Human Neural Stem Cells Normalize Rat Behavior after Hypoxia

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Transplants of cultured neural stem cells from human brain survived, retained multipotent activity, and produced a neuroprotective effect on degenerating neurons in the brain of adult rats subjected to hypoxic hypoxia. They normalized animal behavior and improved conditioning in two-way avoidance response paradigm in a shuttle box.

Key Words: *human brain stem cells; xenotransplantation; hypoxia; behavior; immunohistochemistry*

Studies of neural stem cells (NSC) set new task of evaluating the role of these cells in brain processes under normal and pathological conditions [5,9,11]. Isolation of NSC from various regions of embryonic and mature brain and experiments with long-living cultures provided the basis for studies of the possibility of using NSC for neurotransplantation in cellular and gene therapy of experimental brain diseases and brought us close to clinical trials [8,9,13]. New approaches were developed to the therapy of previously incurable diseases (*e.g.*, Parkinson's disease and Alzheimer's disease) [6,7]. Brain damage with diffuse degeneration and death of neurons is a common neuropsychiatric phenomenon and typical postresuscitation complication. Hypoxic hypoxia is one of the models that reproduce brain trauma in animals. The effects of hypoxia were studied in details. Hypoxic hypoxia is a convenient model for evaluation of the efficiency of various methods for the treatment of these diseases [1,4].

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Here we studied the effect of transplantation of cultured NSC from human embryonic brain on the brain and behavior of adult rats exposed to hypoxic hypoxia.

MATERIALS AND METHODS

Experiments were performed on adult female Wistar rats weighing 200-250 g. The animals were divided into 4 groups: group 1, hypoxic hypoxia (HH, n=5); group 2, hypoxic hypoxia and transplantation of cultured NSC from human fetal brain (HH+T, n=7); group 3, hypoxic hypoxia and administration of physiological saline (HH+PS, n=6); and group 4, no treatment (Normal, n=5).

Hypoxic hypoxia was modeled in an altitude chamber. The pressure was reduced to 180 mm Hg over 1 min [1]. The rats were maintained in the chamber for 3 min; clonic convulsions and seizures developed over this period. About 30% animals died. Air was admitted to survived rats over 30 sec to produce normal atmospheric pressure.

One day after hypoxia the rats received NSC or physiological saline. The suspension of whole brain cells from human embryo (9.5 weeks' gestation, 5×10^5 cells/ml) were cultured in selective growth medium DMEM/F12 with N-2-supplement. The medium con-

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tained various growth factors, including basic human fibroblast growth factor (bFGF), epidermal growth factor (EGF), and leukemia-inhibiting factor (LIF).

Before transplantation the rats were narcotized with 300 mg/kg chloral hydrate and placed in a stereotaxis. The suspension (3 μl, 500,000 cells/μl) was implanted by the following coordinates: 3 mm from the bregma, 2 mm laterally, depth 4-5 mm. Physiological saline was administered to animals of the HH+PS group.

Open-field behavior was studied 3 days after HH. On day 4 the animals were trained conditioned two-way avoidance response (CTWAR) in a shuttle box. CTWAR performance was tested on days 9 and 23. Learning ability was scored (1-8) using the scale developed at the Institute of Higher Nervous Activity and Neurophysiology [3]. Intergroup differences were analyzed using STATISTICA software (*t* test for independent variables). The arithmetic mean for each group was calculated. We compared mean scores in each test for each group or combined the results of 3 tests.

On day 27 after hypoxia the rats were perfused with 4% paraformaldehyde in 0.1 M phosphate buffer. The brain was removed and treated with 30% sucrose overnight. Brain sections (20-40 μ) were prepared on a freezing microtome. Preparations were stained by histological (Nissl procedure) and immunohistochemical methods using primary antibodies against human cell nestin (anti Human Nestin, 1:20, Chemicon), human cell nuclei (anti Human Nuclei, 1:30, Chemicon), glial fibrillary acidic protein (anti GFAP, 1:250, DAKO), β-tubulin III (anti β-Tubulin III, 1:200, Abcam), vimentin (anti Vimentin Santa Cruz, 1:100), and nuclear protein of differentiated neurons (anti-Neu N, 1:30, Chemicon). Samples were treated with biotinylated secondary antibodies (Jackson Immuno Research Laboratories) and streptavidin labeled with Texas Red fluorescent dye (Jackson). Double immunohistochemical staining involved secondary antibodies labeled with fluorescent dyes Texas Red and Cy-2 (Jackson). Sections were examined in luminescent or combined light.

