GENERAL PATHOLOGY AND PATHOLOGICAL PHYSIOLOGY

The State of Cholinergic Structures in Forebrain of Bulbectomized Mice

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The effects of bulbectomy on the state of basal forebrain cholinergic structures and spatial memory in Morris water maze were studied. Immunostaining with polyclonal antibodies to choline acetyltransferase AB144P revealed decreased density of immunoreactive cells in the horizontal limb of the diagonal band of Broca (55% of control), basal magnocellular preoptic nucleus (58.9%), and the caudate nucleus–putamen complex (68.2%). No significant changes in the vertical limb of the diagonal band of Broca and globus pallidus were observed. These findings and published data allow us to assume that pathology of the olfactory bulb can underlie memory impairment during Alzheimer's disease associated with dysfunction of acetylcholine-synthesizing structures in the forebrain.

Key words: *acetylcholinergic system of basal forebrain structures; choline acetyltransferase; bulbectomy; spatial memory*

Inhibition of the acetylcholinergic system accompanied by decreased content of choline acetyltransferase (ChAT), acetylcholinesterase, and decreased density of high affinity acetylcholine (AC) reuptake sites, as well as the death of AC-synthesizing neurons in basal forebrain structures (BFS) are the key factors of Alzheimer's disease characterized by progressive dementia. The fact that some drugs stimulating cholinergic function attenuate cognitive disorders in Alzheimer patients attests to a causal relationship between these processes [12]. Neurochemical mechanisms involved in the regulation of AC-synthesizing neurons, their dysfunction, and the possibility of their correction are now extensively studied [13,14]. The olfactory bulb is the source of trophic factors [9,11,13], in particular, nerve growth factor [14] regulating activity of AC-synthesizing neurons in the basal forebrain [9,13]. These factors can be retrogradely transported via afferent fibers arising from BFS [1,4]. However, functional interaction between AC-synthesizing and olfactory bulb neurons has not been studied in detail. Here we studied delayed effects of bulbectomy on the state of AC-synthesizing BFS and spatial memory in operated animals.

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MATERIALS AND METHODS

The experiments were carried out on 6-month-old male NMRI mice kept at 21-23°C with free access to water and food. The operation was performed under hexenal narcosis (40 mg/kg intraperitoneally), 0.5% novocain was used for local anesthesia [11]. Bilateral bulbectomy was performed by aspiration of olfactory bulbs through a trepanation opening. Sham operated animals were subjected to the same procedure without bulbectomy. Four weeks postoperation the animals were tested for compartment preference in a Morris water maze [6]. Thereafter, the mice were trained (6 days, 4 sessions daily) in finding water-submerged rescue platform in a certain maze compartment. The rate of learning was evaluated by the latency to find the platform. Trained animals were tested for spatial

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TABLE 1. Latency to Find Rescue Platform in Morris Water Maze in Bulbectomized and Control Animals (*M*±*m*)

Animal group	Days of training					
	1st	2nd	3rd	4th	5th	6th
Bulbectomized (*n*=9)	50.8±4.3*	27.0±5.3	23.7±6.3	25.8±6.1**	19.0±2.2**	15.7±4.0
Control (*n*=12)	25.6±5.5	29.2±7.1	27.2±7.7	10.4±2.6	8.9±2.0	11.1±3.0

Note. *p<0.01; **p<0.05 compared to the control.

memory for 1 min in the absence of rescue platform. The time spent in compartments and the number of visits were analyzed.

