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Research Article

# Comparative Analysis of Functional State after Intracerebral and Subcutaneous Implantations of Neural Stem Cells in Rat Pups Subjected to Perinatal Brain Damage

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- Efficacy of cellular therapy

**Abstract**

Cellular preparations (CP) of embryonic nerve tissue previously derived form 9-day-old rat embryos (rENT) and human olfactory neural stem cells. (hoNSC) were used for single injection into the brain or under the skin of rat pups three days after inducing various-severity perinatal injury of the CNS (CNS PI). Cultivated rat fibroblasts (rF), i. e. non-progenitor cells, were implanted as cellular control. We used the animal models of PIs most adequate to clinical manifestations of severe cerebral palsy - a model of perinatal hypoxic ischaemic brain damage (PHIBD) by the Rice and a model of minimal cerebral dysfunctions syndrome – neonatal anoxia (NA) according to the technique suggested by Speiser. All the mentioned cell types labelled with vital dye CFDA-SE preserved their vitality for not less than 28 days following intracerebral or subcutaneous administration.

Two-month-long weekly monitoring of the motor activity (the "Rotarod" tests, "hanging") and cognitive functions (Passive avoidance, Y-maze) showed that implantation of cell preparations of rat fibroblasts (rFCP) (cellular control) exerted no appreciable effect on the functional state of the rat pups. In contrast, implantation of hoNSC CP and rENT CP into the brain and under the skin resulted in considerable restoration of the impaired functions both in rats with PHIBD and those with NA. Subcutaneous implantation turned out to be not less efficient than implantation of cells directly into the brain. No differences in efficacy of implantation of xenogenic hoNSC and allogenic rENT cells were observed. The findings obtained in the present study suggest a possibility of non-specific neuroprotective effect of cell preparations implanted intracerebrally or subcutaneously to rat pups with perinatal lesions of the brain.

## ABBREVIATIONS

CP: Cellular Preparation; Rent: Embryonic Nerve Tissue; Rf: Rat Fibroblasts; hoNSC: Human Olfactory Neural Stem Cells; PI: Perinatal Injury; SC: Stem Cell; PHIBD: Perinatal Hypoxic Ischaemic Brain Damage; NA: Neonatal Anoxia

## INTRODUCTION

Cellular therapy is currently considered to be one of the promising trends in searching for novel neuroprotective and neuroreparative means and modalities [1]. The world experience with implantation of stem cells and/or progenitor cells (hereinafter referred to as SCs) to laboratory animals raised a series of fundamental problems regarding, first of all, the mechanisms of their action on pathological processes in the CNS [2].

The existing hypotheses related thereto are based on the notion that in order to display their neurotropic properties the implanted SCs should be present in the damaged brain's tissue. Particularly, the case at hand is a possibility of replacing the damaged cells lost resulting from a pathological process with exogenous (implanted) SCs followed by their tissue-specific differentiation and incorporation into the host brain cerebral neuronal circuits [3-5]. The therapeutic (functional) effect of SCs observed in preclinical studies is also explained by their capabilities to migrate towards the focus of nervous tissue damage [4-6], and to express growth factors on their surface [7]. It is supposed that such paracrine mechanism ensures neuroprotection [8], and activation of self-repair processes in nervous tissue at the expense of neoangiogenesis [9], and constitutive neurogenesis [10].

However, there is an ever growing body of scientific evidence that are beyond these conventional stem cell replacement concepts. In particular, it was noted that only a small number of the

transplanted SCs migrate to the lesion site (up to 30 %), and even fewer cells do differentiate into neurons (up to 5 %), whereas the majority of SCs remain within the site of implantation in the non-differentiated state [11]. Following intravenous administration to rats with experimental stroke, most haemopoietic SCs having penetrated into the brain parenchyma either die or differentiate into glial cells with transdifferentiation of SCs into mature neurons being extremely rare, if at all confirmed [12-15]. Mention should also be made that there are considerable differences in the number of exogenous SCs detected in the cerebral parenchyma following intracerebral, intra-liquor, intra-arterial, intravenous and intraperitoneal administration thereof while modelling ischaemic stroke [16-18], PI [19], cerebral injuries [20], and spinal cord injuries in rats [21]. At the same time, efficacy of cellular therapy assessed by restoration of the integral systemic functions of the laboratory animals did not differ substantially. No relationship between the type (source) of the implanted SCs and the level of reparation was revealed in CNS lesions [22].

