

# Changes of Hippocampal Neurons after Perinatal Exposure to Ethanol

M. MILOTOVÁ, V. RILJAK, K. JANDOVÁ, J. BORTELOVÁ, D. MAREŠOVÁ,  
J. POKORNÝ, M. LANGMEIER

Institute of Physiology, First Faculty of Medicine, Charles University, Prague, Czech Republic

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## Summary

The effect of ethanol on the structural development of the central nervous system was studied in offspring of Wistar rats, drinking 20 % ethanol during pregnancy and till the 28<sup>th</sup> day of their postnatal life. The structural changes in the hippocampus and dentate gyrus were analyzed at the age of 18, 35 and 90 days. A lower width of pyramidal and granular cell layers, cell extinction and fragmentation of numerous nuclei were found in all experimental animals compared to control animals. The extent of neural cell loss was similar in all monitored areas and in all age groups. At the age of 18 and 35 days, the degenerating cells were observed in the CA1 and CA3 area of the hippocampus and in the ventral and dorsal blade of the dentate gyrus. Numerous glial cells replaced the neuronal population of this region. Some degenerating cells with fragmented nuclei were observed at the age of 90 days. Our experiments confirmed the vulnerability of the developing central nervous system by ethanol intake during the perinatal period and revealed a long-lasting degeneration process in the hippocampus and dentate gyrus.

## Key words

Ethanol • Degeneration • Apoptosis • Hippocampus • Dentate gyrus

## Corresponding author

M. Langmeier, Institute of Physiology, First Faculty of Medicine, Charles University, Albertov 5, 128 00 Prague 2, Czech Republic.  
E-mail: milos.langmeier@lf1.cuni.cz

## Introduction

Ethanol is a psychotropic drug (Nutt 1996) and its chronic abuse causes impairments of brain function, such as visual, motor and memory dysfunctions (Schummers *et*

*al.* 1997, Alling 1999, Dahchour *et al.* 2000).

The brain structure followed in our experiments was hippocampus which is involved in several aspects of memory and other functions. Hippocampal pyramidal neurons are generated during late gestation from the ventricular zone (Schlessinger *et al.* 1975, 1978, Bayer 1980a,b) and they may remain vulnerable when exposed to different neurotoxins during early life (Hort *et al.* 1999, Langmeier *et al.* 2003, Miki *et al.* 2004, Milotová *et al.* 2006, Riljak *et al.* 2007). The long postnatal development of hippocampus, resulting from prolonged proliferation of granular cells (Bayer *et al.* 1982), allows studying changes that arise from interference with the development during prenatal and early postnatal life (Pokorný 1982).

Our study was aimed at the identification of changes of the structure which can persist from the perinatal period till early adulthood (18-day, 35-day, and 90-day). We focused on changes in CA1, CA3 areas of the hippocampus and in dorsal and ventral blades of the dentate gyrus after a long-term perinatal exposure to ethanol.

## Methods

All experiments were carried out in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC) and in agreement with the guidelines of the Animal Protection Law of the Czech Republic. Female Wistar rats of our own breed and six groups of their male offspring were used in the experiments. Each group consisted of ten male rats.

Pregnant Wistar rats of the experimental group were compelled to drink 20 % ethanol solution (as the only drinking fluid) since conception up to the weaning of their

offspring at the age of 28 days. Pregnant rats of the control group drank tap water. Since the 29th day the offspring were separated from their mothers.

At the age of 18, 35 and 90 days the animals were perfused under deep thiopental anesthesia with 4 % paraformaldehyde in 0.1 M phosphate buffer at pH 7.4. Brains were removed, postfixed for one hour in 4 % buffered paraformaldehyde and then submerged for one hour into 20 % sucrose for cryoprotection. Brains were sliced in the frontal plane into 40  $\mu$ m thin sections with a cryostat and the free-floating sections were placed in 0.1 M phosphate buffer. Tissue sections were mounted onto gelatinized slides and allowed to dry at room temperature. Sections were then stained with combination of DNA staining Hoechst and Fluoro-Jade B, which was originally described by Schmued and Hopkins (2000).

