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# Increased Level of β-Amyloid in the Brain of Bulbectomized Mice

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**Abstract**—Six weeks after bilateral olfactory bulbectomy, a peptide with molecular weight of 4 kD was revealed in extracts of the neocortex and hippocampus from mice. Using monoclonal antibodies 4G8, this peptide was identified as β-amyloid. Its level was significantly higher in the bulbectomized animals than in sham-operated mice. The bulbectomized mice displayed sharp impairment in spatial memory when tested in the Morris water maze. The results suggest that bulbectomy initiates in the brain a pathological process similar to human Alzheimer's disease in location, biochemistry, and behavioral manifestations.

*Key words*: sporadic Alzheimer's disease, animal models of human diseases, bulbectomy, β-amyloid, spatial memory

Olfactory bulbectomy seems to be a promising approach to studies on consequences of disorders in the olfactory system. However, in bulbectomized (BE) animals, not only the olfaction is disturbed, but also a kind of depression is induced. Therefore, bulbectomy may be considered as a model of agitated depression [1]. We and other authors have earlier shown that, in addition to depression, BE animals are characterized by dramatically disturbed acquisition and retention of various behavioral paradigms [2], including impairment of spatial memory in the Morris water maze [3]. Our earlier morphological and functional study on the brain of BE mice has also shown an increased number of degenerated neurons with signs of cytolysis, karyolysis, pycnosis, and vacuolization in the temporal cortex, hippocampus, and nucleus raphe which contained serotonin-producing cells, along with accumulation of intracellular lipofuscin [4-6]. With polyclonal antibodies to choline acetyltransferase, BE mice were also found to have a decreased density of cholinergic neurons in the basal structures of the forebrain [3]. The above-mentioned consequences of bulbectomy are reminiscent in their manifestation and location to the pathology in the brain of patients with Alzheimer's disease (AD), which exemplifies a development of a complete loss of memory and degradation of personality in elderly people [7]. Senile plaques and neurofibrillar tangles in the brain are the main neuropathological signs of AD. In spite of absence of a clear idea on the genesis of AD, most authors suppose that the main component of senile plaques, β-amyloid, plays a key role in the induction of neurodegeneration via initiation and modulation of free radical processes in the brain of the patients [8]. Direct evidence that β-amyloid is central to the pathogenesis of AD is specific mutations in the genes of β-amyloid precursor protein (β-APP) and presenilins, proteins that specifically elevate generation of the β-amyloid from β-APP. Transgenic mice that express the mutated human APP gene and presenilins also exhibit the deficits in memory and abnormal amyloidogenesis, and this allows us to consider them as a model of the genetic form of AD [9]. Bulbectomy seems to be especially interesting as a model of nonhereditary sporadic AD, but to prove its validity, the level of β-amyloid in BE animals should be known.

Thus, the purpose of this work was to determine the level of β-amyloid in the brain of BE animals at the stage of the sharp impairment in their spatial memory.

## MATERIALS AND METHODS

Experiments were performed on 6-month-old male mice of the NMRI line, which were kept in a room with daylight at temperature of 21-23°C and with water and food *ad libitum*. They were operated under hexenal anesthesia (40 mg/kg intraperitoneally), with 0.5% Novocain

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for local anesthesia on the scalp. The operation has been described in detail earlier [2].

The olfactory bulbs were removed bilaterally by aspiration through a hole in the skull. Sham-operated (SO) animals (control) were treated similarly, except for the ablation of the olfactory bulbs. Six weeks after the operation, the animals were tested in the Morris water maze to reveal a spontaneous preference for compartments [10]. Only the animals without any preference were used for the experiments. Then, during six weeks (four sessions daily) the mice were trained to find a saving platform hidden underwater in one of the compartments. The rate of training was determined by latency to locate the platform. The spatial memory was tested in the trained animals for 1 min in the absence of the saving platform. The time spent by the animals in each water maze compartment and also the number of entries into them were determined.

