

Changes of Hippocampal Neurons after Perinatal Exposure to Ethanol

(Perinatal Ethanol Abuse and Hippocampal Neurons)

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Short title: Ethanol and changes of hippocampal neurons

Summary

The effect of ethanol on the structural development of the central nervous system was studied in offspring of Wistar rats, drinking during pregnancy and till the 28th day of their offspring 20% ethanol. The structural changes in the hippocampus and dentate gyrus were analysed at the age of 18, 35 and 90 days.

In all experimental animals a lower width of pyramidal and granular layers in comparison to control animals, cell extinction (Fluoro-Jade B positivity) and a fragmentation of numerous nuclei were found. 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. At the age of 90 days only cells with

fragmented nuclei (= degenerating) were observed. Our experiments confirmed vulnerability of the developing central nervous system to the intake of ethanol during perinatal period and revealed long-lasting degeneration process in hippocampus and dentate gyrus.

Key words: ethanol, degeneration, apoptosis, hippocampus, dentate gyrus

Introduction

Ethanol is a psychotropic drug (Nutt 1996) and its chronic abuse causes impairments of the brain function, such as visual, motor and memory dysfunction (Alling 1999, Dahchour *et al.* 2000, Schummers *et al.* 1997)

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, Schlessinger *et al.* 1978, Byer 1980a, Byer 1980b) and they may remain vulnerable when exposed to different neurotoxins during early life (Hort *et al.* 1999, Miki *et al.* 2004, Milotová *et al.* 2006, Langmeier *et al.* 2003). The long hippocampal postnatal development, resulting from the prolonged proliferation of granular cells (Bayer *et al.* 1982) allows studying changes that arise by the interference in the development during prenatal and early postnatal life (Pokorný *et al.* 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 the long-term perinatal exposure to ethanol.

Methods

All experiments were carried 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 only the solution of 20% ethanol every day since the conception 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 offspring were separated from their mothers.

At the age of 18, 35, and 90 days animals were perfused under deep thiopental anaesthesia 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 1 hour into 20% sucrose for cryoprotection. Brains were sliced in the frontal plane into 40 µm 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 combinations of DNA staining Hoechst and Fluoro – Jade B, which (was originally described by Schmued and Hopkins) (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 staining rack (one slide/slot for even staining) and immersed in 100% ethanol solution for 3 minutes, in 70% ethanol solution for 1 minute, in distilled water for 1 minute, in 0.01%

Potassium Permanganate (KMnO_4) for 15 minutes 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 minutes gently shaking, rinsed in distilled water three times for 1 minute. Slides were then immersed in 0.01% Hoechst 33342 staining solution for 10 minutes 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 the 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[®] 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 mm and 4.0 mm posterior to the bregma, L =2 and P=2 mm according to Fífková 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 10 animals from each age group (18, 35, 90-day-old) always in 3 sections and 3 adjacent regions at 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 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. The level of significance was set at $p < 0.0001$.

Results

The average daily consumption of the 20% ethanol solution was 41.6 ± 1.4 ml per animal. Litters born to the mothers exposed to ethanol were smaller being 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 ± 0.54 g, control group 5.5 ± 0.60 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 they were accompanied with high 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 fine fragmented nuclei nor degenerating (Fluoro-Jade B positive) cells were found (Fig. 1, 2).

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

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 was smaller in experimental animals than in controls in all age groups (Tab. 1, ANOVA $p \leq 0.0001$). There were no apparent differences in the size of the pyramidal and granular cells between the control and experimental animals (Fig. 4, 5, 6).

Discussion

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

Several experimental studies have shown that hippocampus appears to be particularly vulnerable to the effects of ethanol exposure during the 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-, 90-day). Dying neurons were characterized by condensed, fragmented nuclei, as is often associated with apoptotic cell deaths (Wozniak *at al.* 2004, Kerr *at al.* 1972). 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 the all experimental animals (18-, 35- and 90-days old animals), the possible role of apoptotic mechanism in the cell death after ethanol intake can be assumed.

Wozniak and co-workers 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 (Wozniak *at al.* 2004).