Two histological methods were used in our study. Fluoro-Jade B dye is an anionic fluorescein derivative useful for the histological staining of neurons undergoing degeneration; staining with bis-benzimide (Hoechst 33342) was used to detect apoptotic cells according to the status of nuclei (condensed and/or fragmented nuclei indicate cell degeneration). Slides were placed in a staining rack (one slide/slot for even staining) and immersed in 100 % ethanol solution for 3 min, in 70 % ethanol solution for 1 min, in distilled water for 1 min, in 0.01 % potassium permanganate ( $\text{KMnO}_4$ ) for 15 min shaking gently. Slides were washed in distilled water three times. Slides in staining rack were removed in a dim place and immersed in 0.001 % Fluoro-Jade staining solution for 30 min gentle shaking, rinsed in distilled water three times for 1 min. Slides were then immersed in 0.01 % Hoechst 33342 staining solution for 10 min and dehydrated (by ascending concentration of ethanol), cover-slipped using D.P.X. Neutral Mounting Medium and allowed to dry.

Samples taken from the dorsal hippocampus were examined and quantified under the microscope Olympus AX-70 Provis with epifluorescence and taken by digital camera Olympus DP70. In the material processed for DNA staining with Hoechst 33342, the width of the pyramidal layer at standard latero-medial locations (intercept between stratum oriens and stratum radiatum, perpendicular to the plane of stratum pyramidale-oriens border) in CA1, CA3 hippocampal areas and the width of the granular layer in dorsal and ventral blades of dentate gyrus (intercept between stratum moleculare and polymorph layer, perpendicular to the plane of stratum granulosum-moleculare border) was estimated with the aid of computerized analysis (OLYMPUS analySIS<sup>®</sup> image

capture/analysis software). Analysis was limited to the region between the AP planes given by standard coordinates. Values for coordinates in adult animals (AP planes between 2.5 and 4.0 mm posterior to the bregma, L=2 and P=2 mm according to Fiková and Maršala 1960) were recalculated for immature rats on the basis of the bregma-lambda distance which was taken as 8 mm in adult rats.

We measured morphological changes in ten animals from each age group (18-, 35-, 90-day-old) always in 3 sections and 3 adjacent regions on both sides from each brain (at the beginning, in the middle and at the end of the relevant CA1 and CA3 areas of the hippocampus, dorsal (DB DG) and ventral blades (VB DG) of dentate gyrus).

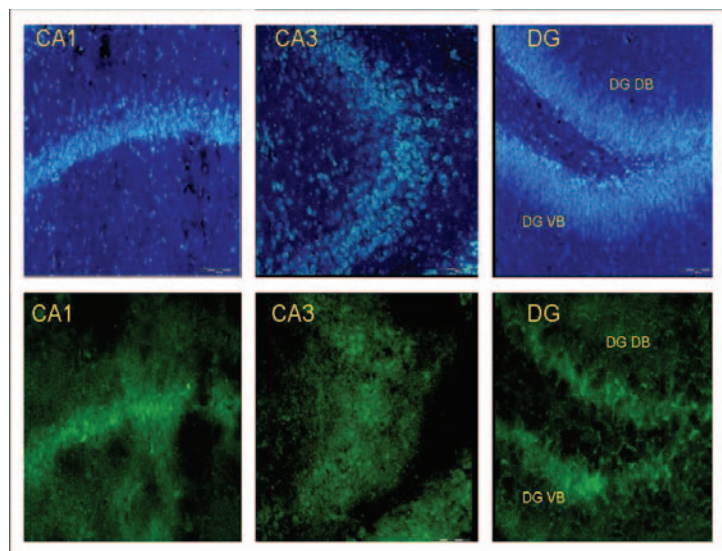
The results were statistically evaluated by the unpaired t-test and one-way ANOVA with Dunnett's post test using GraphPad Prism version 4.00 for Windows, GraphPad Software, San Diego California USA, [www.graphpad.com](http://www.graphpad.com). The level of significance was set at  $p < 0.0001$ .

