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BIOCHEMISTRY, BIOPHYSICS, AND MOLECULAR BIOLOGY

Gene Expression and Content of Enzymes of Noradrenaline Synthesis in the Rat Organ of Zuckerkandl at the Critical Period of Morphogenesis

A. R. Murtazina[a, *], Yu. O. Nikishina[a], N. S. Bondarenko[a], A. Ya. Sapronova[a], E. V. Volina[a], and Academician of the RAS M. V. Ugrumov[a, b]

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Abstract—Gene expression and content of the key enzymes involved in the synthesis of noradrenaline—tyrosine hydroxylase and dopamine beta-hydroxylase—was evaluated in the organ of Zuckerkandl of rats in the critical period of morphogenesis. High levels of mRNA and protein of both enzymes in the perinatal period of development and their sharp decline on day 30 of postnatal development were detected. These data indicate that the synthesis of noradrenaline in the organ of Zuckerkandl is maximum during the critical period of morphogenesis and decreases during the involution of this paraganglion.

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At all stages of ontogeny, the functions of the Organism are ensured by endogenous chemical signals acting through specific receptors on target cells. Noradrenaline (NA) is one of such chemical signals, which has a wide range of physiological effects in adult organisms and functions as a morphogenic factor in the development of vital organs [1, 2]. Noradrenaline is synthesized in the neurons of the brain, sympathetic nervous system, adrenal chromaffin tissue, and paraganglia [3, 4]. These organs at different stages of ontogeny, to a greater or lesser extent, are sources of NA in the general circulation system. During the perinatal development, which is a critical period of morphogenesis, there exist both permanent sources of NA in blood (primarily, the adrenal glands) and temporary sources. One of such transient sources of NA in blood, according to our data [5], are developing brain neurons before the closure of the blood–brain barrier (BBB) (in rodents, until the end of the second week of life [6]). An important source of NA in the perinatal ontogeny is extraadrenal chromaffin cells located in paraganglia, the largest of which is the organ of Zuckerkandl (OZ), which is located in the abdominal aortic bifurcation zone [7]. The chromaffin cells of OZ produce all enzymes of synthesis of NA as a final product: tyrosine hydroxylase (TH), L-aromatic amino acid decarboxylase (AAAD), and dopamine beta-hydroxylase (DBH). These enzymes are found already in the migrating neural crest primordium on day 11 of prenatal development [8]. The organ of Zuckerkandl in rats reaches maximum development in the perinatal period and undergoes involution by the end of the second week of postnatal development. The causes and mechanism of its involution are not clear [7].

An important characteristic of the OZ in ontogeny as a source of NA in blood is its ability to synthesize NA, which is determined by the content and activity of the enzymes involved in its synthesis.

Based on this, the goal of this paper was to assess the molecular genetic mechanisms of NA synthesis in the OZ in ontogeny of rats in the critical period of morphogenesis.

The objectives of the study were to determine the expression level of the genes encoding the enzymes of NA synthesis (TH and DBH) and the content of enzymes of NA synthesis (TH and DBH) in rats during ontogeny.

Experiments were performed on male Wistar rats on embryonic day 21 (E21) and on postnatal days 3 (P3) and 30 (P30). The rats were kept under standard vivarium conditions with an ad libitum access to food and water. The OZ was isolated using the published data on its location in rats [7]. The animals were anesthetized with chloral hydrate (Sigma-Aldrich, United States) at a dose of 400 mg/kg.

The content of TH and DBH mRNA in the OZ was determined by real-time PCR, which was performed in an automated thermocycler 7500 Real-Time PCR System (Applied Biosystems, United States) using the qPCRmix-HS SYBR + ROX mixture (Fermentas,

[a] *Koltzov Institute of Developmental Biology, Russian Academy of Sciences, Moscow, 119334 Russia*
[b] *Department of Psychology, Faculty of Social Sciences, The National Research University Higher School of Economics, Moscow, 101000 Russia*
* *e-mail: aliya_mr89@mail.ru*

United States) and specific oligonucleotide primers (Evrogen and Litekh, Russia): the forward primer 5'-AGTCTGGCCTTCCGCGTGTTTCAA-3' and the reverse primer 5'-CCCCCAGAGATGCAAGTCCAATG-3' for TH and the forward primer 5'-GCCGAAATCTGGAATCCGCATCTT-3' and the reverse primer 5'-TGGGGGCTGTAGTGGTTGTCTCTG-3' for DBH. The expression values of the genes encoding these enzymes were normalized to the expression level of the housekeeping gene *GAPDH*, which was determined using the forward primer 5'-CTGACATGCCGCCTGGAGAAA-3' and the reverse primer 5'-TGGAAGAATGGGAGTTGCTGTTGA-3'. Relative gene expression values were calculated by the ΔΔCt method taking into account the PCR efficiency, which was determined by constructing standard curves [9].