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 of the width of the nerve cell layer in the 35-day-old animals. However, we didn't observe any statistically significant difference in the width of the followed areas between 35-day and 90-day-old animals. As there were no apparent differences in the size of the 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 the all studied areas (CA1, CA3, VB DG, DB DG). The rate of loss of the neural cells was similar (from 35% to 49%) in the all monitored areas and in all age groups. It could mean that the long-term ethanol consumption is accompanied by the same type of degeneration of the neural cells in all age groups and all experimental groups.

Effects of ethanol on the cerebellum have been documented in great details, and changes in neurogenesis, neuronal morphology and enhanced cell death of differentiated neurons was demonstrated (Ward and Wesst 1992). Changes in the gross anatomy of the forebrain have been described (Ward and Wesst 1992, Roebuck *at 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 *at al.* 2000, Nowoslawski *at 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 foetal ethanol syndrome (Ikonomidou *at al.* 2000).

Degenerated neurons were present in the all observed areas of hippocampus. The highest density of the degenerating cells was observed in the group of the 18-day-old animals, lower density of the degenerating neurones was 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 exposition to ethanol during perinatal period induces serious morphological changes in hippocampal system and majority of those changes persists till adulthood.

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Age of animals	Group	CA1(μm)	CA2 (μm)	VB DG (μm)	DB DG (μm)
18 days	experimental group	53.594 \pm 9.1607	69.249 \pm 12.2773	68.307 \pm 9.3809	61.774 \pm 7.1572
	control group	89.773 \pm 7.3237	111.992 \pm 11.2491	100.970 \pm 7.4932	99.824 \pm 7.1612
	decrease	40. 00%	37. 73%	32. 35%	38. 12%
35 days	experimental group	42.276 \pm 4.8123	60.478 \pm 8.6918	59.355 \pm 5.8266	49.271 \pm 10.1052
	control group	64.703 \pm 5.5662	97.250 \pm 9.0949	91.533 \pm 7.0693	87.834 \pm 6.8897
	decrease	34. 67%	37. 81%	35. 15%	49. 91%
90 days	experimental group	38.751 \pm 5.9868	51.174 \pm 6.8259	59.442 \pm 7.3987	48.279 \pm 7.0065
	control group	72.041 \pm 9.6430	90.119 \pm 8.0235	88.851 \pm 9.1531	74.600 \pm 7.3247
	decrease	46. 21%	43. 22%	34. 57%	35. 29%

Tab. 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.

