PHYSIOLOGY

Enhancement of Optical Transmission Capacity of Isolated Structures in the Brain of Mature Mice

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We developed an efficient method of optical clarification of isolated structures in the brain of mature mice. The method decreases optical density and opens the way to use novel methods of optical tomography in the study of the brain tissue in mature organism. Specimens dehydrated with 2-butoxyethanol, destained with Dent solution, and cleared in benzyl benzoate:benzyl alcohol or in water solution of iothalamate had the lowest optical density. The clearing effect did not directly depend on the refraction index of the composition. Possible mechanisms of increasing optical transmission of myelinated structures in the brain are discussed.

Key Words: *optical clearing; nervous tissue; hippocampus; olfactory bulbs; neocortex*

Potentials of the synthesis and testing of novel classes of neurotropic agents are limited by the absence of efficient methods for detecting their molecular targets in large specimens of the nervous tissue such as the whole brain of experimental animals. The prerequisites to implement the research at the higher level of neuroimaging are the development of modern methods of biomedical imaging such as optical projection tomography [5,7] and ultramicroscopy [3], as well as synthesis of the new generation of fluorescent probes and transgenic reporter proteins [1,4]. However, the present methods of 3D visualization of the molecular markers in specimens of the nervous tissue are limited in the choice of the experimental objects usually employing the embryos at the early developmental stage or the semitransparent small fry. Unfortunately, the nervous system of mature animals is optically opaque due to high myelin content and its structural peculiarities in the mature age. Detection of the molecular targets in a non-cleared specimen of myelinated nervous tissue is limited to a depth of 100-200 μ.

Our aim was to develop an efficient method of optical clearing of large structures isolated from the brain of mature mice (hippocampus, olfactory bulbs, neocortex, and cerebellum).

MATERIALS AND METHODS

The study used 3-6-month laboratory C57B1/6 male mice. The animals were kept in the standard cages (by 5 mice in each case sizing 36×21×13.5 cm) with an 12-hour day-night cycle and food and water *ad libitum*. The brain specimens were fixed by any of the following methods: 1) immersion in 1% or 4% paraformaldehyde dissolved in 0.1 M phosphate-saline buffer (PSB) or 2) perfusion with 4% paraformaldehyde by the routine protocol.

The increase in cell permeability included 5 cycles of low-temperature freezing (-70°C) and defrosting at room temperature followed by incubation in Dent solution (methanol:DMSO:30% H_2O_2

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4:1:1) [2]. Dehydration was performed in ethanol or 2-butoxyethanol solutions of increasing concentrations (50, 70, and 90%, 1 h in each concentration, 12-14 h incubation in 100% 2-butoxyethanol) followed by incubation in hexane for 1 h. All incubation procedures were performed at room temperature with constant stirring.

For clarification of specimens we used solutions with various refraction indices: 75% water solution of diatrizoate meglumine/sodium (1.43); 1,3-propranolol (1.44); 75% water solution of iothalamate meglumine/sodium (1.44); glycerol (1.47); methyl salicylate (1.53), and the mixture benzyl benzoate: benzyl alcohol 2:1 (1.55) (Murray clarifier) [2].

The images were formed in transmitted light with a Leica M420 microscope and recorded with a Nikon DXM-1200 CCD camera. After a series of physicochemical processing stages, the optical density of cerebral structures was determined using Image-Pro Plus 3.0 software.

The data were processed statistically using Mann—Whitney non-parametric test at $p<0.05$.

RESULTS

Fixation with formaldehyde affects the refraction index via changes in conformation properties of proteins and via modification of their interaction with the clarifiers. Comparison of various routine fixation protocols showed that perfusion of brain specimens with 4% paraformaldehyde decreased optical density (0.56±0.10) in comparison with immersion in 1% paraformaldehyde (0.65±0.01) or in 4% paraformaldehyde (0.71±0.05; Fig. 1, *a*). However, in the course of further clearing under identical conditions (immersion in iothalamate water solution), the specimens fixed by perfusion in 4% paraformaldehyde were less translucent (0.15±0.04) than after immersion in 1% or 4% paraformaldehyde (0.09±0.02 and 0.10±0.03, respectively). The difference between two latter groups became insignificant (Fig. 1, *b*).