Morphometric study was performed with Nissl-stained brain sections from rats of HH+T, HH+PS, and Normal groups. The number of normal, shrunken, and edematous (vacuolated and hypochromic) large pyramidal neurons in neocortical somatosensory layer V of the contralateral cerebral hemisphere was determined (100 cells from each rat, 25 cells in each slice were analyzed). We examined 4 sections from each rat. Treated and control groups were compared using methods of variational statistics (*t* test for independent variables, STATISTICA software).

RESULTS

The rats did not differ by the number of crossed central, middle, and peripheral squares, number of vertical rearing postures, time of grooming, freezing behavior, and period of locomotor activity in the open field.

Test I (4 days after hypoxia) showed that none of the rats in HH, HH-PS, and HH+T attained learning criterion. Avoidance of painful stimulation was not observed in 3 of 5 animals of the HH group and 5 of 6 rats of the HH+PS group. However, only 2 of 6 rats from the HH-T group did not avoid painful stimulus (one rat was excluded from the experiment because of the absence of the transplant and the corresponding behavioral data were excluded from statistical analysis).

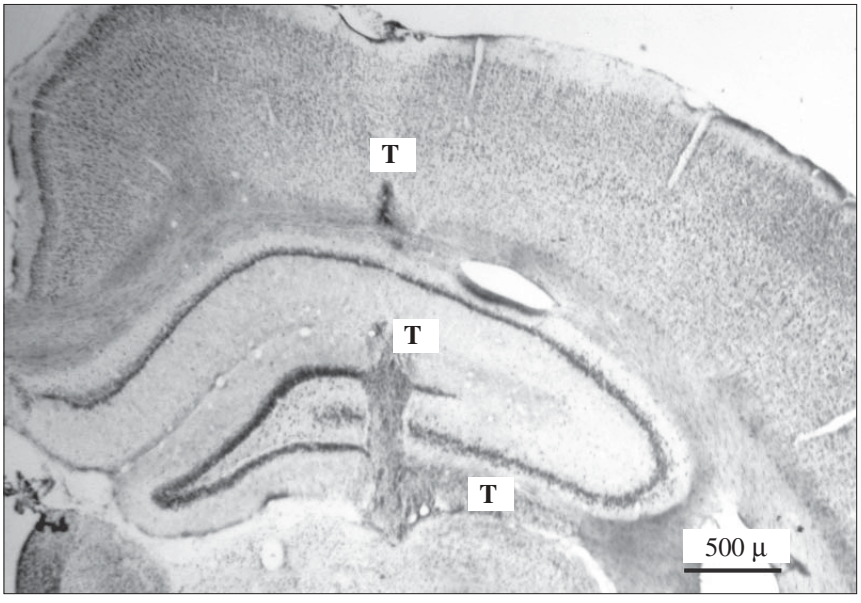

Fig. 1. Transplant of neural stem cells from human fetal brain (T) in the brain of the rat exposed to hypoxia; 27 days after transplantation; cresyl violet staining.

TABLE 1. CTWAR Learning (Mean Score): after 3 (HH, HH+PS, and HH+T) or 2 Tests (Normal, *m*±*SEM*)

Group	Number of terms in variational series	Mean score
Normal (*n*=5)	34	5.30±0.33
HH (*n*=5)	15	2.02±0.35*°
HH+PS (*n*=6)	18	1.6±0.2*
HH+T (*n*=6)	18	2.90±0.66*+

Note. *compared to normal; +compared to HH+PS; °compared to HH+T (tendency).

TABLE 2. Percentage of Various Neurons in Rats (*n*=14, *m*±*SEM*)

Group	Normal	Shrunken	Edematous
Normal	89.0±0.27	2.0±0.2	9.00±0.33
HH+PS	51.00±1.37	30.00±1.37	19.00±0.75
HH+T	71.00±0.96	18.00±0.87	11.00±0.52

Note. *n*, number of terms in the variational series. Each term was calculated as the number of various cells in 25 randomly analyzed neurons.