## Results

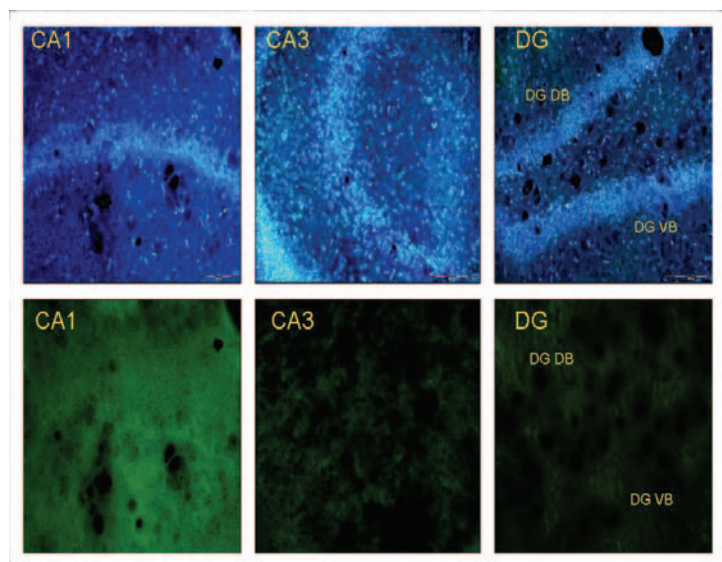
The average daily consumption of the 20 % ethanol solution was  $41.6 \pm 1.4$  ml per animal. Litters born to the mothers exposed to ethanol were smaller by about one half of the control group. There were no differences in the proportion of male and female offspring and the birth-weight of all offspring was similar (experimental group:  $5.7 \pm 0.5$  g, control group  $5.5 \pm 0.6$  g).

At the age of 18 and 35 days, groups of degenerating (Fluoro-Jade B-positive) cells were observed in the CA1 and CA3 area of the hippocampus. Some cells had fragmented nuclei and were accompanied with large numbers of glial cells. The glial scavenging reaction was also prominent in other hippocampal regions, being most intense in the areas CA1 and CA3. In the dorsal and ventral blades of the dentate gyrus many cells with fine fragmented nucleus in DNA staining Hoechst and some Fluoro-Jade B-positive neurons were observed. In the control group, neither cells with a fine fragmented nuclei nor degenerating (Fluoro-Jade B-positive) cells were found (Figs 1 and 2).

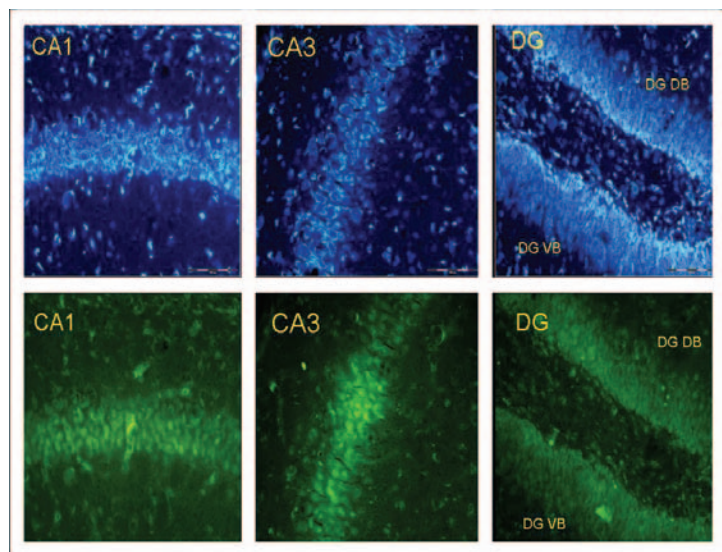
At the age of 90 days no Fluoro-Jade B-positive neurons in any of the studied areas were observed, but some cells with fine fragmented nuclei were identified in all areas (Fig. 3).



**Fig. 1.** Experimental 18-day-old animal. Detail of the CA1 and CA3 area of the hippocampus (CA1, CA3) and ventral and dorsal blade of the dentate gyrus (VB DG, DB DG) in the AP plane 3 mm posterior to bregma. Hoechst and Fluoro-Jade B staining. Neuronal degeneration (FJ-B positive neurons) – green figures, bis-benzimide, Hoechst 33342 staining – blue figures. The microphotographs were made using the microscope OLYMPUS AX70 Provis with digital camera OLYMPUS DP70. Magnification: 40x