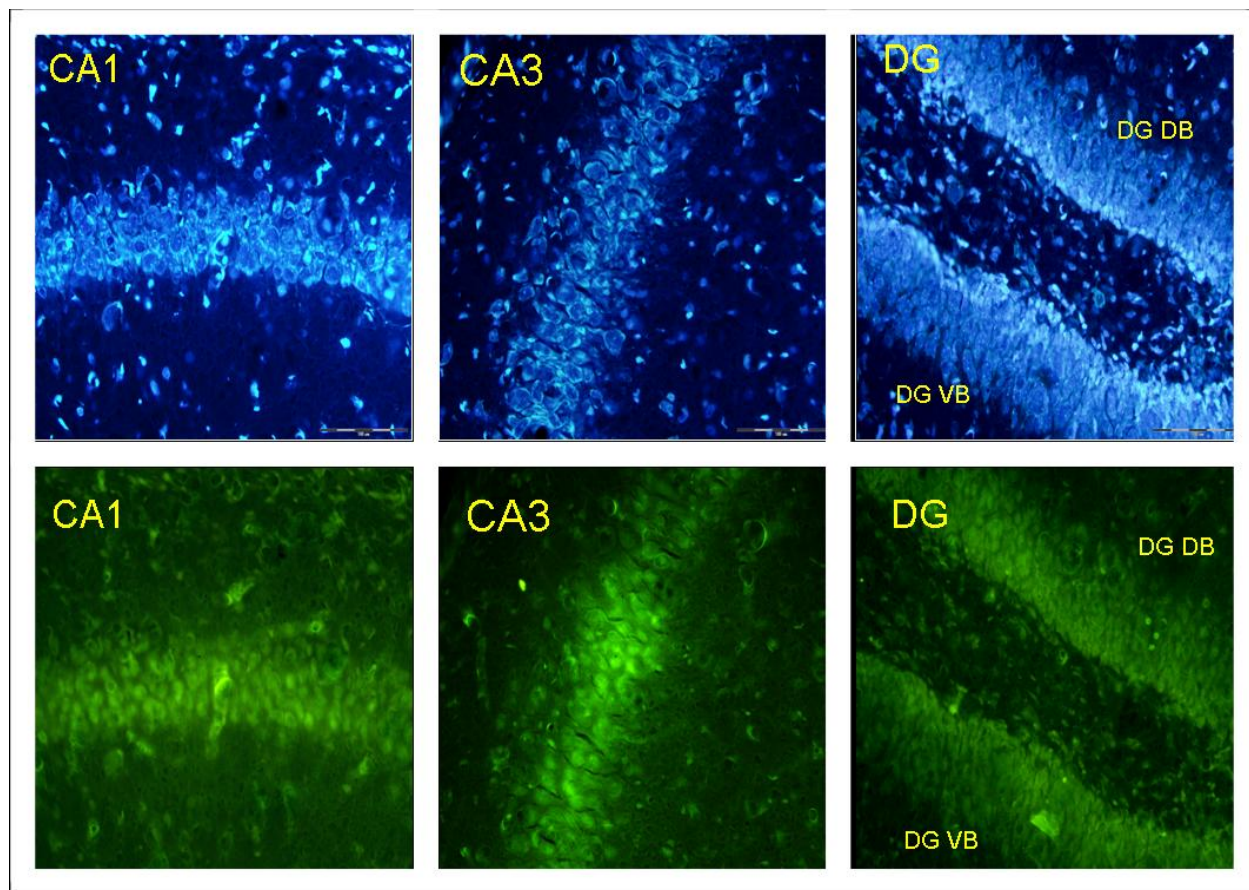


Fig. 1: Experimental 18-day-old animal. Detail of the CA1 and CA3 area of the hippocampus (CA1, CA3) and ventral and dorsal blade of the dentate gyrus (VB DG, DB DG) in the AP plane 3 mm posterior to bregma. Hoechst and Fluoro-Jade B staining. Neuronal degeneration (FJ-B positive neurons) – green colour,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