The protein (β-amyloid) from the brain tissue of the BE and SO mice was isolated and purified as described in [11] as follows: 400-500 mg of the brain tissue (the cortex and hippocampus) was cut into very small pieces and suspended in 5 ml of 50 mM Tris-HCl (pH 7.4) supplemented with 2 mM EDTA, 2% SDS, and 1% 2-mercaptoethanol. After continuous stirring for 16 h at room temperature, the suspension was filtered through an 80-μm filter to remove large particles. The filtrate was centrifuged at 40,000*g* for 45 min. The supernatant fluid was removed, and the precipitate was suspended in 5 ml of the same buffer and centrifuged at 12,000*g* for 1 h. The precipitate was washed twice with bidistilled water from SDS, with centrifugation under the same conditions, and then suspended in Tris buffer (pH 7.5) containing 2 mM $CaCl_2$. A small amount of collagen was washed out by its treatment with collagenase at the concentration of 0.3 mg/ml. To remove the enzyme and the product of proteolysis, the specimen was washed thrice with twice-distilled water, with centrifugation at 10,000*g* for 15 min. The precipitate was supplemented with 0.5 ml of 70% formic acid, maintained for 20 min with stirring, and then centrifuged at 10,000*g* for 15 min. The solution of the peptide in formic acid was used for chromatography and electrophoresis.

The protein was purified by HPLC using a Pharmacia-FPLC-2500 system with a Superose-12 column (10 × 300 mm) equilibrated with 70% formic acid. Chromatography was performed in the same solution of formic acid at the flow rate of 0.2 ml/min and pressure of 3 atm [12].

The isolated protein was identified by SDS-PAGE [13], the gel being stained with Serva Blue R-250.

For immunological DOT-analysis, the brain specimens from the BE and SO animals were prepared as follows: 200-270 mg of the brain tissue (the cortex and hippocampus) was homogenized in 0.5 ml of 70% formic acid, maintained for 1 h, centrifuged at 100,000*g* for 40 min, then the supernatant fluid was evaporated on a rotor evaporator to the minimum volume, supplemented with 1 ml of twice-distilled water, and the solution was neutralized to pH 7.4 with NaOH and lyophilized.

Results of factor analysis of parameters of spatial memory in bulbectomized (BE) and sham-operated (SO) animals

| Group | Search time in the compartments | Number of entries into the compartments |
|---|---|---|
| SO | $n = 12$<br>$F(3.44) = 13.52$<br>$p < 0.001$ | $n = 12$<br>$F(3.44) = 12.3$<br>$p < 0.001$ |
| BE | $n = 9$<br>$F(3.32) = 2.65$<br>$p = 0.066$ | $n = 9$<br>$F(3.32) = 0.178$<br>$p = 0.907$ |

DOT-analysis was performed by a modification of a method with significantly increased sensitivity [14]. This was due to the previous 1-min treatment of the nitrocellulose membrane with 4% ovalbumin in phosphate buffer

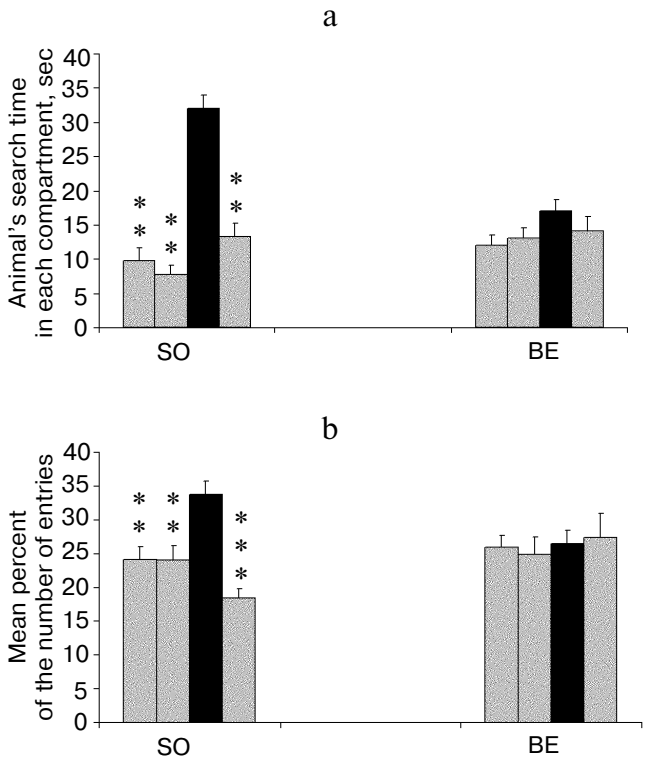


**Fig. 1.** Parameters of the spatial memory tested with the Morris water maze in bulbectomized (BE) and sham-operated (SO) animals: a) search time in each compartment; b) number of entries into the compartment (in % of the total number of entries). The compartment containing the rescue platform during the training is shown by black.