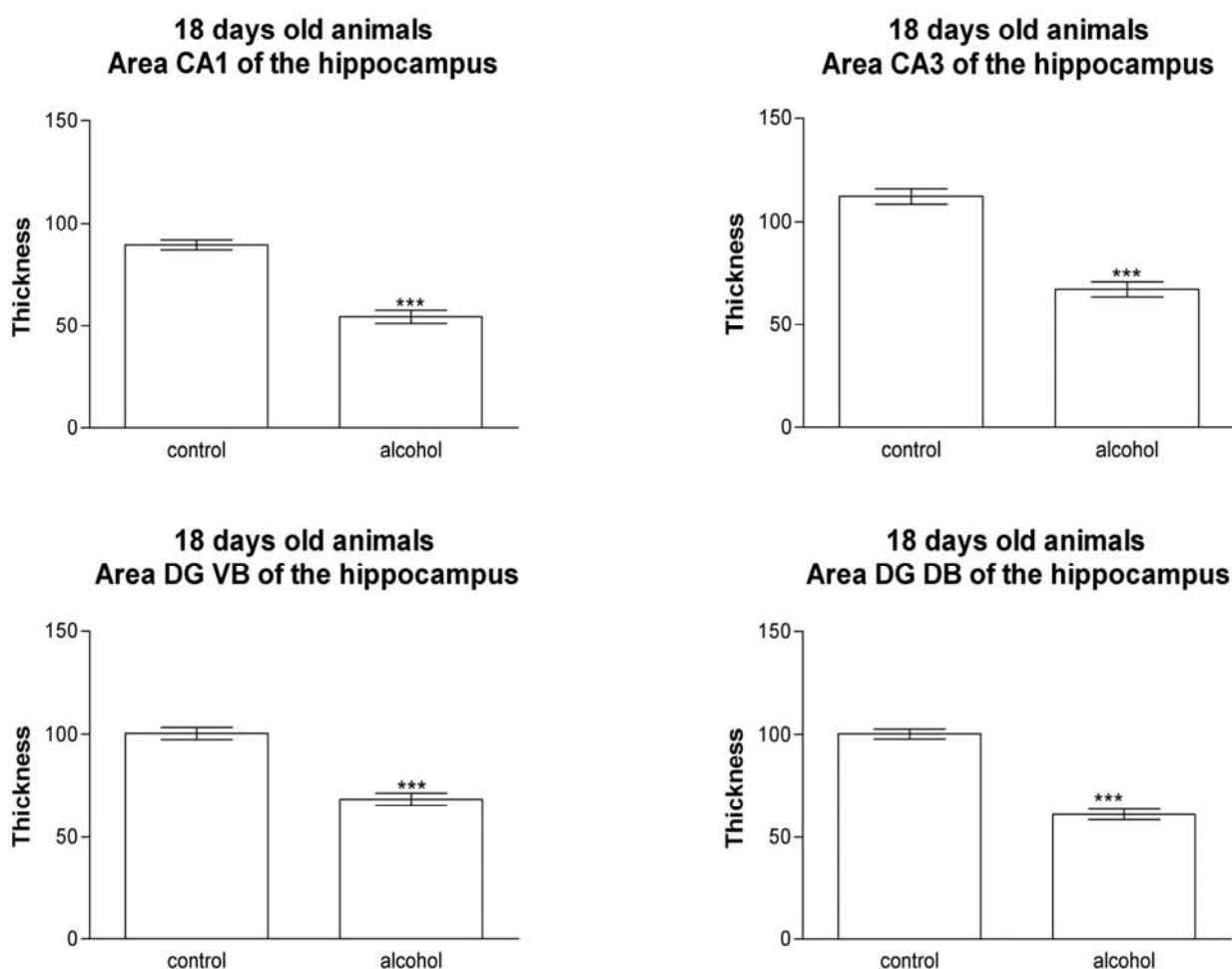
**Fig. 2.** Experimental 35-day-old animal. Detail of the CA1 and CA3 area of the hippocampus (CA1, CA3) and ventral and dorsal blade of the dentate gyrus (VB DG, DB DG) in the AP plane 3 mm posterior to bregma. Hoechst and Fluoro-Jade B staining. Neuronal degeneration (FJ-B positive neurons) – green figures, bis-benzimide, Hoechst 33342 staining – blue figures. The microphotographs were made using the microscope OLYMPUS AX70 Provis with digital camera OLYMPUS DP70. Magnification: 40x

