04 ± 0.28	10.80 ± 1.72	7.62 ± 0.95	11.96 ± 0.62	< 0.01	< 0.05
VIII	6.22 ± 0.39	10.18 ± 1.46	7.50 ±1.25	13.42 ± 1.23	< 0.01	< 0.05
IX	5.60 ± 1.31	11.34 ± 2.56	7.76 ± 0.78	13.32 ± 1.62	< 0.01	< 0.05
X	5.64 ± 1.50	10.58 ± 3.02	8.58 ± 1.17	12.94 ± 3.03	*	< 0.05

The values are the mean ± SD
*P< 0.05 for the 24 h groups and P< 0.01 for the 2 h groups.
2 hE = experimental alcohol-treated group sacrificed 2 h after BrdU administration; **24 hE** = experimental alcohol-treated group sacrificed 24 h after BrdU administration.

There was a significant difference (P<0.01) between the average number of stained granule cells per unit area of cerebellar lobule in groups 2 hE (experimental 12-day postnatal rats sacrificed 2 h after BrdU administration) and 2 hC (control 12-day postnatal rats sacrificed 2 h after BrdU administration), with the experimental group having a smaller number of cells than the control (5.87 ± 0.99 *versus* 10.86 ± 1.71) (Table 1). There was also a significant difference (P<0.01) between groups 24 hE (experimental 12-day postnatal rats sacrificed 24 h after BrdU administration) and 24 hC (control 12-day postnatal rats sacrificed 24 h after BrdU administration), with the latter having a larger number of stained granule cells per unit area of cerebellar lobule than the group treated with alcohol (12.70 ± 1.71 *versus* 7.92 ± 1.18) (Table 1).

The average number of stained granule cells per unit area of cerebellar lobule in 12-day postnatal rats of group 2 hE was significantly lower than in the corresponding control group (2 hC) in all ten lobules (Table 1). A comparison of groups 24 hC and 24 hE showed that the latter group had a lower number of cells than the control group (Table 1). In group 2 hC, 1 - 2 juxtaposed external granular cell layers were observed in all ten cerebellar lobules (Fig. 2A). In group 2 hE, lobules I, II, V, VI, VII, VIII, IX, and X had an external granular cell layer separated from the others (Fig. 2B), whereas lobules III and IV had two cell layers separated in the EGL (Fig. 2E). In lobules VI and VII of group 24 hC, 3 - 4 cell layers were seen close to each other in the EGL (Fig. 2G), whereas in lobules II, III, V, and IX, there were 4 - 5 labeled cell layers in the EGL (Fig. 2H), and five cell layers in lobules I, IV, VIII and X (Fig. 2C). In group 24 hE, 1 - 2 labeled external granular cells that were separated from each other were observed in lobules I, III, and VI (Fig. 2D). However, in the other sections, two labeled cell layers that were separated from each other were observed in the EGL (Fig. 2F).

