MORPHOLOGY AND PATHOMORPHOLOGY

Immunohistochemical Analysis of Active Caspase-3 Expression in Structures of Neonatal Brain

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The hippocampal fields of neonatal rats differ by the level of active caspase-3: dentate gyrus >CA3>CA1>CA2. In the dentate gyrus it was 70% of its maximum value in the cortex, while in CA2 it corresponded to the minimum level in the brain stem. Taking into account the role of caspase-3 in apoptosis, these differences can indicate different intensity of programmed cell death in different fields of the forming hippocampus.

Key Words: *active caspase-3; brain; hippocampus; ontogeny*

Natural programmed cell death (apoptosis) is essential for the formation of orderly structure of the brain during ontogeny [13]. Caspase-3 is the key figure in apoptosis. This cystein protease is present in cells in the form a proenzyme; limited proteolysis results in the formation of active caspase-3, which leads the cell to death. During ontogeny, the processes of proliferation, migration, and differentiation of brain neurons paralleled by intensive elimination of excessive cells start in the caudal compartments and then are unfolded in the rostral structures [7]. During the early postnatal period, these processes are close to completion in the brainstem, but are still in progress in the cortex, hippocampus, and cerebellum. This is seen from different levels of active caspase-3 detected by immunoblotting in these regions [9]. At the same time, it is impossible to evaluate the expression of the enzyme in individual fields of the hippocampus (a structure with an intricate organization) by immunoblotting. These regions can vary by cell viability [3,5,8,10] and by heretofore not studied expression of active caspase-3 during the early ontogeny.

The aim of our study was immunohistochemical detection of active caspase-3 in individual fields of the hippocampus and comparison of the expression of this protease in different brain compartments of neonatal rats.

MATERIALS AND METHODS

The study was carried out on 4-day-old Wistar rats. The brain was removed in animals in a state of deep anabiosis induced by cooling to 10°C after transcardial perfusion with phosphate buffer with 0.9% NaCl (PBS pH 7.4) and then with 4% paraformaldehyde diluted with PBS (pH 7.4). The brain was postfixed in the same fixative for 4 h, washed in several portions of PBS, submerged completely in 10 and then 30% sucrose (at 4°C), and frozen on dry ice with isopentane cooled to -42°C. Brain sections (15 μ) were sliced on a cryostat at -20°C in the frontal plane. Sections of different brain compartments (frontal: pyramidal layer of the hippocampal gyruses, brain cortex above the hippocampus; caudal: nuclei caeruleus of the brainstem and

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cerebellar cortex) were mounted on polylysine-coated slides, dried, and stored at -70°C until immunohistochemical reactions. The borders of anatomical structures were visualized after Nissl staining using rat brain atlas [11].

Active caspase-3 was detected by the peroxidase-antiperoxidase immunohistochemical method. Before the reaction, the slides with sections were dried at ambient temperature, washed in PBS with 0.2% Triton X-100 (PBST), treated with 0.3% H_2O_2 in 100% methanol for 30 min, and washed in 3 portions of PBS, after which treated with blocking solution for 1 h (3% BSA in PBST).

Brain sections were incubated for 12 h at ambient temperature with first polyclonal rabbit antibodies (Abcam) specific to active caspase-3 form diluted 1:100 in blocking solution. After washing in two portions of PBST, the sections were incubated for 12 h with biotinilated goat anti-rabbit IgG antibodies (1:500; Abcam). After washing in PBS, the sections were incubated with streptavidin conjugated with horseradish peroxidase (Abcam) diluted 1:1000, washed in PBS, and peroxidase activity was detected with 3,3'-diaminobenzidene solution (5 mg/ml) containing 0.03% H_2O_2 in 0.2 M Tris buffer (pH 7.6).

Digital photographs of the preparations were made on an Axioskop 2 Plus microscope (Carl Zeiss) with an AxioCam camera. The content of active caspase-3 was evaluated by optical density of immunoreactive substance in the cells, estimated using Scion Image software. A total of 300 cells were analyzed for each structure of the brain from 8 animals.

The results of densitometry were statistically processed by one-way dispersion analysis.

RESULTS

Immunohistochemistry revealed differences in the expression of active caspase-3 in neonatal brain structures (Fig. 1). This parameter also varied in different hippocampal fields. The highest level in the hippocampal structures was detected in the dentate gyrus: 70% of the maximum level in the cortex. The level of active caspase-3 in CA2 field was as low as in the brainstem: 5-fold lower than in the cortex and 3-fold lower than in the dentate gyrus. The levels of active caspase-3 expression in the hippocampal CA1 and CA3 fields were intermediate between the dentate gyrus and CA2 field. Judging from the morphology of stained cells, this protein is located mainly in neuronal bodies and processes. Caspase-3 locally activates the apoptosis in dendrites and synaptic terminals (which is observed in brain injuries [12]) and is presumably involved in the synaptic plasticity processes [1]. High expression of caspase-3 in the neonatal brain is associated with intensive fragmentation of DNA, the key sign of apoptosis [6,9].

The distribution of active caspase-3 in compartments of the neonatal brain clearly reflects their heterochronic formation in the ontogeny. The development of brain structures is characterized by cell proliferation and migration to definitive sites of location, synaptogenesis, and gliogenesis. These processes paralleled by elimination of excessive cells with participation of active caspase-3 start and are completed during the ontogeny in the brainstem and then develop in more rostral brain compartments [7]. The level of caspase-3 mRNA in the brain of newborn rats is significantly higher than in adult animals [2,4]. Reduction of the expression of this protease in the postnatal ontogeny coincides with completion of synaptogliogenesis. Synaptogenesis in the hippocampus, cerebellum, and brain cortex of rats starts during the 2nd week of life, when it is close to completion in the brainstem. The caudorostral gradient of brain formation manifests in differences in the levels of active caspase-3 (detected by immunoblotting [9]) in brain structures and is confirmed by our present findings.

