Content of Apoptotic Enzyme Caspase-3 mRNA in Brain Stem and Cortex in Rats during Postnatal Ontogeny

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The content of caspase-3 mRNA in rat brain stem decreased from birth to postnatal week 3 and dropped below reverse transcription-PCR sensitivity limit in 1.5-month-old animals. The number of brain stem cells in 2-40-day-old rats was constant. The content of caspase-3 mRNA in the cortex was higher than in the brain stem and decreased by one-third by postnatal day 40. The number of cells in the cortex decreased 2-fold during postnatal week 1 and then remained unchanged. Changes in the content of caspase-3 mRNA did not correlate directly with variations in the number of brain cells during postnatal ontogeny.

Key words: *apoptosis; caspase-3; mRNA; ontogeny; brain*

Normally, more than half of initially formed brain neurons undergo apoptosis during ontogeny of the mammalian nervous system [6]. Activation of a cascade of proteolytic enzymes caspases (cystein proteases) is an obligatory component of apoptosis and an indicator of its activity [12]. In the nervous tissue, irreversible cell death is determined by activation of caspase-3 (*cpp 32*), which serves as the main messenger and terminal effector of apoptosis [6,12]. Enhanced expression and activity of this enzyme were observed during apoptosis induced by brain ischemia, hypoxia, and trauma [5,9,11,14]. Caspase-3 knockout mice have hypertrophied brain with abnormal structure and die during perinatal ontogeny [7]. It is known that brain development in characterized by the caudo-rostral gradient [1]. However, little is known about changes in caspase-3 mRNA expression in brain structures maturating at different terms. Here we studied caspase-3 mRNA expression in the brain stem and cortex in rats and correlation of this parameter with the number of cells in these brain structures.

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MATERIALS AND METHODS

The study was performed on 21-day-old fetuses and 2-40-day-old Wistar rats. The animals were kept under standard vivarium conditions. Brain stem including hindbrain and pons region (caudal from the posterior colliculi and rostral from the oval orifice excluding the cerebellum), and frontal cortex (1.5-3.0 mm thick layer) were isolated. These specimens had the same anatomical borders, but increased in weight with brain growth. The number of cells in the isolated brain specimens was evaluated by DNA content [8]. Brain tissue was homogenized in cold 2 M NaCl on 0.05 M phosphate buffer (pH 7.4) and fluorescent intercalating dye Hoechst 33258 (Fluka) was added to a final concentration of 1 μg/ml. Fluorescence was recorded at excitation and emission wavelengths of 458 and 356 nm, respectively. DNA content was calculated using standard DNA solution (10 μg/ml, Calbiochem).

Caspase-3 mRNA level was determined by semiquantitative reverse transcription-PCR of total RNA isolated from the brain tissue by single-step guanidine-isothiocyanate precipitation [2]. cDNA was obtained using Oligo(dT) primer and Gibco BRL revertase (Life Technologies). PCR was carried out in a buffer containing 100 mM Tris-HCl (pH 8.0), 2.5 mM $MgCl_2$, 0.01 M 2-mercaptoethanol, 0.01% Tween-20, 0.5 mM

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dNTP mixture, 100 pm caspase-3 primers [11] (direct 5'-aagccgaaactcttcatc-3' and reverse 5'-tgagcattgacac aatacac-3'), 50 pm β-actin primers [10] (direct 5'-tccctcatgccatcctgcgt-3' and reverse 5'-gga accgctcatt gccgata-3'), and 2 U Taq-polymerase (SibEnzim). The lengths of caspase-3 and β-actin fragments were to 349 and 255 base pairs (b.p.), respectively. PCR was performed in a Master Cycler Gradient amplifier (Appendorf): 30 sec at 95°C, 20 sec at 62°C, and 30 sec at 72°C. Preliminary experiments showed that under these conditions, the amount of caspase-3 and β-actin PCR products progressively increased from the 25th to 40th cycles. Thirty-five amplification cycles under selected conditions provided linear dependency between the amount of cDNA and the yield of PCR product for both genes. The level of caspase-3 was evaluated in relation to β-actin expression. PCR products were separated by electrophoresis in 1.5% agarose gel, the gels were stained with ethidium bromide and scanned in UV (BioDoc II, Biometra GmbH) with subsequent computer densitometry.

The dependence of the examined parameters on animal age was analyzed by single-factor dispersion analysis (ANOVA), significance of intergroup differences was evaluated by multiple Scheffe test.

RESULTS

The content of caspase-3 mRNA in rat brain stem changes significantly during postnatal ontogeny (Fig. 1, *a*; $F_{(3,9)}$=58.01, p<0.00001): it is high in 2- and 5-day-old rat pups, sharply decreases in 24-day-old rats, while in 1.5-month-old animals caspase-3 mRNA cannot be detected by reverse transcription-PCR in the selected regimen. This age-related decrease in caspase-3 expression in the rat brain stem agrees with the data of *in situ* hybridization demonstrating decreased signal of caspase-3 mRNA hybridization in mouse brain stem structures starting from postnatal day 12 [3]. In addition to *in situ* hybridization data, our findings suggest that the content of caspase-3 mRNA in the brain stem sharply decreases by the end of rearing period and that expression of caspase-3 gene is terminated after completion of morphogenetic processes in the central nervous system [1].

