Alcohol Abuse in Mothers during Gravidity and Breastfeeding Brings Changes of Hippocampal Neurons in their Offspring

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Abstract: Neurotoxic effect of ethanol on the CNS of laboratory rats in the prenatal and postnatal period was studied. Another aim of the experiment was to analyse structure of the hippocampus after the prenatal and postnatal exposure to alcohol and to identify the most vulnerable hippocampal regions. Pregnant Wistar rats of our own breed received 20% alcohol p.o. *ad libitum* every day since the conception to the 18th day of postnatal life of their offspring. Since the birth (the day 1) till the age of 18 days offspring were kept together with their mother and were exposed to postnatal alcohol effect (alcohol in the breast milk). At the age of 18 days animals were perfused under deep thiopental anaesthesia with buffered solution of paraformaldehyde. Serial sections were stained with Fluoro-Jade B and DNA specific dye bis-benzimide (Hoechst No 33258).
Brains of young rats aged 18 days were analysed under the light microscope Olympus Provis AX-70 with epifluorescence. In CA1 and CA3 areas and in Gyrus dentatus of the hippocampus, groups of degenerating cells were observed. In all offspring some cells with fine granulated karyons were identified, which were accompanied with high numbers of glial cells. Our results demonstrate the neurotoxic effects of alcohol and the high vulnerability of the developing CNS. The identification of cells with segmented karyons indicates the role of apoptotic mechanism in the cell death.

Introduction

It is known that alcohol is a neurotoxic substance with teratogenic effect. It can cause changes of some molecular, neurochemical and cell processes. Alcohol abuse has many long-term effects that result in premature death and in increased propensity for serious illnesses [1]. The most vulnerable regions of the Central Nervous System (CNS) are neocortex, cerebellum and hippocampus. The hippocampus is a structure that lies deep within the temporal lobe of the brain and is involved in memory. Although the precise function of the hippocampus in specific aspects of memory is controversial, it probably plays a role in the consolidation of memories [2]. It is well known that the hippocampus is one of the target sites for neurotoxic effect of ethanol during brain development [3, 4, 5, 6, 7, 8, 9]. Hippocampal pyramidal neurons are generated during late gestation from the ventricular zone [10, 11, 12, 13] and they may remain vulnerable when exposed to ethanol during early life [14]. The long hippocampal postnatal development allows studying changes that arise by the interference in the development during prenatal and early postnatal life. In this study we focused on specific defects in the CA1, CA3 area of the hippocampus and in the dorsal and ventral blade of dentate gyrus after long-term prenatal exposure to ethanol.

Methods

Female Wistar rats of our own breed were used for the experiments. Two animal groups used in the experiment:

- animals exposed to alcohol (experimental group)
- animals not exposed to alcohol (control group)

Each group consisted of 24 animals. Pregnant Wistar rats of our own breed received 20% ethanol, p.o. *ad libitum*, every day since the conception to the 18th day of postnatal life of their offspring. Since the birth (the day 1) till the age of 18 days offspring were kept together with their mother and were exposed to postnatal alcohol effect (alcohol in breast milk). 20% concentration of ethanol was selected on the basis of preliminary experiments. Animals of the control group of pregnant Wistar rats were not exposed to alcohol, they drank pure water. At the age of 18 days animals (only males) 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 than stained with combinations of DNA staining Hoechst and Fluoro-Jade B. DNA staining Fluoro-Jade B as originally described by Schmued and Hopkins and this staining enables identification of dying neurons [16]. 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 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 staining solution for 10 minutes and dehydrated (by alcohol line), cover-slipped using D.P.X. Neutral Mounting Medium and allowed to dry. Fluoro-Jade B positive neurons were observed in four regions of the hippocampal formation: i) in CA1 area of the hippocampus, ii) in CA3 area of the hippocampus, iii) in the dorsal blade of the dentate gyrus, iv) in the ventral blade of the dentate gyrus. Material was examined and Fluoro-Jade B positive neurons quantified under the light microscope Olympus Provis AX-70 with epifluorescence.

Results

At the age of 18 days, positive groups of degenerating cells were observed in the CA1 area of the hippocampus as visualized by DNA staining Hoechst (Colour figure 3) and Fluoro-Jade B staining (Colour figure 4). In the CA3 area of the hippocampus degenerating cells were also found (Colour figure 6). Some cells with fine granulated karyons were identified, which were accompanied with high numbers of glial cells (Colour figure 5). In the dorsal and ventral blades of the

dentate gyrus we identified many cells with fine granulated nucleus in DNA staining Hoechst (Colour figure 7) and Fluoro-Jade B positive neurons (Colour figure 8).

In the control group neither cells with fine granulated nuclei nor degenerating cells were found. The thickness of the pyramidal cell layer in the areas CA1, CA3 and the thickness of granule cell layer in gyrus dentatus was smaller in experimental animals than in controls. In the experimental group the decrease of cells density was found in all areas of the hippocampus.

Discussion

At present, the effects of ethanol on the immature nervous system are incompletely understood [17]. Several experimental studies have shown that hippocampus appears to be particularly vulnerable to the effects of ethanol exposure during early life [3, 16]. Observation of the cell loss in all areas of the hippocampus in the experimental group and detection of degenerating cells with fine segmented karyons confirm and demonstrate the neurotoxic effects of alcohol and the high vulnerability of the developing CNS. It is know that chronic ethanol treatment induced death of hippocampal neurons. Dying neurons were characterized by condensed, fragmented nuclei, which are often associated with apoptotic process [17, 18].

Our identification of cells with fine segmented nucleus indicates the role of apoptotic mechanism in the cell death but further research is required to confirm or deny this hypothesis.

Effects of ethanol on the cerebellum have been documented in great details, and the previous work has demonstrated changes in neurogenesis, neuronal morphology and revealed enhanced cell death of differentiated neurons [19]. Changes in the gross anatomy of the forebrain have been described [19, 20]. The details of cellular changes in the forebrain in response to ethanol are unclear but recent evidence suggests that in neonatal rats exposed to ethanol apoptosis during the period of physiological neuronal death in the forebrain is enhanced [21].

Because the first two postnatal weeks in rats roughly correspond to the last trimester of human intrauterine development [22] the increased apoptosis can be expected to be also an important component of human foetal alcohol syndrome [21].

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