Despite simultaneous formation, the neonatal hippocampal fields exhibit different intensity of staining by antibodies to active caspase-3: maximum in the pyramidal neurons of the dentate gyrus and CA3 field, weaker in CA1 field, and very low in CA2 field. This can indicate different liability of cells in these brain structures to apoptosis. Loss of neurons caused by kainate [5], trauma [8], or epileptogenic convulsive activity [3] is more intensive in the hippocampal fields with high expression of caspase-3. On the other hand, CA2 field neurons retain viability in spontaneous [3] and pilocarpine-induced convulsive activity [10]. These results indicate that ranking of hippocampal fields by the level of active caspase-3 expression and hence, by liability of their cells to death, is determined during the neonatal ontogeny. It is presumably retained during later periods of life, because distribution of active caspase-3 in the hippocampus of adult monkeys detected recently [14] was similar to that obtained in the present study.

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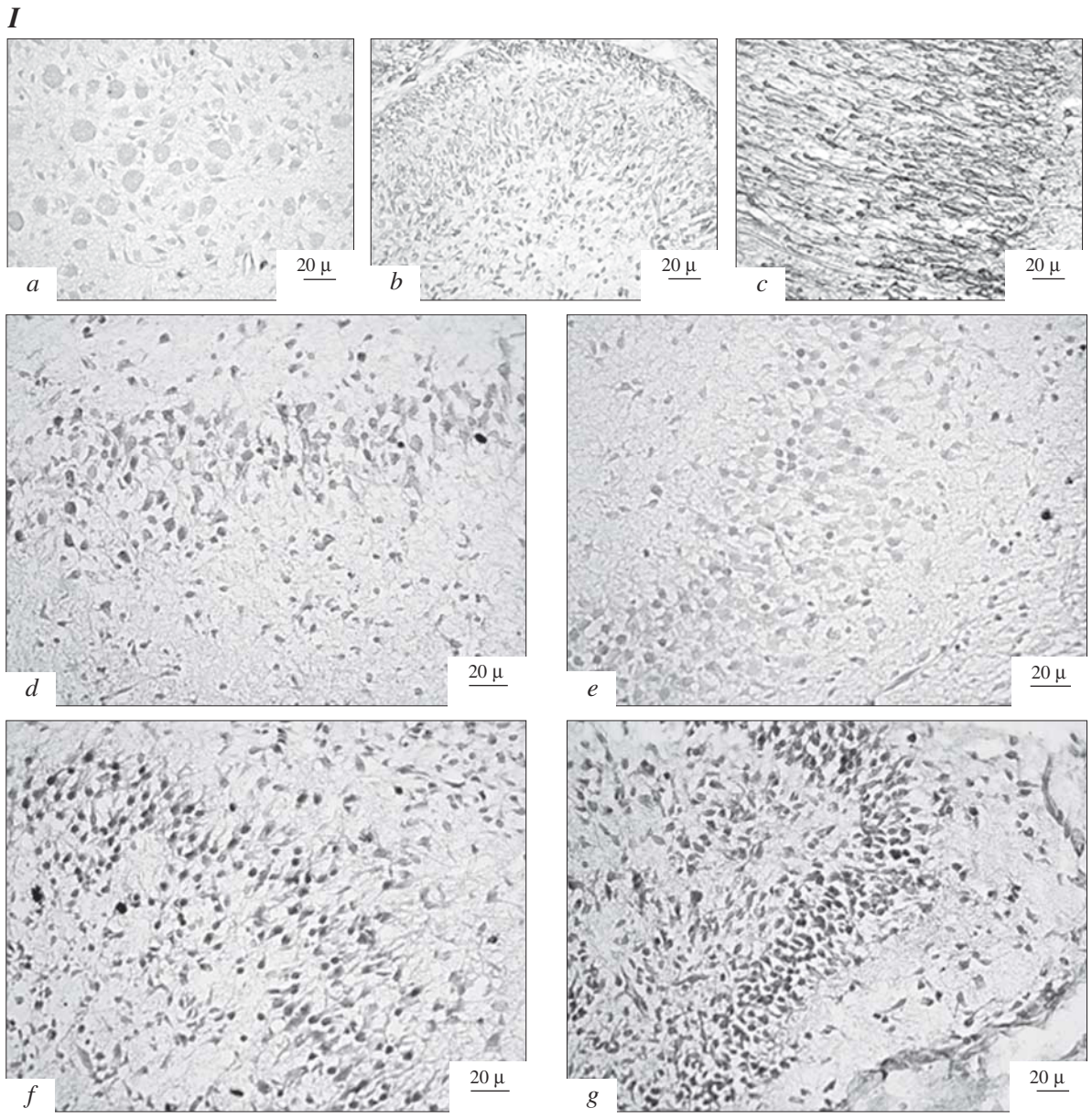

Fig. 1. Immunohistochemical staining (*1*) and results of densitometry (*II*) of active caspase-3 in the brain of 4-day-old Wistar rats. *I*: *a*) brainstem; *b*) cerebellum; *c*) brain cortex; *d-g*) hippocampus. *II*: *1*) brainstem; *2*) cerebellum; *3*) brain cortex. DG: dentate gyrus. $p<0.05$ compared to: *brainstem and hippocampal CA2 field, **all structures except DG. The line on the photographs is 20 μ-long.

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