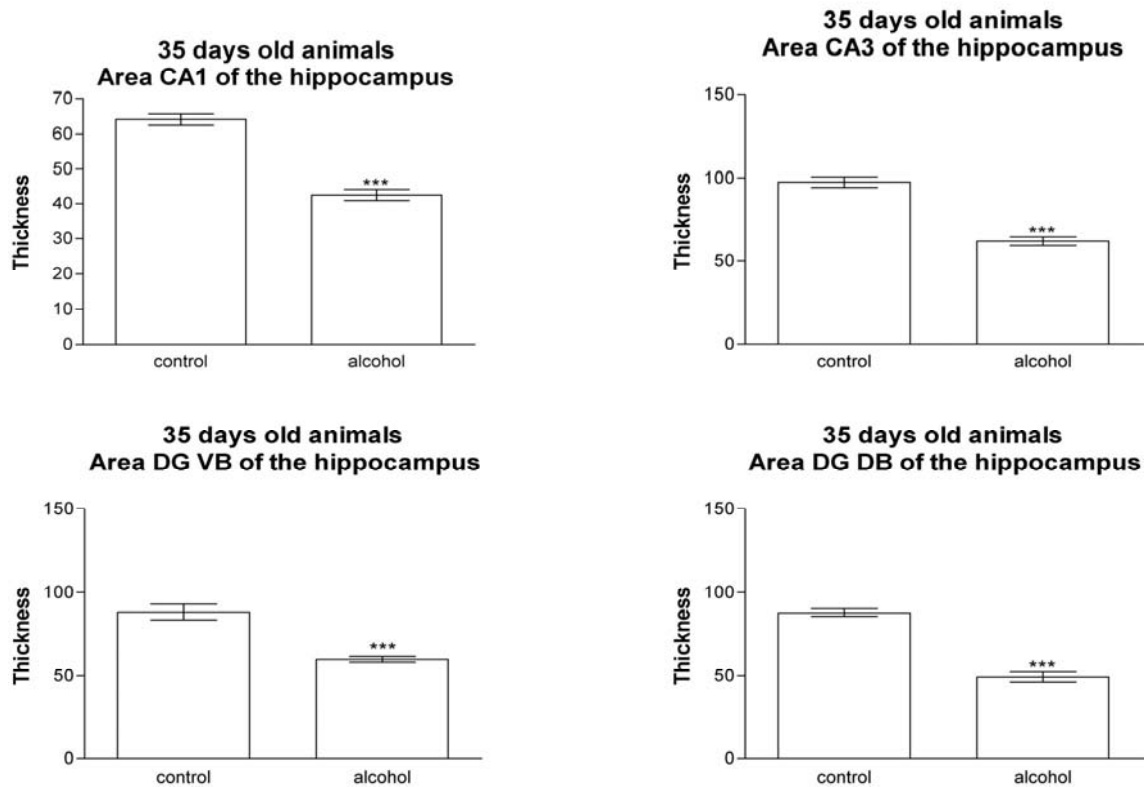


**Fig. 3.** Experimental 90-day-old animal. Detail of the CA1 and CA3 area of the hippocampus (CA1, CA3) and ventral and dorsal blade of the dentate gyrus (VB DG, DB DG) in the AP plane 3 mm posterior to bregma. Hoechst and Fluoro-Jade B staining. Neuronal degeneration (FJ-B positive neurons) – green figures, bis-benzimide, Hoechst 33342 staining – blue figures. The microphotographs were made using the microscope OLYMPUS AX70 Provis with digital camera OLYMPUS DP70. Magnification: 40x