These data are strongly suggestive of the necessity to carry out experiments that could answer the question of whether the presence of therapeutic SCs in the brain parenchyma is the obligatory condition for manifestation of their neuroprotective and neuroreparative action. Apparently, a deliberately minimal penetration of SCs into the CNS might be simulated by means of their subcutaneous implantation in pathological states.

Our studies were aimed at comparing efficacy of implanting allogenic and xenogenic neural stem cells into the brain parenchyma and under the skin to rat pups with perinatal hypoxic-ischaemic lesions leading to persistent impairments of motor and cognitive functions. In our experiments, we employed preparations of progenitor cells of rat embryonic nerve tissue (rENT) and human olfactory neuronal stem cells (hoNSC) – one of the most adequate types of SCs for cellular therapy in neurological clinical practice [23], as well as preparations of rat fibroblasts (rF – non-progenitor cells) as "cellular" control. To objectively characterise the effect of cellular therapy we monitored the functional state of rats prior to their puberty by means of assessment of cognitive functions and functional loading tests. We previously demonstrated the suitability and utility of these loading tests [24-26].

## RESULTS

### General condition of the animals

Experimental perinatal injuries to the CNS were accompanied and followed by death of a relatively small portion of the rat pups, in particular while modelling PHIBD – 4 out of 95 rats (4.2 %) died and while modelling NA only 1 rat pup died out of 39 (2.6 %). The values of weight gain during the 1st month of animals' life tended to decrease. Thereafter (the second month of animals' life), the dynamics of the body weight of rats with CNS PI did not differ from that of intact rats. Implantation of cell preparations in intracerebral and subcutaneous implantation administration to the animals with PHIBD neither exerted influenced significantly substantially the body weight indices.

### *In vivo* implanted cell viability survival

Labelled with vital dye CFDA-SE hoNSC, rF and rENT cells were observed detected in fluorescent analysis in the sites of their administration in the brain and under the skin after 3, 7, 14 and 28 days after implantation (Figure 1). We failed to visualize these cells in more remote terms due to biodegradation of CFDA-SE and decreased intensity of the characteristic fluorescence in the green spectrum.

14 and 28 days after implantation of hoNSC and rENT they were found at a distance of 2-3 mm from the administration site and following implantation under the skin they remained in site of injection. rF implanted both into the brain and subcutaneously remained in site of injection as a compact clusters. No hoNSC, rF and rENT cells injected under the skin were found on serial slices of the brain at all terms of fluorescent analysis.

### Cerebral morphometry

Animals with PHIBD were found to have atrophy of the cerebral hemisphere on the side of occlusion of the common carotid artery (Figure 2). The area of the parenchyma of the mentioned hemisphere at the age of 8 weeks of these rats was by 15% less than in intact animals ($P < 0.001$). Neither intracerebral nor subcutaneous implantation of cell preparation of CPs exerted substantial influence on the value of this parameter. Mention should only be made of a certain tendency towards decreased atrophy of the brain in implantation of neural SC (Figure 2). Microscopy of the Nissl-stained brain slices, at the end of the experiment revealed cellular implants of rF, rENT and hoNSC as a conglomeration of cells in the CA1 zone of the hippocampus and the adjacent structures with impaired architectonics characteristic of them.

### Functional state of animals with PHIBD

Rats with PHIBD not subjected to implantation of cell preparations demonstrated significant alterations in the parameters ($P < 0.05$) at all terms of the study in the "Rotarod" and "passive avoidance" tests, thus suggesting impairments of the integral motor and cognitive functions (Table I). There was virtually no self-restoration of these functions seen within 2-month monitoring. The "hanging" test demonstrated a significant decrease in the parameter only on week 3-4 of life (Table I), suggesting pronounced restoration of the rats' ability to hold onto the rod by puberty (8 weeks).

Implantation of rF CP (cell control) into the brain and under the skin to rats with PHIBD exerted no appreciable effect according to the values of all parameters (Figure 3). In contrast, implantations of hoNSC CP and rENT CP both into the brain and under the skin resulted in improved functional state of the rats, with the indices of the tests in rats with implanted hoNSC CP and rENT CP systematically (at each term of studies) exceeding the values in the animals receiving no implantation and with administration of fibroblasts (cell control). Differenced reached statistically significant levels on week 6-7 of life (Table 2).

The final assessment of the effect of CPs implantations of CP on functional restoration in rats was performed carried out by means of analyzing the value of functional deficiency deficit and efficiency of cellular therapy at the end of the experiment (8 weeks).