One of the two mechanisms underlying the method of clearing with a mixture of similar refraction index ($\Delta \leq 0.05$), dehydration, is required for maximum removal of water from the specimen, because refraction index of water (1.33) significantly differs from that of cell membrane (1.46-1.54) or organelles (1.38-1.41) [6]. Optical density of tissue specimens dehydrated in a series of 2-butoxyethanol solutions was lower than after dehydration in a series of ethanol solutions (0.34±0.02 and 0.37±0.02, respectively; Fig. 1, *c*).

The major problem in clearing of cerebral tissues of mature animals originates from lipid-protein complexes of mature myelin, because low pH promotes their dissociation [6]. However, variation in pH of clarifier (iothalamate solution) had no effect on the optical density of the specimens: 0.11±0.02 at pH 6.0 and 0.11±0.02 at pH 7.4 (Fig. 1, *d*).

After incubation in Dent solution (methanol, DMSO, H_2O_2) followed by additional clearing (benzyl benzoate:benzyl alcohol), the optical density of brain specimens was significantly lower (0.03±0.01) than that of specimens not exposed to permeabilization and clearing (0.12±0.01). Supplementing the protocol with 5 freezing–defrosting cycles in Dent solution did not decrease the optical density of the specimens (0.02±0.01, Fig. 1, *e*).

To test the hypothesis that coincidence of refraction indices increases optical transmission capacity, we compared the optical density of brain specimens after immersion into clearing media with various refraction indices. Optical density did not depend on the refraction index of the clearing mixture (Fig. 1, *f*). Lower values of optical density were obtained after clearing the specimens in water solution of iothalamate (0.10±0.03) and in mixture benzyl benzoate:benzyl alcohol (0.02±0.01), the corresponding refraction indices being 1.44 and 1.55. The specimens cleared in the media with intermediate values of the refraction index (glycerol, 1.47 and methyl salicylate, 1.53) had significantly higher optical density (respectively, 0.25±0.04 and 0.12±0.05). The clearing media with identical refraction index of 1.43 (water solution of diatrizoate and 1,3-propanediol) had different effects on the optical density (0.13±0.02 and 0.36±0.03, respectively). The substances possessing the strong polarizing groups such as aromatic rings and iodine atom (benzyl benzoate, iothalamate, diatrizoate, and methyl salicylate) produced a pronounced clearing effect.

The data obtained yielded the following optimal protocol of optical clearing of the isolated brain structures of mature mice:

1. Fix the brain in fresh 4% paraformaldehyde for 24 h at 4°C. Wash it in PSB for 1 h and make the preparations of isolated brain structures (hippocampus, neocortex, cerebellum, and olfactory bulb), postfix them in 4% paraformaldehyde for 2 h (Fig. 2, *a*, *c*, *e*, *g*).

2. Dehydrate the preparations in 2-butoxyethanol solutions of increasing concentrations: 50, 70, 90% for 1 h in each and then in 100% solution for 12-14 h. Incubate in hexane for 1 h and then in fresh Dent solution (methanol:DMSO:30% H_2O_2 4:1:1) at bright light for 1 h. Replace paraformaldehyde and methanol with ethanol for the specimens containing endogenous fluorescent proteins. If immunohistochemical detection is needed, the

