Regional Features of the Expression of Genes Involved in Neurogenesis and Apoptosis in the Brain of Adult Rats

V. V. Kolobov, Z. I. Storozheva, M. A. Gruden, and V. V. Sherstnev

Translated from *Byulleten' Eksperimental'noi Biologii i Meditsiny*, Vol. 153, No. 5, pp. 707-711, May, 2012
Original article submitted March 5, 2011

The expression of mRNA of genes involved in neurogenesis and apoptosis (*Apaf1*, *Ascl1*, *Bax*, *Bcl2*, *Casp3*, *Casp8*, *Casp9*, *Dffb*, *Myh10*, *Naip2*, *Napa*, *Notch2*, *Numb*, *Pura*, *S100a6*, *Tnf*) in the prefrontal cortex, hippocampus, and cerebellum was studied in adult rats. The content of mRNA of these genes (except *Apaf1*) was several-fold higher in the cerebellum than in the hippocampus and brain cortex. In the hippocampus, the expression of *Apaf1* was significantly lower than in the prefrontal cortex, while the expression of *Ascl1*, *Pura*, *S100b*, and *Tnf* was higher. Regional differences in the direction, strength, and numbers of significant correlations between the expression of the studied genes were detected. Documented differences in gene expression were regarded as validation of the structural and functional cooperation of neurogenesis and apoptosis at the molecular genetic level.

Key Words: *mRNA expression; neurogenesis; apoptosis; adult brain; Wistar rats*

Numerous studies have demonstrated the continuity and mutual support of the processes of neuronal and glial cell proliferation, migration, differentiation, and programmed death in human and animal nervous system. Neurogenesis and neuroapoptosis have been documented for many compartments of the brain and spinal cord of adult mammals [2,6,9]. Thousands of genes are expressed in the cerebral structures, including the genes regulating neurogenesis and apoptosis [5,12-14]. However, the intra- and inter-regional expression of the genes involved in the formation, development, survival, and death of new cells in the adult brain has not been studied, while studies of these specific features of gene expression seem to be highly significant for the detection of the molecular genetic mechanisms of the structural and functional interactions of neoneurogenesis and neuroapoptosis and for understanding of their role in the integrative functions of CNS in health and disease.

We studied the profiles of expression of 17 neurogenesis and apoptosis genes and the relationships between the expression of these genes in the prefrontal cortex, hippocampus, and cerebellum of adult male rats.

P. K. Anokhin Institute of Normal Physiology, Russian Academy of Medical Sciences, Moscow, Russia. ***Address for correspondence:*** f.neurochemistry@gmail.com. V. V. Kolobov

MATERIALS AND METHODS

The study was carried out on 3-month-old male Wistar rats (n=9; 220-250 g) from Stolbovaya Breeding Center. The animals were kept in cages 4-5 per cage with free access to fodder and water at 12:12 h light:darkness(regimen. The rats were adapted to vivarium conditions for 2 weeks before the study. The animals were kept and handled in accordance with regulations of the EC Council (Directive 86/609/EEC of November 24, 1986).

The prefrontal cortex, hippocampus, and cerebellum (median horizontal fragment including both hemispheres and the vermis) were rapidly isolated in the cold (4°C) after decapitation. In accordance with the recommended protocols, total RNA fraction was isolated using TRIzol (Invitrogen), the RNA preparations were then purified from genome DNA admixture by DNase treatment (RQ1 RNase-Free DNase, Promega). The concentration of RNA was measured on a Qubit fluorometer (Invitrogen).

Reverse transcription was carried out with 50 ng total RNA, 200 U revertase M-MLV and oligo-$(dT)_{15}$ with 0.5 U RNAse inhibitor (Promega) for 1.75 h at 37°C. The resultant complementary DNA was diluted 10-fold with deionized water and stored at -80°C. Samples with deionized water instead of RNA served as the negative control.

The real-time PCR was carried out repeatedly for every sample, the test volume 25 µl, with 1 µl diluted cDNA, 0.5 µl ready primer mixture (SABiosciences), 5 µl qPCRmix-HS SYBR (Eurogen), and 18.5 µl deionized water. The protocol was as follows: amplification at 94°C, 1.5 min; 50 cycles: denaturation at 94°C, 30 sec; primer annealing at 58°C (-64°C for ***Actb***), 15 sec; elongation at 72°C, 30 sec.