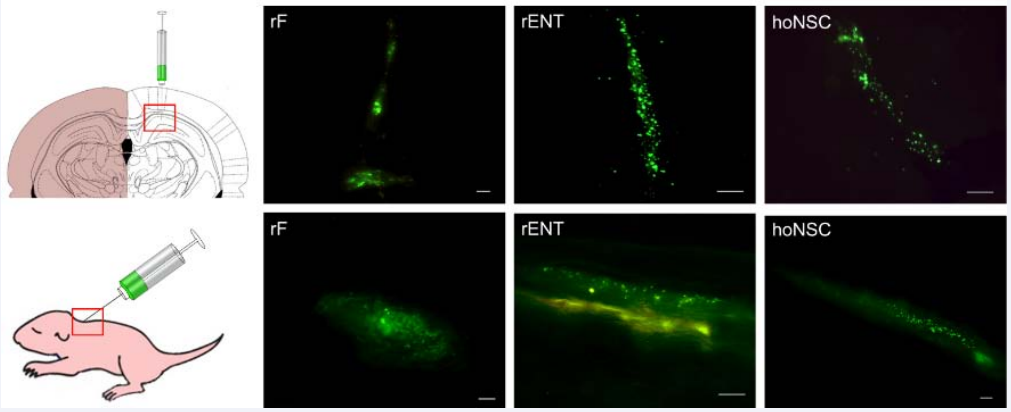


**Figure 1** Detection of cells labelled with vital dye CFDA-SE 28 days after implantation into the CA-1 zone of the hippocampus of the contralateral hemisphere of the brain (left part of the Figure) and under the skin of the interscapular region (right part of the Figure) to rat pups with PHIBD. Cryoslices, fluorescent microscopy. Scale bar is 120μm.

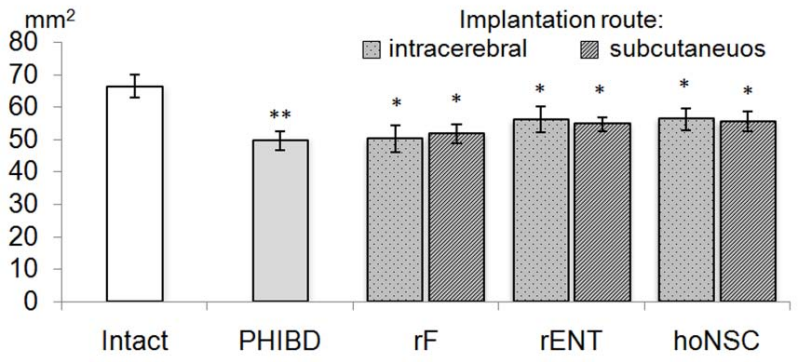


**Figure 2** Areas (mean ± S.E.M, mm2) of the cerebral ipsilateral hemispheres parenchyma 8 weeks after implantation of rF, rENT and hoNSC to rat pups in acute stage of PHIBD. Coronal cryoslices of AP -0.2 mm.

**Table 1:** Dynamics of functional impairments in rats during modelling of PHIBD.

| Groups of animals | Parameters (mean ± S.E.M.) of the rats' functional state, age (weeks) | | | | | |
|---|---|---|---|---|---|---|
| | 3 | 4 | 5 | 6 | 7 | 8 |
| Time of holding on the string, s | | | | | | |
| Intact | 162.7 ± 29.7 | 161.4 ± 95.4 | 122.6 ± 143.6 | 106.4 ± 118.9 | 112.3 ± 20.8 | 98.7 ± 79.3 |
| PHIBD | 84.1 ± 15.9* | 26.9 ± 16.6* | 19.9 ± 25.0 | 16.8 ± 20.4 | 17.3 ± 21.0 | 17.4 ± 14.0 |
| Time of holding on the rotating rod, "Rotarod" test, s | | | | | | |
| Intact | | 68.5 ± 9.0 | 76.8 ± 8.4 | 105.9 ± 15.7 | 97.7 ± 13.3 | 86.2 ± 12.2 |
| PHIBD | | 39.5 ± 8.6* | 41.8 ± 5.0* | 42.7 ± 6.5* | 45.5 ± 5.8* | 40.0 ± 4.4* |
| Latent period of passive avoidance, s | | | | | | |
| Intact | | | 196.5 ± 19.7 | 182.9 ± 21.4 | 230.7 ± 18.6 | 248.7 ± 16.9 |
| PHIBD | | | 35.3 ± 10.7 * | 35.8 ± 12.0 * | 107.7 ± 22.9* | 125.5 ± 22.9 * |