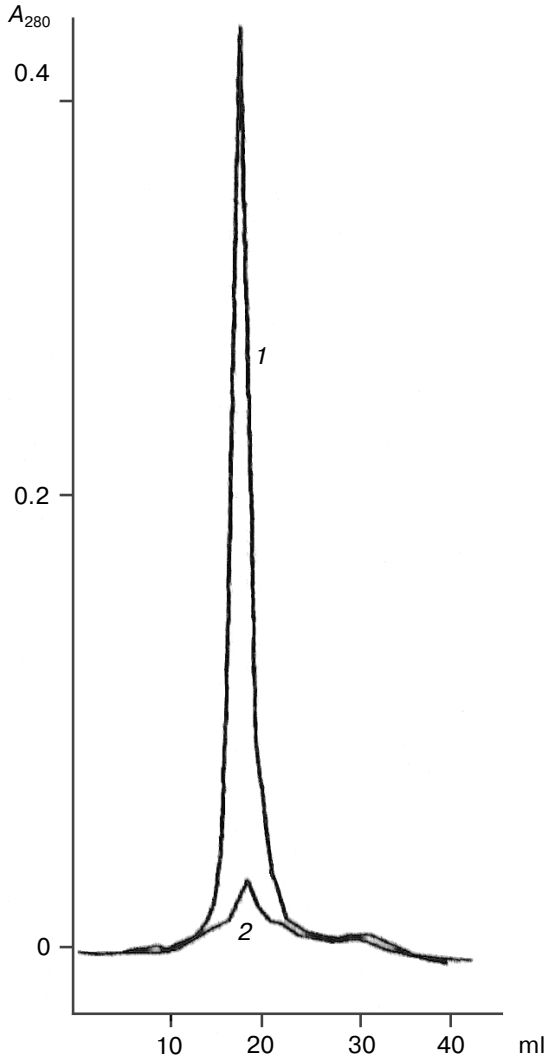


**Fig. 2.** Chromatographic profile of the brain extract in formic acid from the bulbectomized (*1*) and sham-operated (*2*) animals on a Superose-12 column (10 × 300 mm).

followed by a subsequent 10-min treatment with 2.5% glutaraldehyde. The membrane with the specimens placed on it was kept for 1 h in 4% ovalbumin in phosphate buffer supplemented with 0.1% $NaN_3$. The DOT-analysis was performed routinely [14].

During the work, the following control reactions were performed: to determine the endogenous peroxidase activity, nonspecific sorption of secondary antibodies, and also the activity of immune reagents and the antigen presence under conditions of the experiment. For the calibration curve and subsequent quantitative determination of β-amyloid in the brain extracts, the intensity of staining of different concentrations of β-amyloid was used.

The staining intensity of spots on the membrane was determined using the ONEDSCAN program, version 1.3, copyright 1994-1997.

The following antibodies were used:

– monoclonal antibodies 4G8 reacting with (17-24) amino acid sequence of β-amyloid which has the same structure in human and mouse β-amyloid (dilution 1 : 1000), thus, these antibodies were highly specific for determination of these protein substances;

– biotinylated horse antibodies to mouse IgG (dilution 1 : 3500) from Vector (USA);

– monoclonal antibodies to biotin (the clone BN-34) conjugated with peroxidase (1 : 4000) from Sigma (USA).

## RESULTS

The intragroup analysis of variance was used to determine the difference in the spatial memory between the BE and SO animals, and only the trained SO animals could find the training compartment, as it was revealed by the number of entries into this compartment and the search time. The BE mice had pronounced deficit in special memory (table and Fig. 1).