The number of brain stem cells evaluated by DNA content remained constant during the examined period of ontogeny ($F_{(4,26)}$=0.54, p<0.716). Brain stem cells proliferate only during embryogenesis [1], therefore, our data revealed no changes in their total number after birth. Thus, the decrease in caspase-3 mRNA content in the brain stem during postnatal ontogeny occurs against the background of unchanged cell number.

Intensive elimination of cells in rat brain cortex occurs during the first week after birth [3]. We revealed a sharp and highly significant ($F_{(4,29)}$=10.67, p<0.00002) decrease in cell number in the cortex between postnatal days 2 and 7 (Fig. 1, *b*). The expression of caspase-3 mRNA in the brain cortex during this period significantly surpassed that in the brain stem ($F_{(1,25)}$=175.3, p<0.00001). However, it seems unlikely that these regional differences in caspase-3 mRNA are related to peculiarities of the formation of cell population in the examined brain regions, because high expression of caspase-3 gene persisted from postnatal day 7 through 40, when the cell number remained practically unchanged. The content of this transcript in 1.5-month-old rats decreased by only 30%

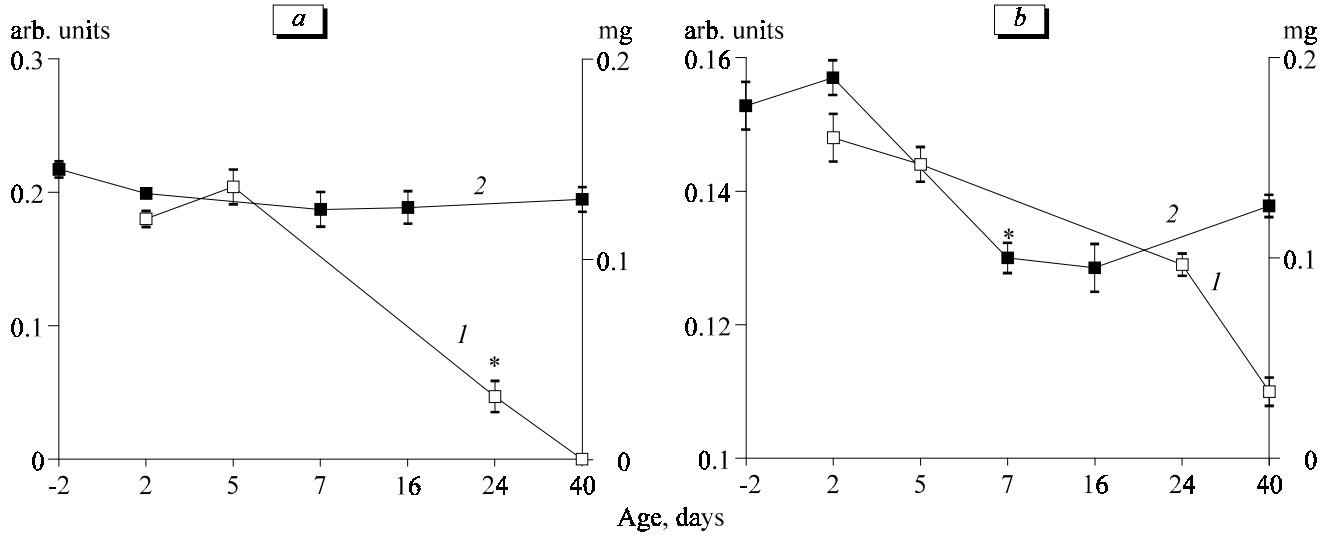

Fig. 1. Content of caspase-3 mRNA (*1*, left ordinate) and DNA (*2*, right ordinate) in the brain stem (*a*) and cortex (*b*) during ontogeny. *p<0.05 compared to previous age.

compared to 2-day-old pups. However, these age-related differences in the expression of caspase-3 gene in the brain cortex were significant ($F_{(3,10)}$=5.91, p<0.014).

According to the current views, brain cortex of adult mammals contains polypotent cells, which can proliferate and differentiate into neurons, astro- and oligodendrocytes [4]. This implies the existence of mechanisms eliminating excessive young cells or cells destined to be replaced by newly formed cells [6]. This can explain high content level of caspase-3 mRNA in the brain stem compared to that at all examined terms of ontogeny, which agrees with the results obtained on adult brain [13]. High content of caspase-3 mRNA after postnatal day 7 against the background of unchanged cell number attests to inactive state of this enzyme in cortical cells. On the other hand, high transcript content probably indicates higher potencies to apoptosis in cortical cells compared to brain stem cells.

Thus, the present study revealed pronounced regional peculiarities in the dynamics of caspase-3 mRNA level during ontogeny. Whereas in the brain stem, transcript content by postnatal month 1.5 decreased below the reverse transcription-PCR sensitivity threshold, it persisted at a high level in the cortex. The level of caspase-3 mRNA showed no correlations with changes in the cell number in brain structures during ontogeny. The peculiarities of caspase-3 mRNA expression in the brain cortex can underlie enhanced neurodegeneration in the forebrain after ischemia, stroke, and hypoxia.

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