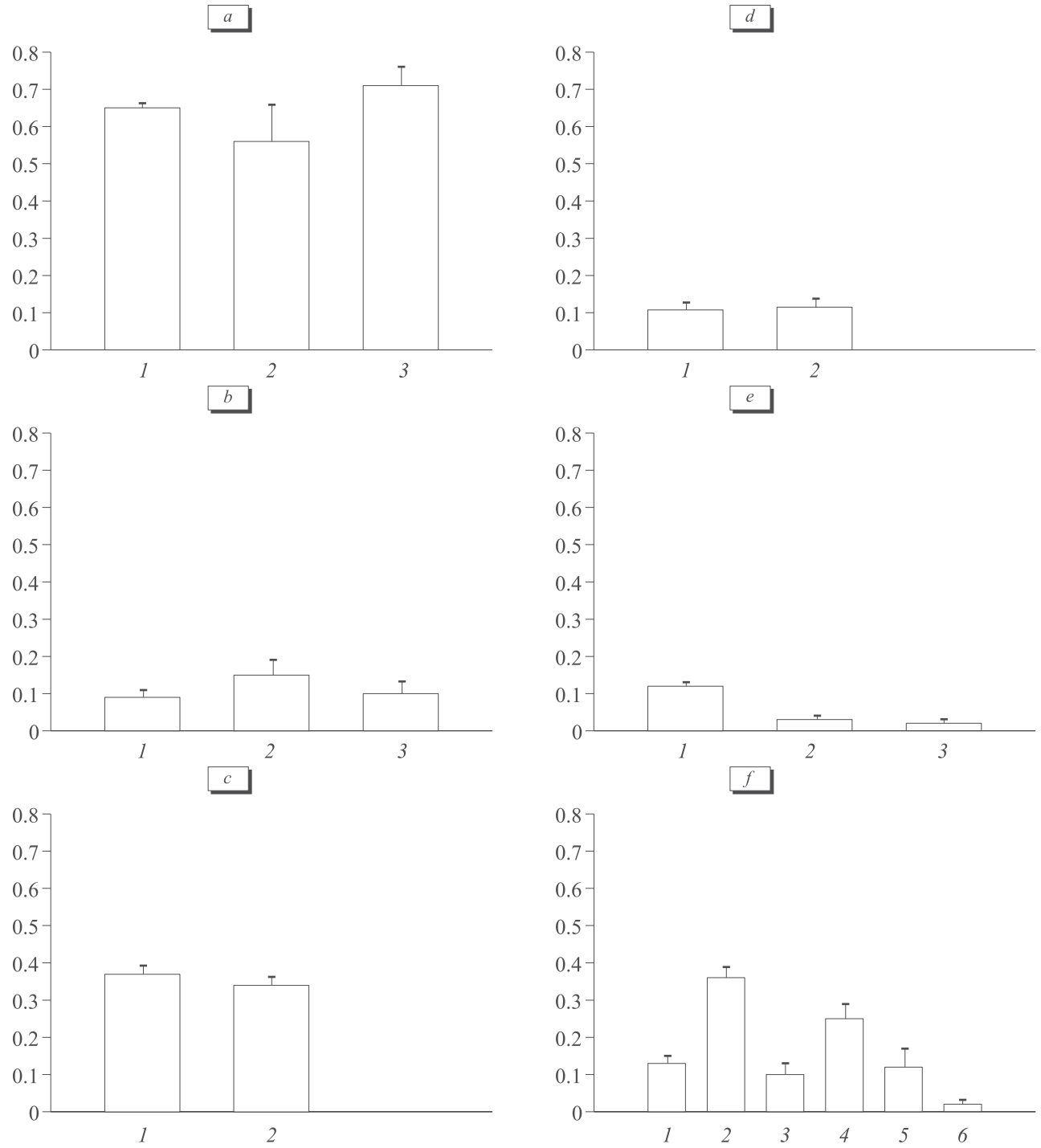

Fig. 1. Effect of physicochemical processing on the optical density of the olfactory bulbs of mature mice. *a*) after fixation: *1*) immersion in 1% formaldehyde; *2*) perfusion in 4% formaldehyde; *3*) immersion in 4% formaldehyde; *b*) after clearing with iothalamate: *1*) immersion in 1% formaldehyde; *2*) perfusion in 4% formaldehyde; *3*) immersion in 4% formaldehyde; *c*) after dehydration: *1*) in increasing ethanol concentrations; 2) in series of 2-butoxyethanol; *d*) after clearing with iothalamate: *1*) pH 6.0; *2*) pH 7.4; *e*) after clearing in benzyl benzoate:benzyl alcohol: *1*) without clearing and permeabilization; *2*) incubation in Dent solution; *3*) incubation in Dent solution and 5 freezing-defreezing cycles; *f*) after clearing in various substances; *1*) 75% water solution of diatrizoate; *2*) 1,3-propandiol; *3*) 75% water solution of iothalamate; *4*) glycerol; *5*) methyl salicylate; *6*) benzyl benzoate:benzyl alcohol.

specimens should be rehydrated in PSB, and after termination of the reaction, they should be dehydrated in 2-butoxyethanol.