The brain extract in formic acid was subjected to chromatography on a Superose-12 column, and a single peak with molecular weight of 4 kD was found, as deter-

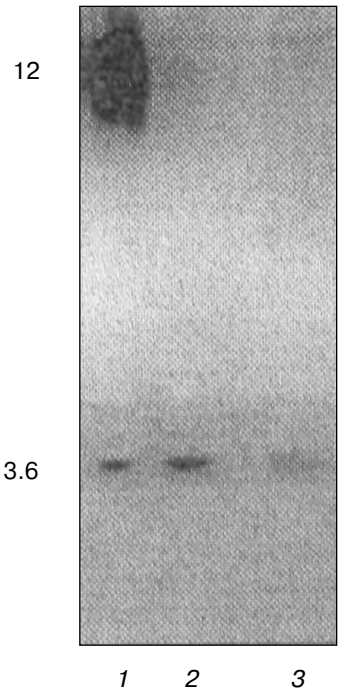


**Fig. 3.** SDS electrophoresis in polyacrylamide gel of the brain extract in formic acid from the BE and SO animals: *1*) marker proteins (to the right their molecular weight in kD): cytochrome *c* (12 kD), B-chain of insulin (3.6 kD); *2*) brain extract in formic acid of the BE mice; *3*) brain extract in formic acid of the SO mice.

mined by location of the peak of the insulin B-chain (molecular weight of 3.6 kD) chosen as a marker (Fig. 2).

By electrophoresis of the extract in formic acid of the brain tissue from the BE animals before and after chromatography on a Superose-12 column, a single spot was revealed in all specimens (Fig. 3). The spot location in the experimental specimen in gel obviously corresponds to location of the spot of the insulin B-chain (molecular weight of 3.6 kD) chosen as a marker.

Results of DOT-analysis as histograms (Fig. 4) show a significantly increased level of β-amyloid in all samples from the BE compared to the SO mice. Specificity of the antibody binding to β-amyloid was confirmed by the control results. Absence of staining of the membrane with the samples placed during interaction with 3,3′-diaminobenzidine (DAB) suggested the absence of endogenous peroxidase activity. Absence in the samples of immunoglobulins cross-reacting to secondary antibodies was shown by testing nonspecific sorption of secondary antibodies: the reaction was negative when the membrane with the samples on it was treated with secondary antibodies and stained with DAB. The activity of immune reagents and antigen validity under the experiment conditions were also monitored. The membrane with the marker of β-amyloid was treated at first with primary antibodies, then with secondary antibodies, and stained with DAB.

The mean levels of β-amyloid in the brain of the BE and SO animals were 33.8 ± 6.78 ($n$ = 7) and 5.047 ± 0.39 ng/g ($n$ = 7), respectively. Thus, the level of β-amyloid in the brain of the BE mice was significantly higher than in the control SO animals ($p < 0.001$) (Fig. 4).

## DISCUSSION

A month and a half after ablation of the olfactory bulbs the animals were shown to have spatial memory deficit and a significantly increased level of β-amyloid in extracts of the neocortex and hippocampus compared to the SO animals. Note that the level of β-amyloid in the BE mice was comparable to its concentration in the same structures in transgenic mice with the mutated human