The content of TH and DBH in the OZ was determined by Western blot analysis. The OZ was homogenized in RIPA buffer (50 mM Tris-HCl, 150 mM NaCl, 0.1% sodium dodecyl sulfate, and 1% NP40 (pH 8.0)) using an Labsonic M ultrasonic homogenizer (Sartorius AG, Germany) and centrifuged at 2000*g* and 4°C for 20 min. The protein content in the supernatants was determined with BCA. Then, the supernatant (100 μL) was supplemented with β-mercaptoethanol (5 μL) and boiled for 5 min. The resulting clarified homogenates were used for Western blotting analysis. Electrophoresis was performed according to Laemmli [10]. Each lane contained an equal amount of protein (20 μg of TH and 40 μg of DBH). The commercial kit Thermo Scientific PageRuler Plus Prestained Protein Ladder (Thermo Scientific, United Science) was used as a molecular weight marker. The proteins were transferred onto a nitrocellulose membrane for 1 h 15 min at 290 mA in a transfer buffer (25 mM Tris-HCl (pH 7.5), 192 mM glycine, and 20% ethanol). The transfer quality was assessed after staining with Ponceau S. The membranes were washed from the dye in TNT buffer (10 mM Tris-HCl (pH 7.5), 150 mM NaCl, and 0.1% Tween 20). To prevent the nonspecific adsorption of antibodies, the membranes were incubated in a blocking solution (5% skim milk and 0.1% Tween 20) for 1 h at room temperature and then for 10 h with mouse monoclonal antibody to TH (1 : 1500, Sigma-Aldrich) and sheep polyclonal antibodies to DBH (1 : 1000, Abcam, United Kingdom) at 4°C. After incubation, the membranes were washed several times in TNT buffer and incubated with the secondary antibodies (conjugated to horseradish peroxidase; Thermo Scientific) to mouse IgG (1 : 50 000) and sheep IgG (1 : 50 000) at 20°C for 2 h. After incubation with the second antibodies, the membranes were washed several times with TNT buffer and developed by the standard method using the enhanced chemiluminescence kit ECL Western Blotting Detection Reagent (Amersham, United States). X-ray films were scanned, and the resulting images were processed using the ImageJ software, evaluating the integral absorption of protein bands. The content of DBH and TH was expressed as a percentage of the total protein content in the control sample (in this case, the brain tissue of rats on postnatal day 30).

The results were statistically processed using the GraphPad Prism6 program. The significance of differences was estimated using ANOVA and the Wilcoxon–Mann–Whitney test.

The extraadrenal chromaffin tissue is located in paraganglia, which rank second after adrenal glands as a source of circulating catecholamines. Unlike the adrenal glands, the largest of paraganglia, OZ, secretes primarily NA. It was assumed that the OZ, whose functional activity is maximum in the prenatal and early postnatal periods, it essential for the normal development of the cardiovascular system [11]. Given that the number of TH-immunopositive chromaffin cells in the OZ tissue in mice and rats is maximum during the perinatal development and decreases many times approximately at the end of the first month of life [7, 8], in this study we evaluated the synthesis of NA in the indicated periods. The expression of genes and the content of two main enzymes of NA synthesis (TH and DBH) in the OZ in E21 and P3 periods, i.e., during the maximum development of the OZ, as well as in the P30 period, when the OZ undergoes involution, were considered as indices characterizing the synthesis of NA in the OZ.

Tyrosine hydroxylase catalyzes the rate-limiting stage of synthesis of all catecholamines. Therefore, the regulation of gene expression and the synthesis and activity of this enzyme is the key stage that controls the synthesis of these important biogenic amines. Dopamine beta-hydroxylase is expressed only in the cells that synthesize NA and adrenaline. In the chromaffin tissue of the OZ, these enzymes detected immunohistochemically already during the prenatal development [8].

The results of the study of expression of TH and DBH genes are shown in Fig. 1. The TH mRNA content in the OZ on E21 was 3.32 ± 0.63 arb. units. On day P3, we observed a nonsignificant decrease in this index, which was probably due to the large sample variability. By day P30, the TH mRNA content significantly decreased 9.7 times, which was most likely due to the involution of the OZ. Given that the OZ in rats undergoes involution at the end of the second week of life, we do not expect to detected NA synthesis enzymes in the OZ on day P30. It should be noted that our data obtained in this series of experiments differ from the data obtained by the authors of [8], where several small paraganglia surrounded by blood vessels were found in rats of the same age. This finding allowed the authors of the cited paper to assume that these paraganglia can function as an endocrine tissue throughout life. The DBH mRNA content in the OZ on E21 was 1.93 ± 0.43 arb. units. Then, on day P3, as in the case of TH, we observed a nonsignificant trend,

