Expression of β-Secretase mRNA in the Brain of Rats with Immunohistochemical Destruction of Basal Forebrain Cholinergic System

E. A. Lushchekina, M. Bigl*, J. Apelt*, C. Lange-Dohna*, S. Rossner*, and R. Schliebs*

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We studied properties of cloned BACE mRNA (β-site of the enzyme cleaving amyloid precursor protein) and evaluated the possibility of using this clone for identification and/or prediction of neurodegenerative disorders associated with cholinergic deficiency. Wistar rats subjected to immunohistochemical destruction of the basal forebrain cholinergic system were used as the experimental model. Nonradioactive *in situ* hybridization and immunohistochemical visualization of the astroglia revealed strong hybridization signal of BACE mRNA in neurons of the cortex, hippocampus, and thalamus. Astrocytes remained unstained. Immunohistochemical destruction of the basal forebrain produced no significant changes in the distribution of BACE mRNA.

Key Words: *β-secretase; in situ hybridization; immunocytochemistry; cholinergic system*

Destruction of the basal forebrain cholinergic system serves as the model of Alzheimer's disease and functional cognitive and memory disturbances. β-Amyloid peptides formed after degradation of the amyloid precursor protein (APP) forming neurotoxic fibrillar complexes play an important role in the pathogenesis of these disorders [7]. A possible way of prevention of neurodegeneration is suppression of β-amyloid deposition. The enzymes cleaving APP are of particular interest in this respect. β-Secretase was identified [11,12,14] as a β-site of the APP-cleaving enzyme (BACE) [11].

Here we studied activity of BACE mRNA in brain cells of rats subjected to immunohistochemical destruction of the basal forebrain using nonradioactive *in situ* hybridization.

MATERIALS AND METHODS

RNA probes were synthesized on the basis of BACE fragments (positions 821-1170 according to [11]) obtained from rat brain cDNA by polymerase chain reaction with primer sets L1 (TAC ACC CAG GGC AAG TGG) and R1 (CTG CCA GTG TAT GGG GAA TGG). The fragments were cloned in pGEM-T vector using T/A cloning Kit (Promega) and transformed into magnesium-competent cells of *Epicurian coli* XL1-Blue MRF' Kan by heat shock [9]. Plasmid DNA (20 μg) was obtained using a Miniprep Kit (Qiagen). Screening and sequencing of plasmid DNA were performed with M13 primers using AmpliTaq FS Big Dye Terminator kit. In experiments with promoters T7 and Sp6 of the pGEM-T vector, plasmids with BACE fragments were used to generate digoxigenin-stained antisense and sense RNA probes by *in vitro* transcription according to the Ambion Megascript protocol.

Immunotoxic destruction of the cholinergic system in the basal forebrain was performed on male Wistar rats. 1921gC-Saporin (4 μg) was stereotactically injected into the left lateral ventricle. Controls received an equivalent amount of dissociated immunotoxin components. Fourteen days after treatment the animals were euthanized with ether and perfused. Physiological saline and 4% paraformaldehyde in 0.1 M

Institute of Higher Nervous Activity and Neurophysiology, Russian Academy of Sciences, Moscow; *P. Flechsig Institute for Brain Research, Leipzig

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phosphate buffer (pH 7.4) were infused into the heart. The brain was postfixed in the same solution at 4°C for 16 h. Sections (50 μ) obtained on a vibrotome were used for nonradioactive *in situ* hybridization. Before hybridization the sections were placed in a mixture of 0.25% acetic acid and 0.1 M triethanolamine and prehybridized at room temperature for 6 h. The sections were covered with a solution containing 50% formamide, 5× Denhardt's solution, 150 mM NaCl, 15 mM sodium citrate (pH 7.0), 250 μg/ml brewer's yeast tRNA, 500 ng/ml DNA from herring sperm, and 500 ng/ml labeled cRNA probe and placed in a humid chamber at 42°C for 16 h. After hybridization the sections were washed with 0.2× SSC at 70°C (2 h) and then at room temperature. Labeled hybridization products were detected using alkaline phosphatase-conjugated anti-digoxigenin antibodies. 5-Bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium served as the substrate. BACE mRNA-positive cells looked dark blue. Hybridization with digoxigenin-labeled sense probe served as the control.