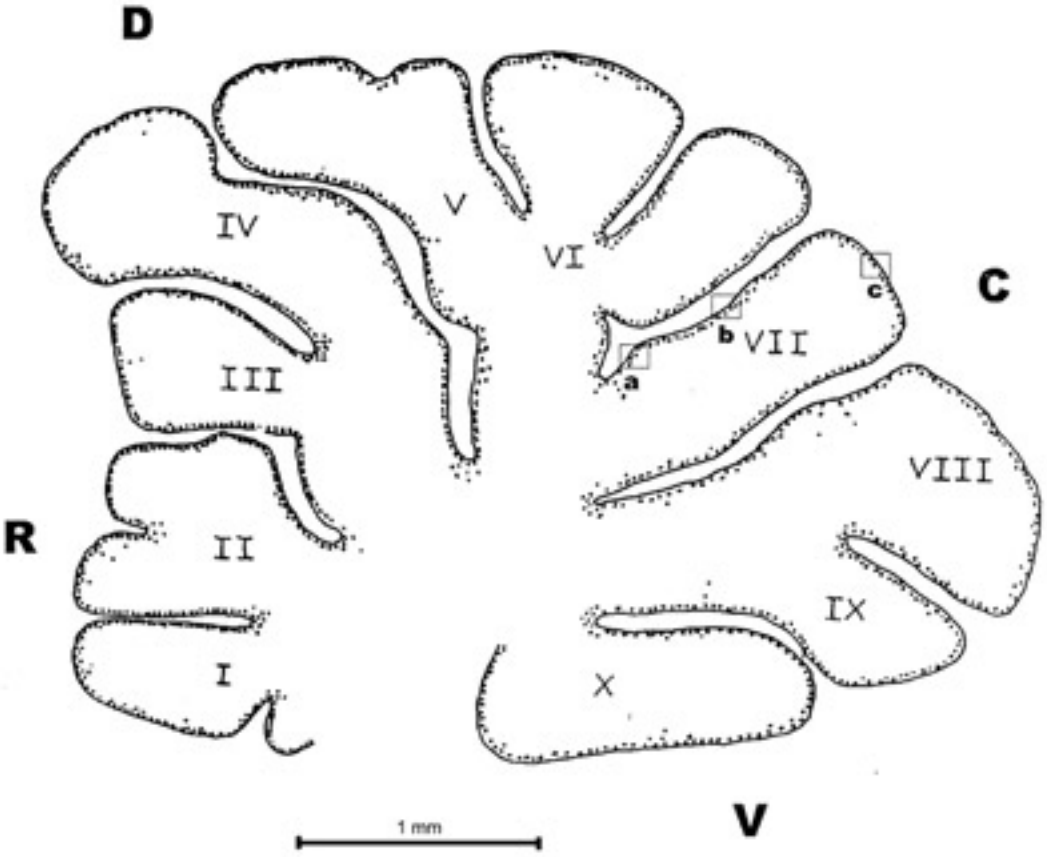
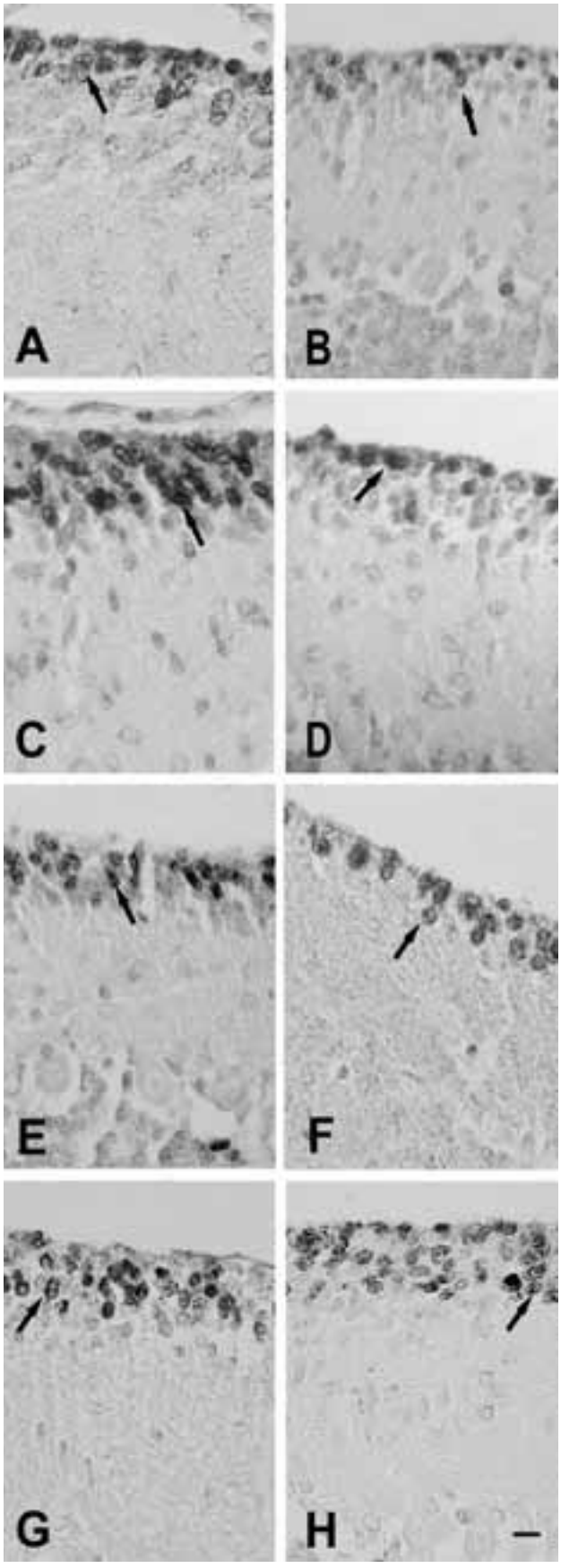


Figure 1. Camera lucida drawing showing the cerebellar vermis with its ten layers (I-X). **R -** rostral, **D** - dorsal, **C** - caudal, **V** - ventral. The external granular layer is visible. Note too the three areas where the images of the immunohistochemically-marked granular cells were taken: **a** - rostro-basal area, **b** - rostral-medial area, and **c** - apical area.



DISCUSSION

The exposure of cerebellar granule-cells to ethanol during development produces abnormalities that result from the disruption of many normal developmental events. Acute postnatal exposure to ethanol changes the schedule of the neuronal migration of granule cells in cerebelar cortex. These changes result from ethanol-induced alterations in the kinetics of neuronal migration because of the increased amount of time that these postmitotic cells remain in the external granular layer. Several studies have shown that developing cerebellar granule cells are especially vulnerable, particularly when exposure to ethanol occurs during the early postnatal period.