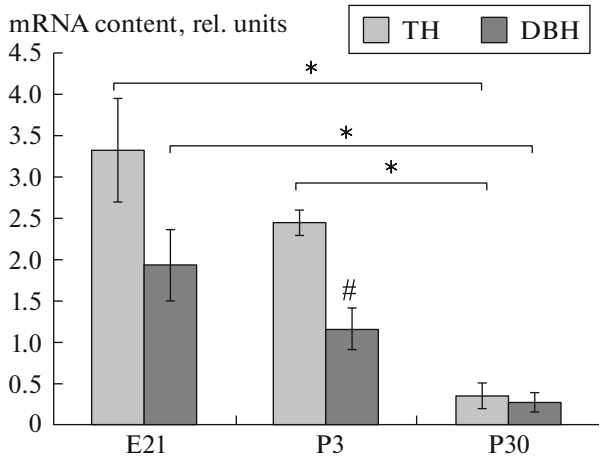

Fig. 1. The content of mRNA of tyrosine hydroxylase (TH) and dopamine beta-hydroxylase (DBH) in the organ of Zuckerkandl (OZ) in rats in ontogeny. Designations: E21—$n = 9$, P3—$n = 6$, P30—$n = 3$. Here and in Fig. 2, *$p < 0.05$ between selected periods of time; #$p < 0.05$ between TH and DBH.

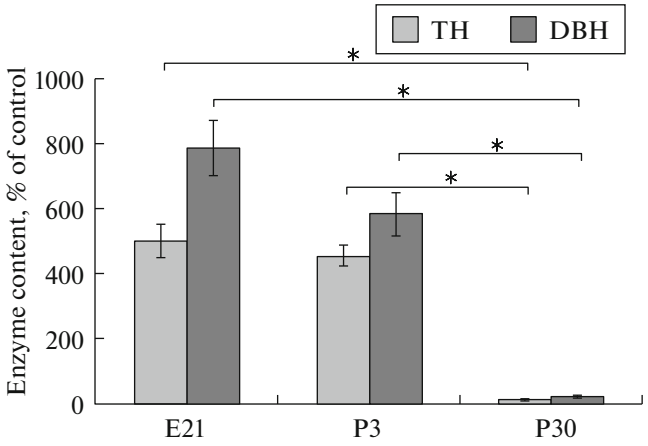

Fig. 2. The content of tyrosine hydroxylase (TH) and dopamine beta-hydroxylase (DBH) in the organ of Zuckerkandl (OZ) in rats in ontogeny. Designations: E21—$n = 12$, P3—$n = 8$, P30—$n = 4$.

and on day of P30 the content of DBH mRNA significantly decreased 7 times.

Thus, in the developmental periods studied, we found a similar dynamics of expression of the genes encoding both enzymes of NA synthesis—a decrease in the mRNA level, which may be due to a similar transcriptional regulation of both enzymes. However, we found no data about the peculiarities of the mechanisms of transcription regulation of these genes in the OZ in the available literature. It is known [12] that the level of TH and its activity are determined by transcriptional and translational mechanisms during ontogeny and that in different tissues they are regulated in different ways.

A comparison of the mRNA content of NA synthesis enzymes showed that on the 21st day of the prenatal development period, the TH mRNA content was 1.7 times higher than DBH, but the differences were nonsignificant, and on the 3rd day of life the expression of the TH gene was significant in 2.1 times higher than DBH. At the 30th day of life, there were no significant differences between mRNA of TH and DBH.

Along with the analysis of expression of the genes encoding the enzymes of NA synthesis, in the next series of experiments (Fig. 2) we estimated the relative content of the enzymes of NA synthesis. The highest TH content was detected on days E21 and P3. Then, this index decreased many times by day P30. Unlike TH, the DBH content on day E21 was slightly higher than on day P3 (by 26%), but these differences were nonsignificant. In the period from day P3 to day P30, the Content of TH decreased many times. The comparison of the dynamics of the level of transcription and translation products of the genes encoding the enzymes of NA synthesis testify to a correlation between these indices: a slight decrease or a constant level on days E21 and P3 and a sharp decline by day P30 in the expression level of genes and proteins of both TH and DBH, which is consistent with the idea of the OZ involution.

When comparing the results of this study on the gene expression and content of TH and DBH proteins with the content of NA in the OZ, measured by us earlier [13], a clear correlation between these indices of NA synthesis can be noted. For example, on days E21 and P3, the content of NA in the OZ reaches its maximum values. The levels of mRNA and the content of enzymes of its synthesis in the same periods are also high. In the period from day P3 to day P30, the content of NA drastically decreases, and the content of mRNA and protein of TH and DBH also sharply declines. Interestingly, the peak of the secretory activity of the OZ, judging by the level of spontaneous NA release, is observed on day P3, and the ability of the OZ to secrete NA also decreases by day P30 [14]. These data correlate with the morphological observations of the OZ in this developmental period and, in general, reflect the involution processes in the OZ occurring in the first months of life [7, 8].

Despite the fact that currently the physiological role of the OZ has not yet been established completely, it is assumed that the NA synthesized in the OZ in the critical period of morphogenesis is required for maintaining the physiologically active concentration of NA in blood until the complete maturation of other sources. By the time of OZ degeneration, the adrenals glands and the sympathetic nervous system acquire the highest functional activity [14, 15].

Thus, the molecular genetic indices of the NA synthesis in rats—the content of mRNA and proteins TH

and DBH—in the OZ are maximum in the prenatal and early postnatal developmental periods (i.e., in the critical period of morphogenesis).

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