Sham operated and bulbectomized mice (5 animals per group) were decapitated under ether anesthesia. The brain was fixed for 1 day in 4% paraformaldehyde on phosphate buffer and then transferred to 25% sucrose for 48 h. Sections (15-20 μm) prepared on a Reiherd cryostat were washed in phosphate buffer to remove cryoprotector and incubated with 1% H_2O_2 on phosphate buffer containing 0.1% Triton X-100 for 30 min for inhibition of endogenous peroxidase activity. After washout the sections were treated with 3% BSA and incubated with AB144P polyclonal antibodies against ChAT (1:100 dilution, 50 μl, Chemicon International Inc.) for 24 h at 4°C. After washout, the sections were incubated with biotinylated secondary antibodies (anti-mouse immunoglobulins, Vector, 1:1000) for 2 h, washed, and treated with Vectastain Elite ABC KIT reagents (Vector Lab.) for the formation of avidin-biotin-peroxidase complexes according to manufacturer's instruction. Peroxidase activity was visualized with 0.01% diaminobenzidine tetrachloride (Research Biochemicals Inc.) on 0.05 M Tris buffer with 0.06% $NiCl_2$ (pH 7.6) in the presence of 0.01% H_2O_2. For evaluation of nonspecific staining some brain sections from bulbectomized and control animals were processed in parallel except incubation with primary antibodies. Immunohistochemical reaction was performed on free floating sections using an orbital shaker (Elmi). The number of ChAT-positive cells in vertical (NVDB) and horizontal (NHDB) limbs of the diagonal band of Broca, basal magnocellular preoptic nucleus (NMP), ventral globus pallidus (GPv) and caudate nucleus–putamen (NCP) complex was determined. To this end stained cells in structures of the left and right hemispheres were counted in 8-12 view fields (magnification 16.3×10) on 5 sections for each structure, and the mean cell density per view field was calculated. Statistical significance of morphological and behavioral differences between bulbectomized and control animals was evaluated using Student *t* test and one-way ANOVA followed by post-hoc Fisher LSD test.

RESULTS

Four weeks postoperation, bulbectomized mice (*n*=9) showed a lower training rate (F(1.114)=6.5, p=0.012) in the Morris water maze compared to controls (*n*=12). Mean times of finding rescue platform in experimental and control groups were 27.5±2.48 and 18.97±2.28 sec, respectively (Table 1).

Analysis of variances within groups used for evaluation of differences in spatial memory between bulbectomized and control animals showed that trained compartment was preferred only by trained controls (as assessed by the number of visits and the time spent in this compartment), while bulbectomized animals demonstrated pronounced spatial learning deficit (Table 2, Fig. 1).

Factor analysis revealed a significant effect of bulbectomy on the density of ChAT-immunoreacti-

TABLE 2. Factor Values of Preference of Water Maze Compartments Assessed by Time of Stay and Number of Visits

Animal group	Time of stay		Number of visits	
	before training	after training	before training	after training
Bulbectomized				
F(3.32)	1.54	2.65	2.44	0.178
p	0.221	0.066	0.082	0.907
Control				
F(3.44)	0.294	13.52*	0.471	12.31*
p	0.831	0.00006	0.708	0.0004

Note. *p<0.001 compared to the control.

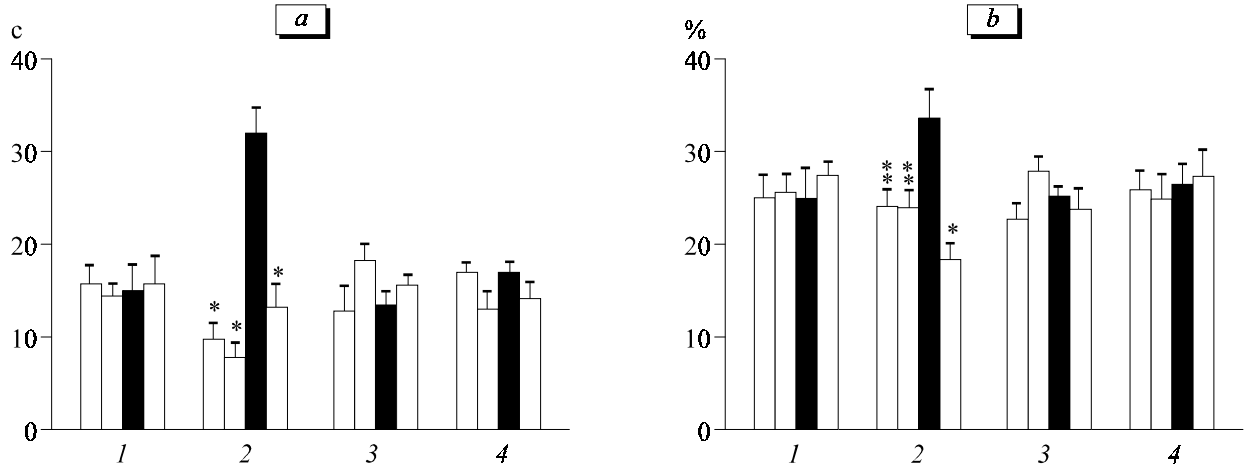

Fig. 1. Time of stay (*a*) and number of visits (*b*) to different water maze compartments during memory testing in control (*1*, *2*) and bulbectomized (*3*, *4*) mice. *1*, *3*) before, *2*, *4*) after training. Open bars: indifferent compartments; dark bars: trained compartment. **p*<0.001, ***p*<0.01 compared to trained compartment.