The counts of immunoreactive cells in the cerebellar cortex revealed the dynamics of cellular proliferation and migration to the internal granular layer (IGL) of control and ethanol–treated rats 2 h and 24 h after the administration of BrdU. Comparison of the 2 hC and 24 hC groups showed that the latter had a greater number of cells, that was significant only in lobules I, V, and VIII. In agreement with Bauer-Moffett and Altman [3], the proliferative compartment of the external germinative cell layer in rats produced granular cells that decreased in number from the tenth postnatal day onwards. Altman [2] and Fujita [9] reported that cells of the external granular layer proliferate quickly during the first two weeks after birth, but that proliferation decreases sharply during the third week, with concomitant postmitotic cell migration and differentiation of the EGL into cortex. The germinative layer undergoes gradual dissolution and disappears almost completely by the end of the third week.

There was a significant reduction in the number of external granular layer cells of the cerebellum in ethanol-treated rats sacrificed 2 h and 24 h after the injection of BrdU when compared to their respective

Figure 2. Photomicrographs of the cerebellar cortex area in 12-day postnatal rats showing granular cells labeled with BrdU (**arrows**). **A)** Control rat sacrificed 2 h after BrdU administration (2 hC) - one or two juxtaposed external granular cell layers can be seen. **B)** Ethanol-treated rat sacrificed 2 h after BrdU administration (2 hE) - Note the external granular cell layer and the external granular cells separated from each other. **C)** Control rat sacrificed 24 h after BrdU administration (24 hC) - Note the EGL with five cell layers. **D)** Ethanol-treated rat sacrificed 24 h after BrdU administration (24 hE) - Note 1 - 2 external labeled granular cell layers separated from each other. **E)** 2 hE – Note the two cell layers separated in the EGL. **F)** 24 hE – Note the two labeled cell layers separated from each other in the EGL. **G)** 24 hC- Note the 3 - 4 cell layers close to each other in EGL. **H)** 24 hC- 4 - 5 labeled cell layers are visible in the EGL. Bar = 10 μm for all panels.

control groups. These results indicate that ethanol reduced the number of granular cells. Similar results were observed by Ferreira *et al*. [8], who reported that exposure to ethanol markedly affected cerebellar histogenesis and showed that the granular layer was severely affected. Natale [21] reported that ethanol caused several abnormalities in the CNS, including alterations in neuronal and glial cell migration. Bonthius and West [4], Marcussen *et al*. [15] and Miller [19] observed that exposure to alcohol during gestation and in the early postnatal period resulted in the selective loss of neuronal cells in the cerebellum, although Maier and West [14] found no decrease in the number of cerebellar Purkinje cells after treatment with ethanol during neurogenesis.

The number of external granular cells in the 2 hE group was lower than in the 2 hC group in all of the lobules. These results were similar to those reported by Bauer-Moffett and Altman [3]. The experimental group (2 hE) had 1 - 2 cell layers, while the control group (2 hC) had 1 - 2 juxtaposed cell layers. This reduction was more accentuated in the 24 h group (1 - 2 cell layers separated from each other in the 24 hE), and 3 - 4 juxtaposed cell layers in the 24 hC. These observations indicate that ethanol affected the number of external granular cells and reduced the number of granular cell layers. These results also suggest that exposure to ethanol damages the cerebellar cortex during the period of proliferation and neuronal migration. Quantitative alterations in EGL neurons can explain the late granular cell migration seen during the development of the cerebellum after exposure to ethanol in the postnatal period.

Ethanol affected the kinetics of cell migration during the time intervals studied here. In the ethanol-treated rats, there was a 34.9% increase in the cells of the EGL between 2 h and 24 h after BrdU administration, compared to 16.9% increase for the control rats. These findings suggest that ethanol causes damage when exposure occurs during the period of neuronal migration.

As shown here, ethanol may reduce the final number of granule cells in the developing cerebellum. This may result from an increase in the rate of cell death amongst granule cells following a reduction in synaptogenesis, from direct neurotoxicity, or from an alteration in the kinetics of migration. Apoptosis of cerebellar granule neurons has been observed *in vitro* [13]. A correlation between the synaptogenesis and the period of sensitivity to ethanol-induced neuronal damage has also been reported [12]. Alcohol or its metabolic products can interfere with cerebellar development by altering the production or function of natural regulatory substances that help promote the orderly growth and differentiation of neurons [17].

Changes in the environmental milieu of a neuron during one of the critical postnatal developmental periods can cause neuronal damage. Much of the data concerning the developmental effects of ethanol on the nervous system relates to the results described here [3,5], and the significant decrease in the number of granule cells [25] provide a further contribution to our understanding of ethanol-induced or -related damage that affects the development of cerebellar granular cells.

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