3. Clear the specimens in 75% water solution of iothalamate or in benzyl benzoate:benzyl alcohol 2:1 mixture (Fig. 2, *b*, *d*, *f*, *h*; the calibration

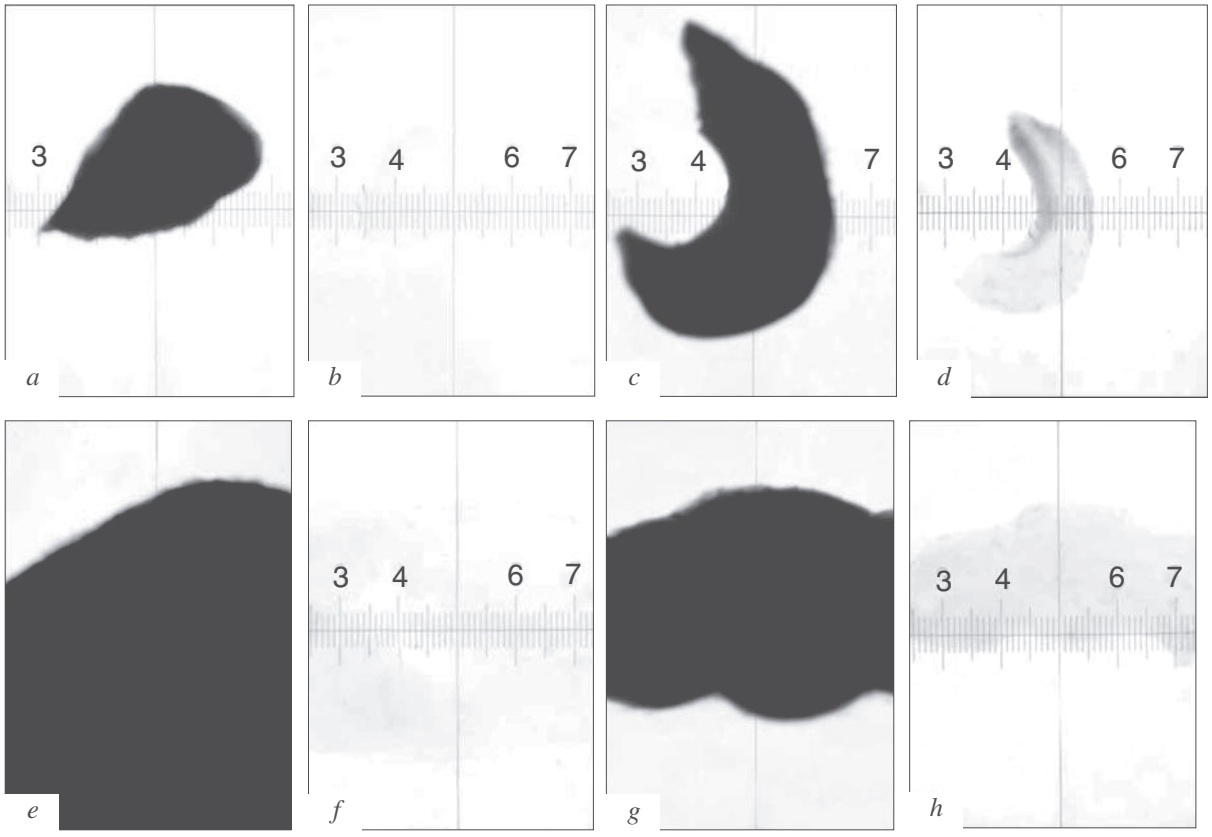

Fig. 2. Cerebral structures of mature mouse: olfactory bulb (*a*, *b*), hippocampus (*c*, *d*), neocortex (*e*,*f*), and cerebellum (*g*, *h*). *a*, *c*, *e*, *g*: the specimens were fixed in 4% formaldehyde; *b*, *d*, *f*, *h*) after fixation, the specimens were cleared according to the developed protocol.

scale was placed under the specimen). The fluorescent markers were not destroyed for several months.

The developed protocols made it possible to obtain optically translucent brain structures of mature mice. Distribution of molecular targets in clarified specimens can be analyzed by light, fluorescent, and confocal microscopy.

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