The amount of mRNA was evaluated by automated registration of the threshold cycle (C_t) by the amplifier. No amplification was found in the negative control samples. Averaged C_t values of each sample's repeats were used to calculate the level of gene mRNA expression (R) by the $2^{-\Delta\Delta Ct}$ and $2^{-\Delta\Delta Ct'}$ methods suggested previously [10]. The R values determined by $2^{-\Delta\Delta Ct}$ method could not be used to detect the correlations between the genes in the prefrontal cortex, as the R values for all test genes were fixed and equal to 1. The R values determined by $2^{-\Delta\Delta Ct'}$ method allowed detection of correlations between the genes in all structures, including the prefrontal cortex, as the R values for all experimental genes varied.

TABLE 1. Structural and Functional Relationships between the Levels of Relative Expression of Gene mRNA (r_s)

Brain compartment	Spearman's rank correlation coefficient	
	positive correlations	negative correlations
Prefrontal cortex	*Apaf/Ascl1* (0.68), Apaf1/Bax **(0.92)**, *Apa1/Naip2* (0.78), *Ascl1/Bax* (0.78), *Bax/Naip2* (0.75), *Bax/Numb* (0.68), Bcl2/Dffb **(0.97)**, *Naip2/Numb* (0.82), Notch2/Tnf **(0.93)**, Notch2/Actb **(0.9)**, *Tnf/Actb* (0.82)	*Bcl2/Napa* (-0.72), Casp3/Casp9 **(-0.83)**, *Casp3/Myh10* (-0.68), *Casp3/Gapdh* (-0.72), *Dffb/Pura* (-0.68), *Napa/Notch2* (-0.7), *Napa/Tnf* (-0.68), *Napa/Actb* (-0.72)
Hippocampus	*Apaf1/Tnf* (0.72), Ascl1/Casp8 **(0.87)**, *Ascl1/Dffb* (0.78), Ascl1/Napa **(0.98)**, Ascl1/Pura **(0.93)**, Bcl2/Casp3 **(0.87)**, Bcl2/Casp9 **(0.85)**, Bcl2/Myh10 **(0.97)**, Bcl2/Naip2 **(0.93)**, Bcl2/Notch2 **(0.9)**, *Bcl2/Numb* (0.78), Bcl2/Gapdh **(0.87)**, Casp3/Casp9 **(0.93)**, Casp3/Myh10 **(0.87)**, Casp3/Naip2 **(0.93)**, Casp3/Notch2 **(0.93)**, Casp3/Gapdh **(0.85)**, Casp8/Dffb **(0.92)**, Casp8/Napa **(0.83)**, Casp8/Pura **(0.88)**, Casp9/Myh10 **(0.88)**, Casp9/Naip2 **(0.92)**, Casp9/Notch2 **(0.9)**, Casp9/Gapdh **(0.89)**, *Dffb/Napa* (0.8), *Dffb/Pura* (0.73), Myh10/Naip2 **(0.88)**, Myh10/Notch2 **(0.83)**, *Myh10/Numb* (0.68), Myh10/Gapdh **(0.87)**, Naip2/Notch2 **(0.97)**, *Naip2/Numb* (0.75), Naip2/Gapdh **(0.88)**, Napa/Pura **(0.92)**, Notch2/Gapdh **(0.88)**	*S100a6/Tnf* (-0.78)
Cerebellum	Apaf1/Bcl2 **(0.87)**, *Apaf1/Casp3* (0.8), *Apaf1/Naip2* (0.73), *Apaf1/Tnf* (0.78), *Ascl1/Dffb* (0.7), *Ascl1/S100a6* (0.82), *Bax/Casp8* (0.73), *Bax/Dffb* (0.77), Bcl2/Casp3 **(0.83)**, Bcl2/Naip2 **(0.8)**, Casp3/Naip2 **(0.83)**, *Casp3/Tnf* (0.68), Casp8/Dffb **(0.87)**, Casp9/Myh10 **(0.83)**, *Casp9/Notch2* (0.73), *Casp9/Numb* (0.82), *Dffb/S100a6* (0.77), *Myh10/Naip2* (0.68), Myh10/Notch2 **(0.87)**, Myh10/Numb **(0.88)**, *Naip2/Notch2* (0.78), *Naip2/Numb* (0.7), *Naip2/Tnf* (0.72), *Napa/Pura* (0.75), Napa/Gapdh **(0.87)**, Notch2/Numb **(0.98)**, Pura/Gapdh **(0.92)**	*Apaf1/Napa* (-0.68), *Ascl1/Casp9* (-0.78), Ascl1/Myh10 **(-0.93)**, *Ascl1/Notch2* (-0.73), *Ascl1/Numb* (-0.75), Bax/Casp9 **(-0.87)**, *Bax/Napa* (-0.72), *Bax/Pura* (-0.68), *Bax/Gapdh* (-0.68), *Casp8/Casp9* (-0.8), *Casp8/Notch2* (-0.68), *Casp8/Numb* (-0.78), Casp9/Dffb **(-0.87)**, Casp9/S100a6 **(-0.88)**, *Dffb/Myh10* (-0.75), *Dffb/Notch2* (-0.72), *Dffb/Numb* (-0.8), Myh10/S100a6 **(-0.95)**, *Napa/Tnf* (-0.72), Notch2/S100a6 **(-0.92)**, Numb/S100a6 **(-0.93)**, *Tnf/Gapdh* (-0.68)