Taking into account that the astroglia play a role in cholinergic dysfunction [1], the sections after *in situ* hybridization were subjected to immunohistochemical assay to reveal astrocytes and evaluate the presence of hybridization products. The sections were incubated with monoclonal antibodies to glial fibrillar acid protein. The ABC complex and diaminobenzidine were used as a chromogen turning the astroglia into a brown color [1]. The sections were examined under a Zeiss Axioplan 2 phase contrast microscope using a Sony DXC-930P color video camera.

RESULTS

Hybridization with antisense BACE mRNA probes revealed intensive staining of neurons in the neocortex, hippocampus, and thalamus. However, this staining was not revealed after hybridization with sense probes. After immunotoxic destruction of the basal forebrain the intensity of BACE mRNA expression revealed by hybridization with antisense probes slightly decreased.

After *in situ* hybridization and astrocyte-specific immunohistochemical reaction BACE mRNA-positive neurons and astrocytes were colored dark blue and brown, respectively (Figs. 1 and 2).

As differentiated from antisense probes, sense probes revealed no BACE mRNA-positive cells on the corresponding sections (Fig. 1, *b*; Fig. 2, *b*). After immunohistochemical destruction of the basal forebrain expression of BACE mRNA insignificantly decreased, while the number of reactive astrocytes increased (Fig. 1, *a*; Fig. 2, *a*). We revealed no colocalization of dark blue BACE mRNA-positive signals and brown signals (astroglia), which attested to the absence of BACE mRNA expression in astrocytes.

Our studies revealed similar patterns of BACE mRNA expression in the brain of intact rats and animals with immunotoxic destruction of the basal forebrain, which is consistent with the results of experiments on intact rats, humans [12], and transgenic Tg2576 mice [2]. Strong hybridization signals were detected in the hippocampus, cerebral cortex, thalamus, medial septum, and diagonal band. It should be emphasized that these signals were observed in neurons, but not in the neuroglia.

After cholinergic destruction the intensity of BACE mRNA expression did not increase, but even decreased. These data suggest that compensatory processes proceed at the biochemical level. Our previous experiments with transgenic Tg2576 mice also showed that the development of cognitive disorders typical of Alzheimer's disease is not accompanied by the increased expression of BACE mRNA [2]. Therefore, high-intensity expression of BACE mRNA is not necessarily associated with pathological changes in the cholinergic system.

Experimental destruction of the cholinergic system is used as the model of cognitive disorders. Electrolytic, immunotoxic, and genetic lesions of the cholinergic system most often simulate Alzheimer's disease [2,7,12]. Published data show that disturbances in the cholinergic system produce various functional disorders. Clinical and experimental observations of patients with attention-deficit hyperactivity disorder (ADHD) and other cognitive disturbances and experiments on animals showed that cholinergic receptors play a role in the development of behavioral disorders [4]. It was demonstrated that nicotine affects locomotor activity and cognitive functions, including attention, learning, and memory, which is mediated by the cholinergic mechanisms [5,8]. ADHD is associated with functional disturbances not only in the dopaminergic and noradrenergic systems, but also in the histaminergic system (via cholinergic transmission) [10]. Observations of healthy volunteers showed that drugs inhibiting cholinergic transmission impair storage of new information in long-term memory and affect time perception [6]. Clinical research demonstrated that cholinergic agents improving cognitive processes hold much promise for the therapy of patients with ADHD [3]. For instance, the ABE-418 cholinergic agonist attenuates ADHD symptoms [13]. Published data indicate that studies of consequences of cholinergic destruction would elucidate the mechanisms of various functional disturbances.