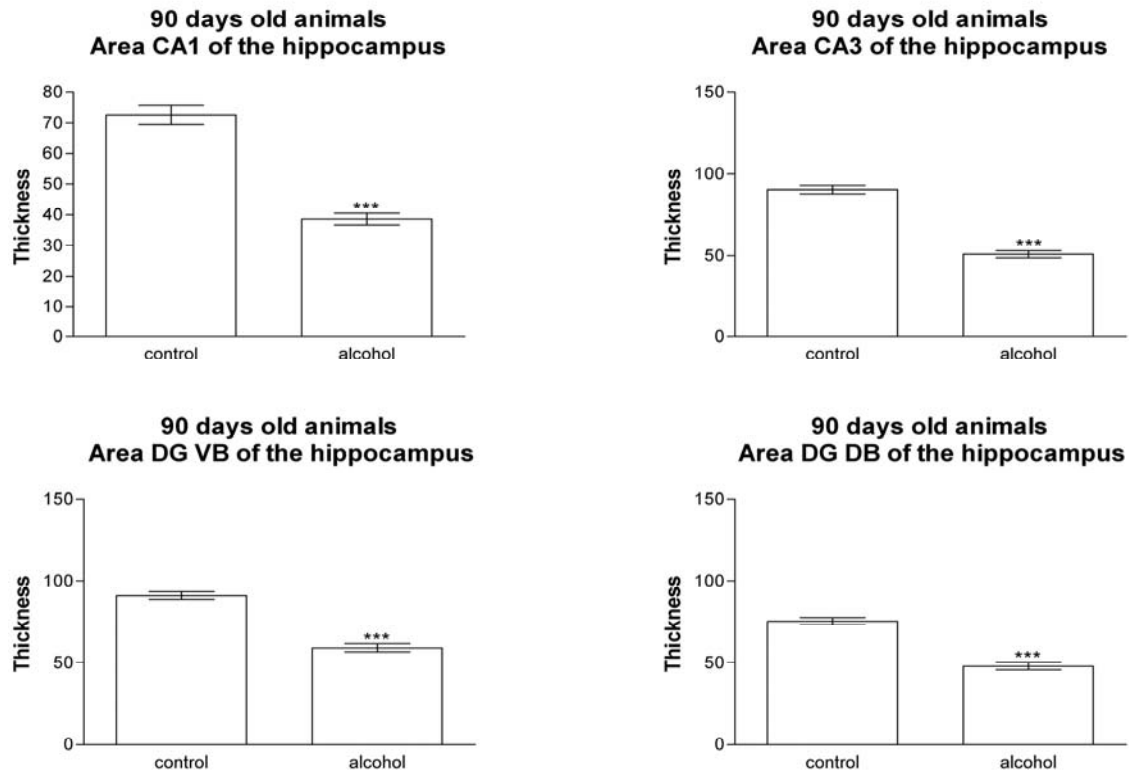
**Table 1.** The width of the pyramidal cell layer in the areas CA1, CA3 and the width of granule cell layer in both blades of the gyrus dentatus in the experimental group (perinatal ethanol consumption) and in the control group.

Age of animals	Group	CA1 ( $\mu\text{m}$ )	CA2 ( $\mu\text{m}$ )	VB DG ( $\mu\text{m}$ )	DB DG ( $\mu\text{m}$ )
18 days	experimental group	$53.594 \pm 9.161$	$69.249 \pm 12.277$	$68.307 \pm 9.381$	$61.774 \pm 7.157$
	control group	$89.773 \pm 7.324$	$111.992 \pm 11.249$	$100.970 \pm 7.493$	$99.824 \pm 7.161$
	decrease	<b>40.00 %</b>	<b>37.73 %</b>	<b>32.35 %</b>	<b>38.12 %</b>
35 days	experimental group	$42.276 \pm 4.812$	$60.478 \pm 8.692$	$59.355 \pm 5.827$	$49.271 \pm 10.105$
	control group	$64.703 \pm 5.566$	$97.250 \pm 9.095$	$91.533 \pm 7.069$	$87.834 \pm 6.890$
	decrease	<b>34.67 %</b>	<b>37.81 %</b>	<b>35.15 %</b>	<b>49.91 %</b>
90 days	experimental group	$38.751 \pm 5.987$	$51.174 \pm 6.826$	$59.442 \pm 7.399$	$48.279 \pm 7.007$
	control group	$72.041 \pm 9.643$	$90.119 \pm 8.024$	$88.851 \pm 9.153$	$74.600 \pm 7.325$
	decrease	<b>46.21 %</b>	<b>43.22 %</b>	<b>34.57 %</b>	<b>35.29 %</b>

**Fig. 4.** Effect of the perinatal ethanol intake on the width of the pyramidal layer in the hippocampus and the granule cell layers in the dentate gyrus of 18-day-old rats. \*\*\* Significance level of differences at  $p \leq 0.0001$ .