Magnification: 40x

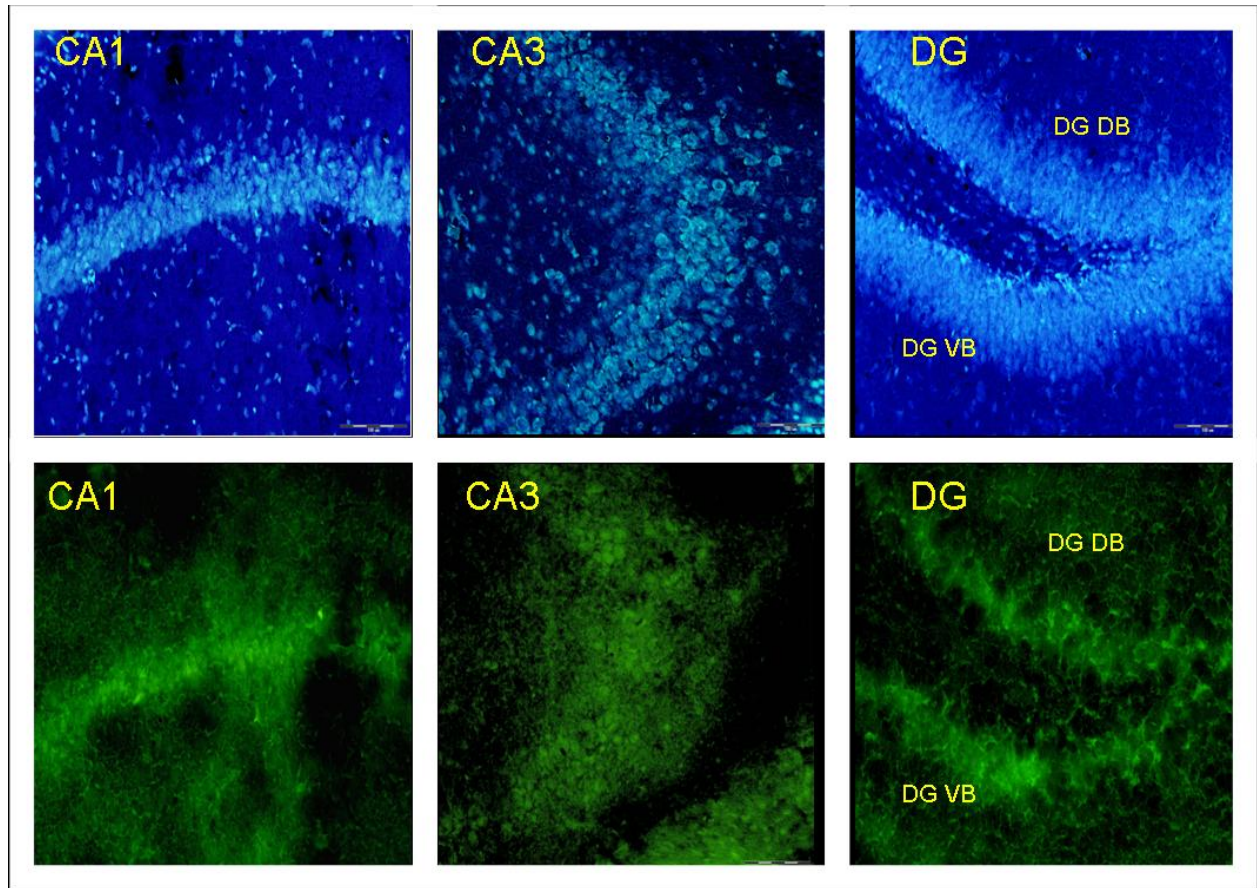


Fig. 2: Experimental 35-day-old animal. Detail of the CA1 and CA3 area of the hippocampus (CA1, CA3) and ventral and dorsal blade of the dentate gyrus (VB DG, DB DG) in the AP plane 3 mm posterior to bregma. Hoechst and Fluoro-Jade B staining. Neuronal degeneration (FJ-B positive neurons) – green colour,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