The absence of pronounced changed in the pattern of BACE mRNA expression after immunotoxic destruction of the basal forebrain suggests that this enzyme plays an ambiguous role in the pathogenesis of

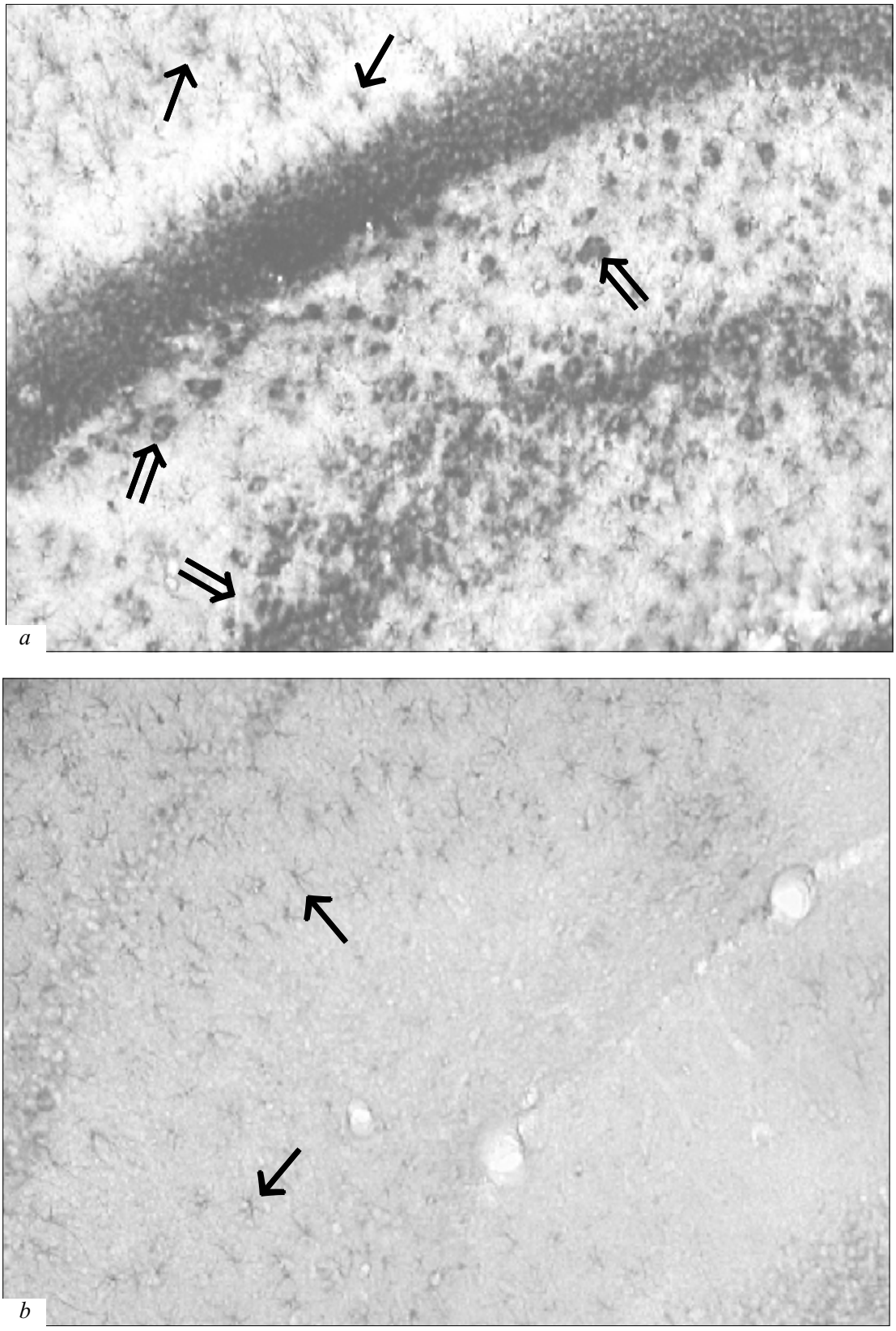

Fig. 1. Distribution of BACE mRNA in brain sections of the hippocampus in intact rat (×30). Here and in Fig. 2: distribution of hybridization product with antisense (*a*) and sense probes (*b*). Double arrows: BACE mRNA-positive neurons. Thin arrows: astrocytes.