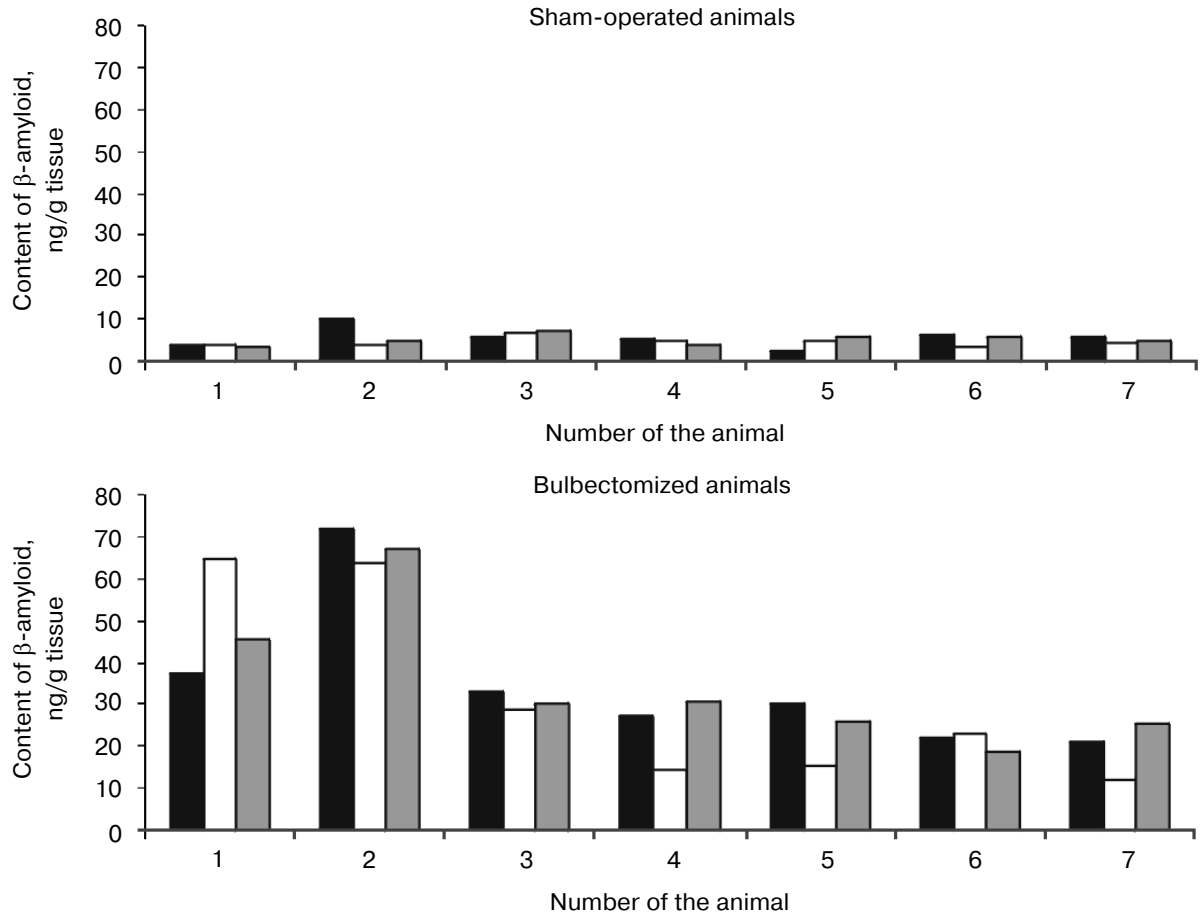


**Fig. 4.** Distribution histograms of β-amyloid levels in the brain extracts from the BE and SO mice determined by DOT-analysis. Columns of different shading show data of repeated determinations of the sample from the same animal.

APP gene when these mice were in the early stage of plaque formation along with spatial memory impairment [15]. However, later the level of β-amyloid in the transgenic animals increased 100-fold and more compared to its level in young animals. In our case the level of β-amyloid in the BE mice increased no more than sixfold compared to the SO mice.

The presence of β-amyloid in the brain of mice is not surprising because it is a product of proteolytic processing of β-APP, the gene of which belongs to the conservative part of the genome and is found in the majority of animal species, including mice. The increased level of β-amyloid after BE may be caused by the brain trauma which is shown to stimulate the synthesis of APP and production of β-amyloid [16]. But a specific result of BE is the promotion of increase in the level of β-amyloid in the cortex and hippocampus, i.e., in the structures which are damaged in human AD. AD is characterized not by a diffuse amyloidogenesis over the whole brain, but only by the increased level of β-amyloid and appearance of amyloid plaques in the structures directly or indirectly associated with the olfactory system [17]. However, the hypothesis of the key role of the olfactory system in pathogenesis of Alzheimer's disease is supported insufficiently, although some clinical data show that morphological changes in the brain of the patients are mainly located in the cortical and subcortical olfactory structures, such as medial amygdala, hippocampus, entorhinal cortex, and temporal cortex [18], which are connected with the olfactory bulbs. Clinical observations also show that, in addition to dementia, patients with AD display changes in behavior, such as depression with periodical agitation along with olfaction impairment [19]. The risk group of AD includes persons with traumas of the brain frontal region and also workers of lacquer and paint factories. Patients with AD, similarly to BE animals, have dysfunction of the acetylcholinergic, serotoninergic, and glutaminergic systems of the brain [20, 21].

Our data suggest that activation of APP synthesis and the subsequent production of β-amyloid in the cortex and hippocampus can be initiated by damage to the peripheral part of the olfactory system, in particular, of the olfactory bulbs, which are clinically shown to be reduced in patients with AD because of degeneration of the mitral cells [22]. Most current hypotheses on genesis of AD suggest that increase in the level of β-amyloid, which has a neurotoxic effect, causes the development of degenerative changes in the brain [21, 23].

Thus, bulbectomy, which results in disorders in the peripheral olfactory system, can induce the development of a complex of behavioral and biochemical changes similar to manifestations of AD in humans, including the increase in the level of β-amyloid, which is one of the main markers of this disease.

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