Magnification: 40x

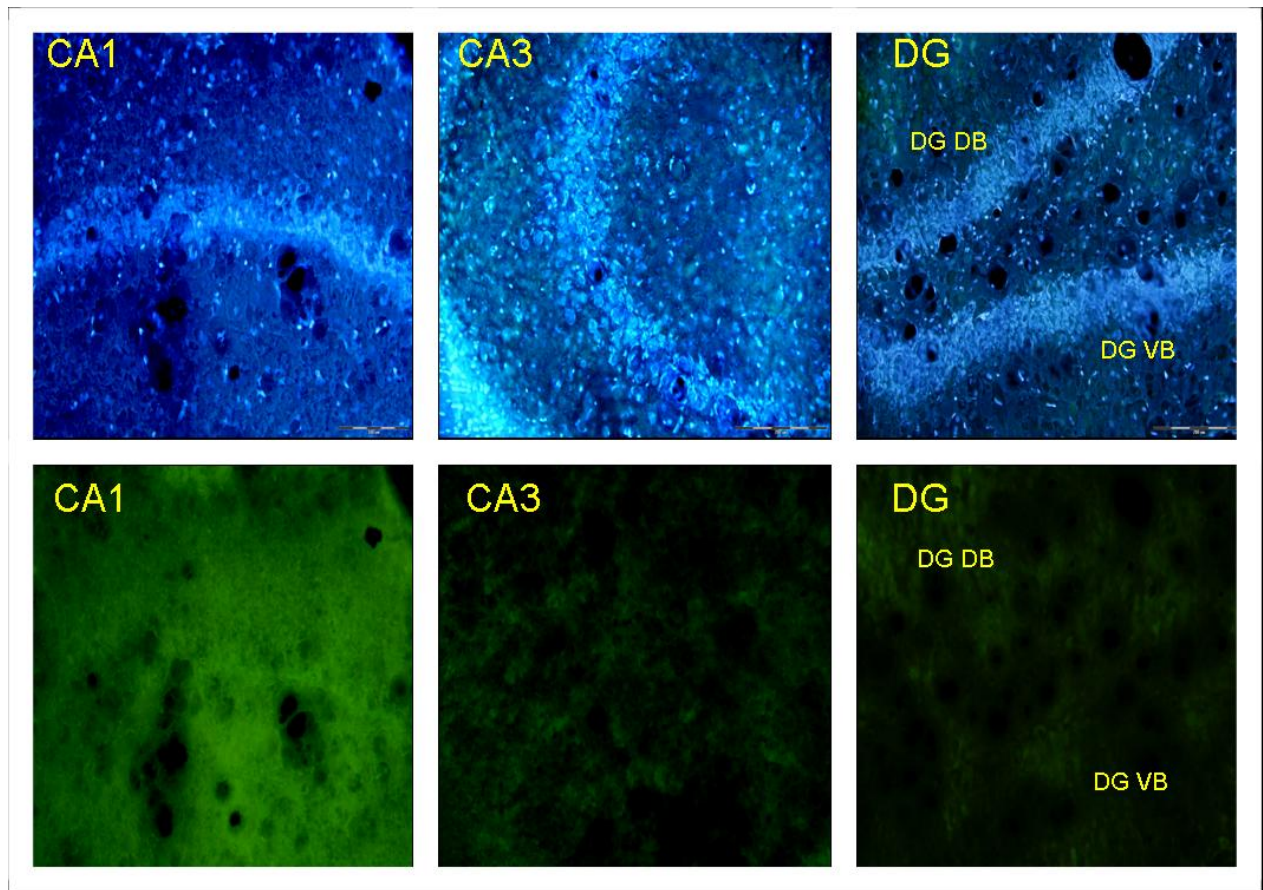


Fig. 3: Experimental 90-day-old animal. Detail of the CA1 and CA3 area of the hippocampus (CA1, CA3) and ventral and dorsal blade of the dentate gyrus (VB DG, DB DG) in the AP plane 3 mm posterior to bregma. Hoechst and Fluoro-Jade B staining. Neuronal degeneration (FJ-B positive neurons) – green colour,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