ve cells in NHDB (F(1.78)=5.32, p=0.0225), NMP (F(1.103)=6.67, p=0.01), which is regarded as an analogue of the nucleus basalis of Meynert in man, and in NCP (F(1.186)=13.6, p=0.0005). No differences in cell density between experimental and control animals was revealed in NVDB and GPv (F(1.108)=1.31, p=0.253 and F(1.92)=1.58, p=0.108, respectively). The density of ChAT-immunopositive cells in NHDB, NMP, and NCP complex of bulbectomized mice decreased to 45, 41.1, and 31.8% of the control, respectively. The bulbectomy-induced decrease of the number of ChAT-immunoreactive cells in NHDB and NMP, but not in NVDB and GPv is seen microphotographs of the corresponding brain sections from bulbectomized and control animals (Fig. 3).

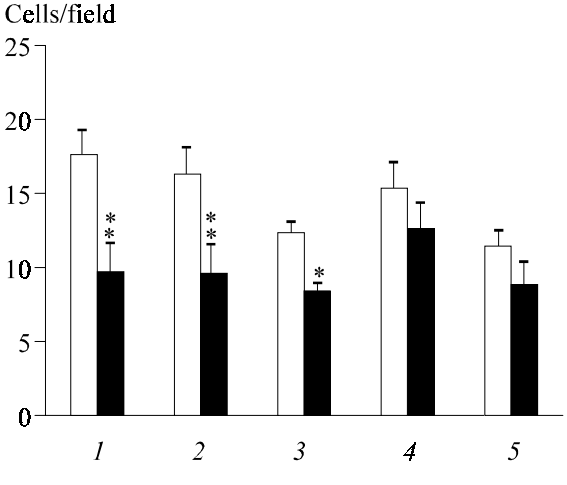

Fig. 2. Density of ChAT-immunoreactive cells in basal forebrain structures of bulbectomized (dark bars) and control (open bars) mice. *1*) horizontal limb of vertical band of Broca, *2*) magnocellular preoptic nucleus, *3*) caudate nucleus and putamen, *4*) vertical limb of diagonal band of Broca, *5*) ventral area of globus pallidus. **p*<0.01, ***p*<0.05 compared to the control.

Memory deficit in bulbectomized animals [11] can result from hypofunction of the AC-system in BFS, since death of AC-synthesizing neurons in BFS is known to cause cognitive deficiency [10]. It is important to note that bulbectomy produces a selective effect on BFS and their afferent fields without changing ChAT activity in other brain structures [14,15]. Reduced density of ChAT-immunopositive cells in NHDM was most pronounced probably due to the absence of trophic factors delivered to this structure via direct afferents from the olfactory bulbs [1,4]. The decrease in the content of ChAT-containing neuron in NMP and NCP is probably associated with enhanced synthesis of galanin, a peptide inhibiting activity of AC-ergic neuron [2]. Increased content of pro-mRNA for this peptide was found in bulbectomized animals [5]. Hypofunction of the AC system in NCP manifests in total hyperactivity typical of bulbectomized animals [11], because damage to AC interneurons sharply enhances motor activity via interaction between AC- and dopaminergic systems [7]. It is important to note that early stages of Alzheimer's disease are characterized by olfactory deficit and degenerative changes in the olfactory bulbs [3,8], which according to our data result in hypofunction of cholinergic BFS and memory impairment.