In test II (9 days after hypoxia) none of the rats of the HH and HH-PS groups demonstrated CTWAR. By contrast, 3 animals of the HH+T group learned CTWAR. It should be emphasized that 1 rat of this group exhibited CTWAR after 15 presentations (maximum score, 8 points).

In test III (24 days after hypoxia) only 1 rat of the HH group slowly attained learning criterion (5 points). None of the animals in the HH+PS group demonstrated CTWAR. At the same time, 3 rats of the HH+T group demonstrated CTWAR. All animals of the Normal group demonstrated good performance of CTWAR in test II.

The rats exposed to hypoxia were compared with intact animals of the Normal group. We summarized the results of 2 tests performed at a 5-day interval. Intact rats differed from animals of other groups (Table 1). It should be emphasized that HH+T rats demonstrated better performance of CTWAR compared to animals of the HH+PS group (p=0.012). The differences between HH+T and HH groups were less pronounced (p=0.088).

Morphological study of brain sections at a level of the ventral hippocampus showed that transplants survived practically in all rats of the HH+T group (except 1 rat). Survived transplants were localized in the hippocampus, thalamus, and/or cerebral cortex (Fig. 1).

Immunohistochemical study showed that transplants contained populations of various cells, including nestin-positive stem cells (Fig. 2, *a*), vimentin-positive committed cells, β-III-tubulin-positive early neuroblasts (Fig. 2, *b*), and GFAP-positive astrocytes. The absence of glial barrier at the boundary between the transplants and recipient brain tissue allowed growth and migration of transplanted cells. It was found that neuroblasts possessed maximum migration capacity and migrated into regions of neuronal degeneration in the recipient brain.

Quantitative analysis of the distribution of pyramidal neurons with various morphofunctional characteristics revealed considerable intergroup differences. Hypoxia was accompanied by the appearance of typical hyperchromic neurons (Fig. 3, *a*) with shrunken nucleus and cytoplasm. They were found not only in focuses of hypoxia, but also diffusely spread over layer V of the cortex. The number of edematous neurons increased (Fig. 3, *b*). These neurons were sometimes seen in intact rats. The percentage of normal, shrunken, and edematous neurons markedly differed in animals of various groups (Table 2).

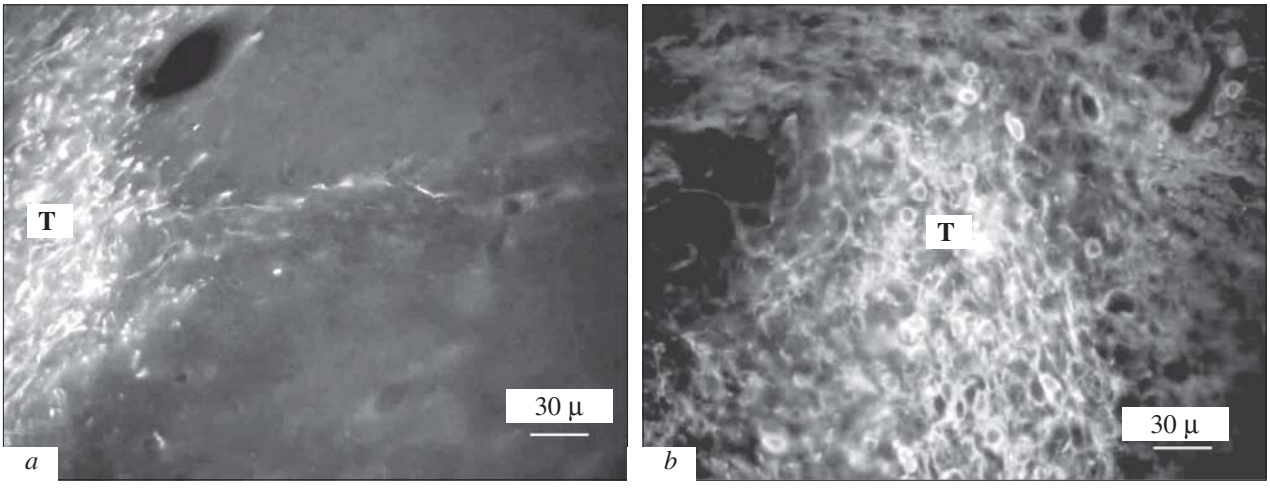

Fig. 2. Transplant of neural stem cells from human brain: nestin-positive stem cells with long processes (*a*) and β-III-tubulin-positive cells differentiating into neurons (*b*). Antibodies against human nestin and β-III-tubulin.