Magnification: 40x

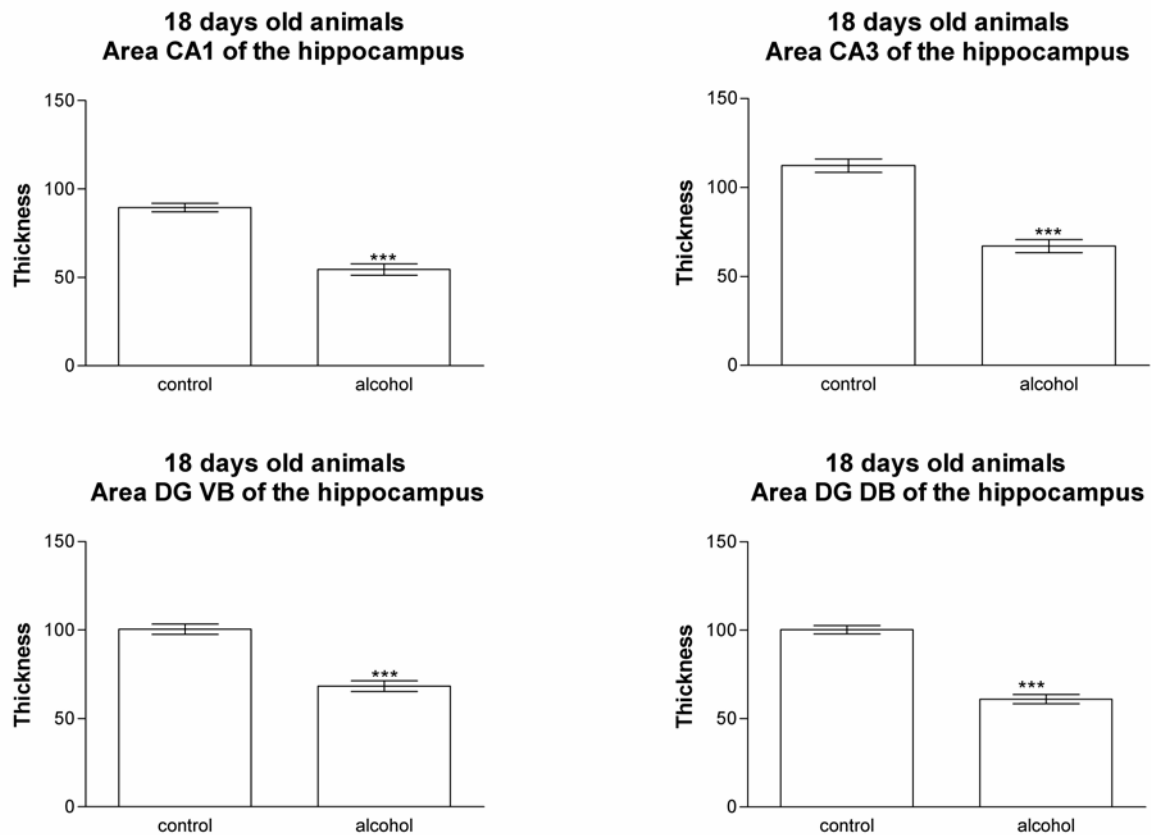


Fig. 4: Effect of the perinatal ethanol abuse on the width of the pyramidal layer in the hippocampus and the granule cell layers in the dentate gyrus of 18-day-old rats.

*** Significance level of differences at $p \leq 0.0001$

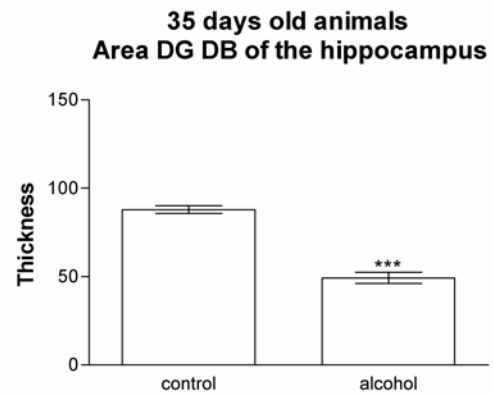
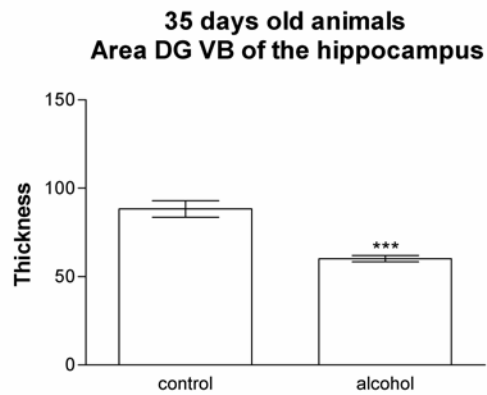
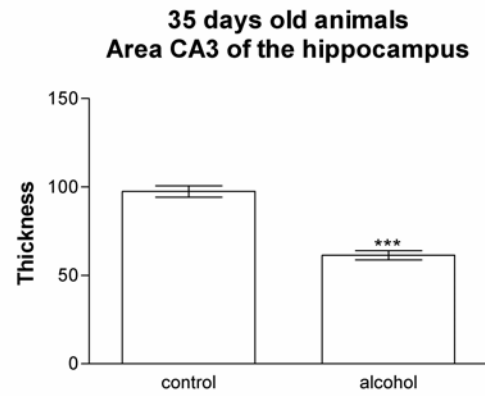
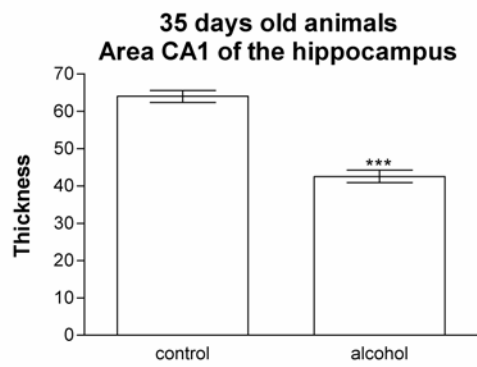


Fig. 5: Effect of the perinatal ethanol abuse on the width of the pyramidal layer in the hippocampus and the granule cell layers in the dentate gyrus of 35-day-old rats.