Note. Significant r_s values at $p<0.05$ are presented. Values at $p<0.01$ are shown with bold letters.

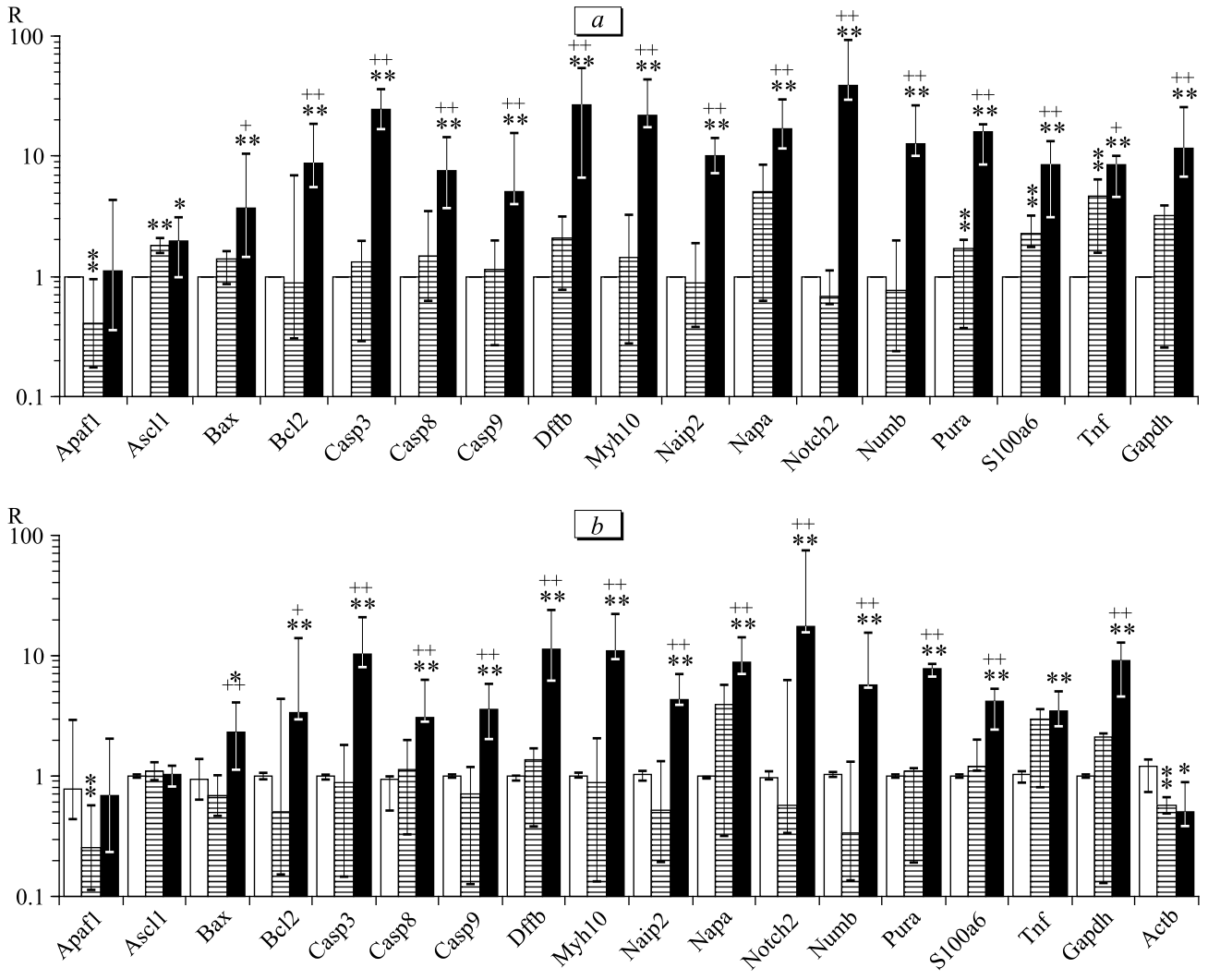

Fig. 1. Regional profiles of neurogenesis and apoptosis genes expression in the rat brain structures (median±25-75 quartiles). Logarithmic scale ordinate: level of gene mRNA expression: *a*) standardization with consideration for *Actb* reference gene by the $2^{-\Delta\Delta Ct}$ method; *b*) standardization without consideration for reference gene by the $2^{-\Delta\Delta Ct}$ method. Light bars: prefrontal cortex; cross-hatched bars: hippocampus; dark bars: cerebellum. *$p<0.05$, **$p<0.01$ in comparison with prefrontal cortex; $^{+}p<0.05$, $^{++}p<0.01$ in comparison with hippocampus.

The data were statistically processed using Statistica 7.0 software by nonparametric statistical methods, as the values of mRNA expression did not conform to the normal distribution law. The significance of differences between the two samples was verified by Mann–Whitney's *U* test. Correlations between the median levels of mRNA expression were detected using Spearman's ranked coefficient (r_S).

In order to determine the group of genes for further studies, *in silico* analysis of Gene Ontology database (GO, www.geneontology.org) [3] was carried out for genes whose protein products were functionally related during neurogenesis and apoptosis. The gene selection algorithm was based on the functional classification of genes (and their products) by biological processes in which they were involved. Three main categories for neurogenesis were chosen as the starting points: neuroblast proliferation (GO:0007405), development of the nervous system (GO:0007399) and brain (GO:0007420); and 6 categories were chosen for apoptosis: apoptosis regulation (GO:0042981), induction (GO:0006917), and repression (GO:0006916), neuronal apoptosis (GO:0051402) with its positive (GO:0043525) and negative (GO:0043524) regulation.