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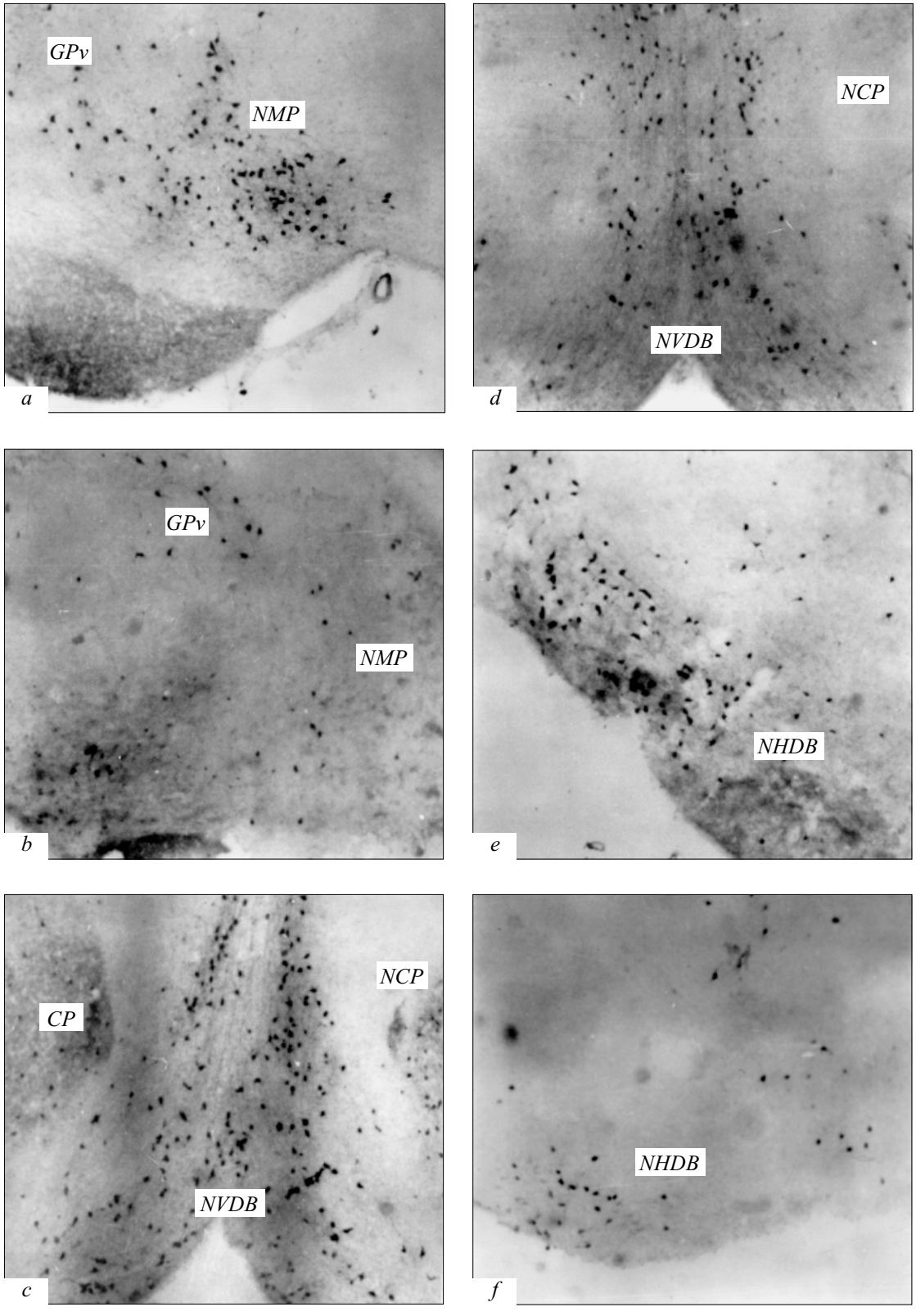

Fig. 3. Distribution of ChAT-immunoreactive cells in control (*a*, *c*, *e*) and bulbectomized (*b*, *d*, *f*) mice (polyclonal antibodies AB144P, ×130). Sections of magnocellular preoptic nucleus (NMP; *a*, *b*), vertical (NMDV; *c*, *d*), and horizontal (NHDB; *e*, *f*) limb of diagonal band of Broca. NCP: caudate nucleus–putamen, GPv: ventral area of globus pallidus.

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