*** Significance level of differences at $p \leq 0.0001$

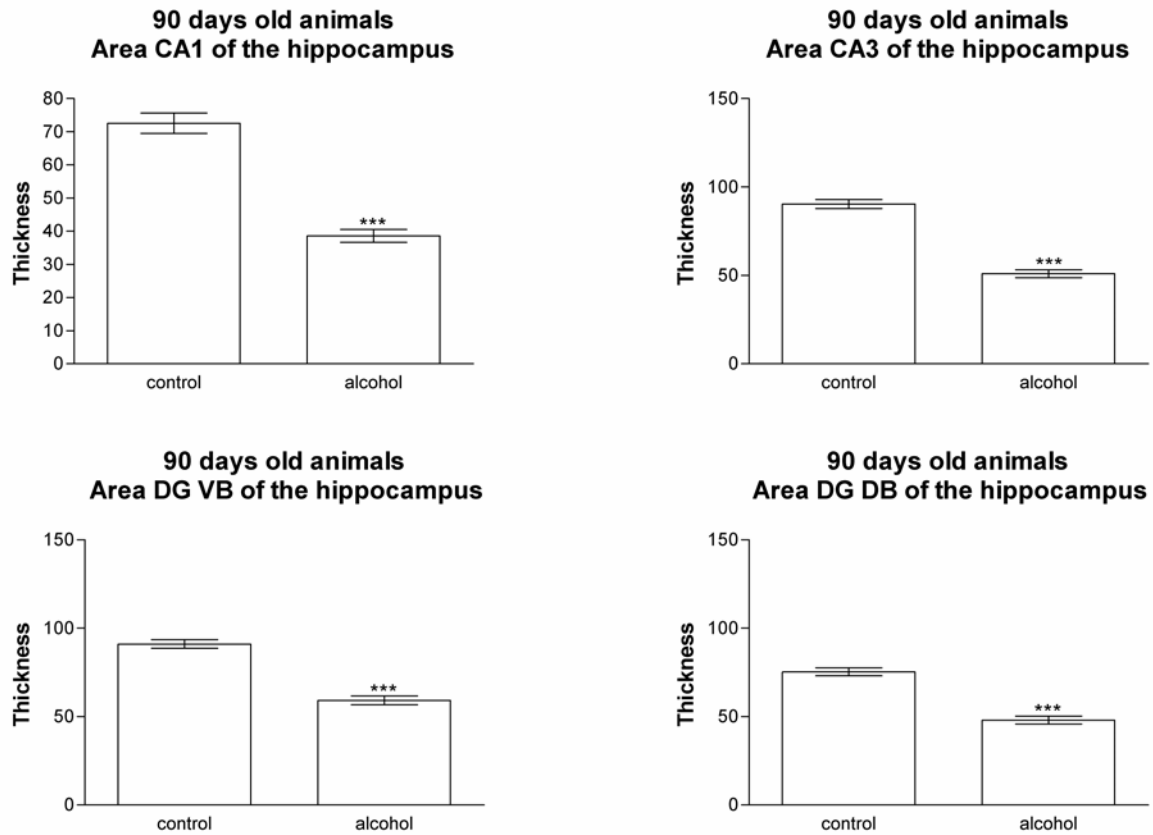


Fig. 6: Effect of the perinatal ethanol abuse on the width of the pyramidal layer in the hippocampus and the granule cell layers in the dentate gyrus of 90-day-old rats.

*** Significance level of differences at $p \leq 0.0001$