The following genes were selected: *Apaf1* (apoptosis peptidase activation factor 1), *Ascl1* (Achaete-scute complex, homolog 1 (Drosophila)), *Bax* (Bcl2-associated X protein), *Bcl2* (B cell, CLL/Lymphoma 2), *Casp3* (caspase-3), *Casp8* (caspase-8), *Casp9* (caspase-9), *Dffb* (fragmenting DNA factor β-polypeptide), *Myh10* (nonmuscle myosin, heavy chain 10), *Naip2* (NLR family, apoptosis inhibitory protein 2), *Napa* (N-ethylmaleimide sensitive factor-α), *Numb* (Numb homolog (Drosophila)), *Pura* (purine-rich domain

binding protein A), and *Tnf* (TNF-α). The study also included *Notch2* and *S100a6* genes, their protein products involved in Ca binding and regulation of Ca^{2+}-dependent neurogenesis and apoptosis processes. The reference gene candidates were β-actin *Actb* and glyceraldehyde-3-phosphate dehydrogenase *Gapdh* genes, the kevels of their mRNA being constant in tissues under conditions of various kinds of exposure. According to GO, *Gapdh* gene is linked to the studied neurogenesis and apoptosis genes in 3 biological processes: 1) neuronal apoptosis (GO:0051402): *Apaf1*, *Bax*, *Bcl2*, and *Casp3*; 2) apoptotic process (GO:0006915): *Bax*, *Bcl2*, *Casp3*, *Casp8*, *Dffb*, and *Tnf*; 3) development of multicellular organism (GO:0007275): *Apaf1*, *Notch2*, and *Tnf*. By contrast, *Actb* gene is involved in just one biological process: axonogenesis (GO:0007409) cross-linked with the studied genes *Bcl2*, *Myh10*, and *Numb*. Hence, *Actb* was selected as the reference gene.

RESULTS

The expression profiles of the studied genes differed significantly in brain structures of adult rats (Fig. 1). In the cerebellum, the levels of mRNA for all the studied genes (except *Apaf1*) were several-fold higher than in the prefrontal cortex and (except *Apaf1* and *Ascl1*) in the hippocampus. The expression of *Casp8*, *Myh10*, *Napa*, and *Notch2* was maximum. In the hippocampus, the level of *Apaf1* mRNA was significantly lower and of *Pura*, *S100a6*, and *Tnf* mRNA higher than in the cortex. High expression of neurogenesis genes in adult brain, specifically, in the hippocampus, striatum, cerebellum, and cortex was described [5,12,14]. Some authors suggested a relationship between the gene transcription activity profiles in the cerebellum and its specific involvement in mechanisms of training and memory, realized simultaneously with involvement of the forebrain, hippocampus, and cortical structures [4,8].