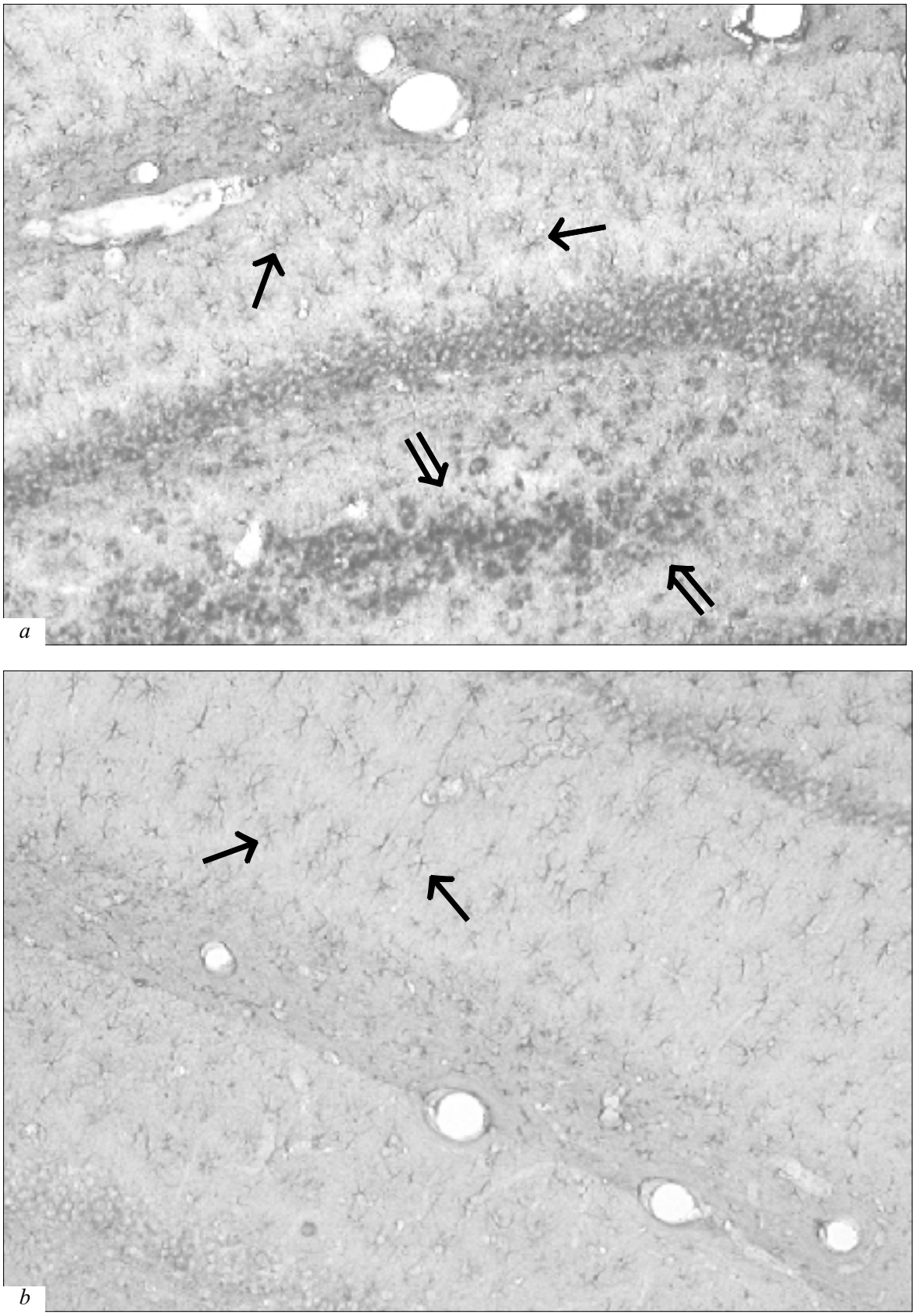

Fig. 2. Distribution of BACE mRNA in brain sections from rat with immunohistochemical destruction of the cholinergic system (×30).

neurodegenerative diseases. The assumptions that destruction of the cholinergic system stimulates expression of BACE mRNA and that intensive expression of BACE mRNA can be used as the marker of pathological changes in this transmitting system were not confirmed. The search for other biochemical parameters that would allow us to reveal and prevent this disorder is of considerable importance.

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