**Fig. 5.** Effect of the perinatal ethanol intake on the width of the pyramidal layer in the hippocampus and the granule cell layers in the dentate gyrus of 35-day-old rats. \*\*\* Significance level of differences at  $p \leq 0.0001$ .



**Fig. 6.** Effect of the perinatal ethanol intake on the width of the pyramidal layer in the hippocampus and the granule cell layers in the dentate gyrus of 90-day-old rats.

The width of the pyramidal cell layer in the areas CA1, CA3 and width of the granule cell layer in both blades of the gyrus dentatus was smaller in experimental animals than in controls in all age groups (Table 1, ANOVA  $p \leq 0.0001$ ). There were no apparent differences in the size of pyramidal and granular cells between the control and experimental animals (Figs 4, 5 and 6).

## Discussion

The effect of ethanol during either prenatal or postnatal brain development has been studied by several authors (Pierce *et al.* 1989, Bothius and West 1990, Miller 1995, Miki *et al.* 2000a,b,c), but the model of the perinatal exposure to ethanol and its immediate and long-term effects in the hippocampal region have not been studied.

Several experimental studies have shown that the hippocampus appears to be particularly vulnerable by ethanol exposure during prenatal life (Barnes and Walker 1981).

Long-lasting intake of ethanol induces the death of hippocampal neurons which was found not only at the end of ethanol exposition (18-day) but also in other age groups (35- and 90-day). Dying neurons were characterized by condensed, fragmented nucleus, which is often associated with apoptotic cell death (Kerr *et al.* 1972, Wozniak *et al.* 2004). Regarding our findings of the fragmented nuclei of the neurons in the hippocampal regions CA1 and CA3 and in the ventral and dorsal blades of the dentate gyrus in all experimental animals (18-, 35- and 90-day-old animals), the possible role of apoptotic mechanism in cell death after ethanol intake can be assumed.

Wozniak *et al.* (2004) found that administration of ethanol to rodents during the period of synaptogenesis induces extensive apoptotic neurodegeneration in the developing brain and that this neurotoxicity can explain the reduced brain mass.

If we compared the width of the granular and pyramidal cell layers in control 18- and 35-day-old animals, we detected a strong decrease in the width of the nerve cell layer in the 35-day-old animals. However, we did not observe any significant difference in the width of the examined areas between 35- and 90-day-old animals. As there were no apparent differences in the size of

pyramidal and granular cells between the control and experimental animals, it was possible to conclude that the number of nerve cells was reduced.

In the ethanol-exposed animals of all ages, we observed smaller width of the nerve cell layer in all the studied areas (CA1, CA3, VB DG, DB DG). The rate of loss of the neural cells was similar (from 35 % to 49 %) in all monitored areas and in all age groups. This could indicate that the long-term ethanol consumption is accompanied by the same type of degeneration of neural cells in all age groups and all experimental groups.

The effects of ethanol on the cerebellum have been documented in great detail, and changes in neurogenesis, neuronal morphology and enhanced cell death of differentiated neurons were demonstrated. Changes in the gross anatomy of the forebrain have been described (Roebuck *et al.* 1998). Recent experimental results suggest that apoptosis during the period of physiological neuronal death in the forebrain is enhanced in neonatal rats exposed to ethanol (Ikonomidou *et al.* 2000, Nowoslawski *et al.* 2005). The first few postnatal weeks in rats roughly correspond to the last trimester of human embryonic development (Dobbing and Sands 1979). This increased apoptosis is likely to be an important component of the human fetal ethanol syndrome (Ikonomidou *et al.* 2000).

Degenerated neurons were present in all the observed areas of the hippocampus. The highest density of the degenerating cells was observed in the group of 18-day-old animals, lower density of the degenerating neurons was present in the group of 35-day-old animals. In the group of the 90-day-old animals no Fluoro-Jade B-positive neurons were observed.

We can conclude that the long-time exposure to ethanol during the perinatal period induces serious morphological changes in the hippocampal system and the majority of these changes persist till adulthood.

## Conflict of Interest

There is no conflict of interest.

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