We carried out analysis of correlations of neurogenesis and apoptosis genes expression in various compartments of adult rat brain. Clear-cut local differences in the direction, strength, and number of correlations between the expressions of the studied genes were detected (Table 1). The maximum number of significant correlations ($p<0.05$) was documented for the cerebellum: 49 (27 positive and 22 negative ones). In the prefrontal cortex and hippocampus, 19 and 36 significant correlations were found, respectively. Of these, 8 significant negative correlations were detected in the prefrontal cortex and 1 in the hippocampus. Importantly that these local quantitative parameters of relationships between gene expression levels were in general comparable to our previous data on correlations between immunobiochemical parameters of neurogenesis/apoptosis processes in the respective cerebral structures of adult male Wistar rats [1]. The following correlations were common for the cerebellum and hippocampus: *Myh10*/*Numb*, *Napa*/*Pura*, *Ascl1*/*Dffb*, *Bcl2*/*Casp3*, *Casp9*/*Myh10*, *Myh10*/*Naip2*, *Apaf1*/*Tnf*, *Bcl2*/*Casp3*, *Bcl2*/*Naip2*, *Casp8*/*Dffb*, *Bcl2*/*Naip2*, *Casp3*/*Naip2*; for the cerebellum and prefrontal cortex: *Apaf1*/*Naip2*, *Napa*/*Tnf*, *Casp3*/*Myh10*, *Casp3*/*Casp9*. Only one gene pair, *Naip2*/*Numb*, exhibited significant positive correlations in all the examined brain structures. The Numb protein is assumed to play the key role in the maintenance of neuronal precursor cell population in the course of brain development [11,15]. The Naip family proteins suppress the neuronal differentiation and apoptosis, which was shown *in vitro* [7]. It seems that direct relationship between transcription activities of *Numb* and *Naip2* genes, detected *in vivo* in our experiments, reflected the evolutionally conservative aspect of the structural and functional neoangiogenesis and neuroapoptosis interactions.

In general, our data confirm the concept of inseparable conjugation of neurogenesis/apoptosis processes in adult brain and indicate the local specificity of their interactions at the molecular genetic level.

REFERENCES

1. V. V. Sherstnev, V. V. Yurasov, Z. I. Storozheva, and M. A. Gruden', *Neirokhimiya*, **25**, No. 4, 309-316 (2008).
2. D. N. Abrous, M. Koehl, and M. Le Moal, *Physiol. Rev.*, **85**, No. 2, 523-569 (2005).
3. M. Ashburner, C. A. Ball, J. A. Blake, *et al.*, *Nat. Genet.*, **25**, No. 1, 25-29 (2000).
4. E. S. Boyden, A. Katoh, and J. L. Raymond, *Annu. Rev. Neurosci.*, **27**, 581-609 (2004).
5. C. Burger, M. C. Lopez, H. V. Baker, *et al.*, *Neurobiol. Learn. Mem.*, **89**, No. 4, 379-396 (2008).
6. H. Frielingsdorf and H. G. Kuhn, *Deb. Neurosci.*, **1**, 33-41 (2007).
7. R. Götz, C. Karch, M. R. Digby, *et al.*, *Hum. Mol. Genet.*, **9**, No. 17, 2479-2489 (2000).
8. O. Hikosaka, K. Nakamura, K. Sakai, and H. Nakahara, *Curr. Opin. Neurobiol.*, **12**, No. 2, 217-222 (2002).
9. H. Landgren and M. A. Curtis, *J. Cell. Physiol.*, **226**, No. 1, 1-7 (2011).
10. K. J. Livak and T. D. Schmittgen, *Methods*, **25**, No. 4, 402-408 (2001).
11. Q. Qu and Y. Shi, *J. Cell. Physiol.*, **221**, No. 1, 5-9 (2009).
12. C. Stansberg, A. O. Vik-Mo, R. Holdhus, *et al.*, *BMC Genomics*, **8**, 94 (2007).
13. D. Torbati, B. R. Totapally, A. Raszynski, *et al.*, *Neuropediatrics*, **39**, No. 4, 219-225 (2008).
14. M. A. Zapala, I. Hovatta, J. A. Ellison, *et al.*, *Proc. Natl. Acad. Sci. USA*, **102**, No. 29, 10,357-10,362 (2005).
15. W. Zhong, M. M. Zhiang, G. Weinmaster, *et al.*, *Development*, **124**, No. 10, 1887-1897 (1997).