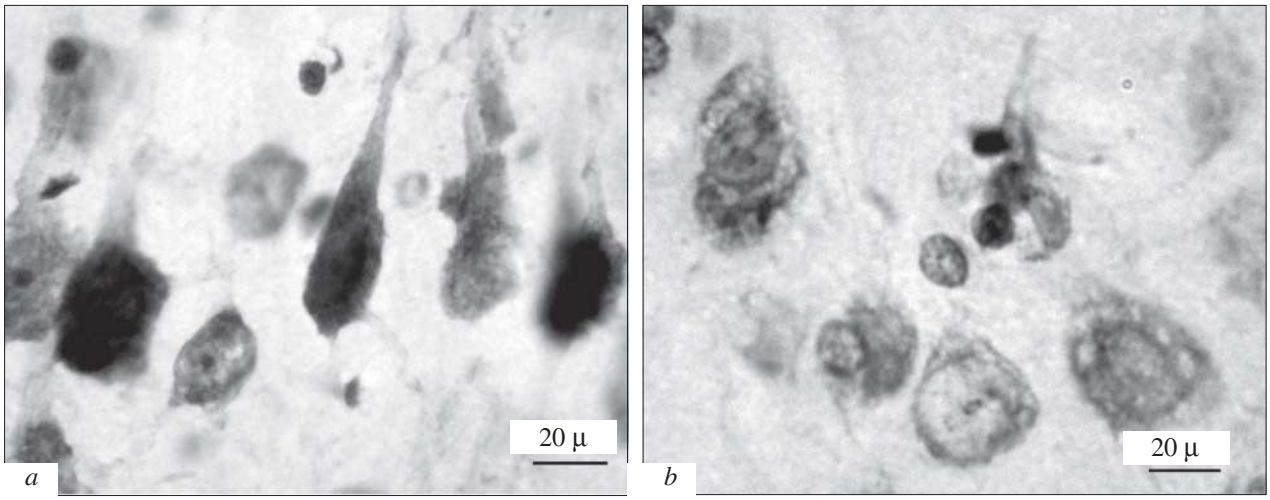

Fig. 3. Hypoxic hypoxia is followed by the appearance of a considerable number of degenerating hyperchromic shrunken (*a*) and hypochromic edematous neurons in the neocortex and hippocampus (*b*). Cresyl violet staining.

The number of normal pyramidal neurons in rats of HH+PS and HH+T groups was much lower than in intact animals (p=0.000006 and p=0.0026, respectively). However, the number of these neurons in HH+T rats was higher than animals of the HH+PS group (p=0.009). The number of shrunken neurons in rats of the HH+PS group was higher than in intact animals (p=0.003), but did not differ from that in HH+T rats (p=0.29). The number of shrunken neurons in HH+PS rats was higher than animals of Normal and HH+T groups (p=0.034 and p=0.013, respectively). However, the count of these cells did not differ in rats of Normal and HH+T groups (p=0.88).

Quantitative analysis of the distribution of pyramidal neurons with various morphofunctional characteristics showed that transplants of human NSC produce a strong neuroprotective effect on the brain of rats subjected to hypoxia.

Intact rats exhibited better CTWAR performance compared to animals exposed to hypoxia. The efficiency of learning did not differ between rats of HH and HH+PS groups. However, HH+T rats demonstrated better learning compared to animals of HH+PS (statistically significant) and HH groups (tendency). This improvement can be related to the presence of transplants in the brain of experimental rats [2].

Our results indicate that transplants of human NSC survive, retain multipotent activity, and produce a neuroprotective effect on degenerating neurons in the brain of adult rats subjected to hypoxic hypoxia. The transplants normalize animal behavior and improve CTWAR learning in the shuttle box.

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