Note: Intact group n = 42, PHIBD group n = 30. *- $P < 0.05$ as compared with intact animals

Functional deficit was calculated as the difference between relative values of the functional value in groups with implanted cells (rENT, hoNSC and rF) and values of the function of intact animals, taken as 100%. Functional deficit in the motor sphere by the "Rotarod" test amounted in untreated animals to 54%, whereas implantation of neurogenic cells decreased it: in the group of rENT cerebral implantation – 37 %, subcutaneous implantation – 25 %, in the group of hoNSC - cerebral implantation – 10 %, subcutaneous implantation – 43 %. A positive statistically significant effect of cell therapy on rats' ability to hold on the string ("hanging" test) was observed only on week 3-4 of rats' life, while later on and at the end of the experiment the therapeutic effect was inconsiderable (Table 2 and Figure 3).

According to the "passive avoidance" test, functional deficit amounted to 49 %. Implantations of hoNSC CP and rENT CP into the brain and under the skin decreased it to 27 % and 20 % in the rENT group, - 20 % and 0 % in the hoNSC group, respectively.

**Table 2:** Monitoring of the indices .(mean ± S.E.M) of functional state of rats after intracerebral and subcutaneous implantation of cell preparation of rat fibroblasts (rF), rat embryonic nervous tissue (rENT) and human olfactory neuronal stem cells (hoNSC) in acute period of PHIBD.

| Groups of animals | Intracerebral implantation, rats' age, weeks | | | | | | Subcutaneous implantation, rats' age, weeks | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 3 | 4 | 5 | 6 | 7 | 8 | 3 | 4 | 5 | 6 | 7 | 8 |
| Time of holding on the string, s | | | | | | | | | | | | |
| rF | 149.4 ± 17.0 b | 154.9 ± 37.5 | 141.4 ± 33.9 | 122.6 ± 33.1 | 106.3 ± 22.7 | 80.5 ± 13.8 | 133.1 ± 31.7 | 127.4 ± 25.6 | 163.6 ± 28.3 | 102.6 ± 27.4 | 97.6 ± 23.9 | 84.4 ± 14.7 |
| rENT | 426.6 ± 66.0 a,b,c,d | 372.6 ± 49.8 a,b,c | 130.9 ± 23.4 | 61.1 ± 3.4 a,b,e,d | 77.9 ± 11.6 b | 109.6 ± 15.2 | 386.4 ± 56.3 a,b,c | 357.9 ± 76.7 b,c | 207.7 ± 60.5 | 145.1 ± 41.8 | 113.2 ± 33.5 e | 91.2 ± 17.7 |
| hoNSC | 185.4 ± 34.9 b,d | 296.3 ± 65.9 a,b,c | 168.7 ± 21.1 e | 89.7 ± 14.1 d | 98.0 ± 12.3 | 89.0 ± 15.8 e | 254.9 ± 43.3 b,c | 211.3 ± 61.8 | 116.4 ± 19.3 | 92.2 ± 24.1 e | 108.6 ± 33.0 | 140.3 ± 21.9 b,c,e |
| Time of holding on the rotating rod, "Rotarod" test, s | | | | | | | | | | | | |
| rF | | 32.1 ± 8.8 a | 35.3 ± 7.2 a | 20.4 ± 7.2 a, b | 31.9 ± 9.0 a | 39.6 ± 9.5 a | | 25.3 ± 7.4 a | 28.6 ± 7.0 a | 20.7 ± 7.4 a | 24.1 ± 8.3 a, b | 31.0 ± 8.3 |
| rENT | | 77.3 ± 29.4 b | 51.2 ± 8.1 a | 44.3 ± 10.5 a, c | 69.2 ± 9.9 b, c,d | 54.0 ± 9.1 | | 72.8 ± 14.1 b,c,d | 59.3 ± 11.8 c | 59.2 ± 10.7 c, d | 61.3 ± 10.1 c | 64.3 ± 11.2 b,c |
| hoNSC | | 40.3 ± 8.7 a | 38.2 ± 6.9 a | 39.8 ± 7.1 a, c | 34.7 ± 8.3 a, d | 77.6 ± 28.8 a | | 40.3 ± 7.8 d | 38.9 ± 13.4 a | 26.0 ± 9.6 a, d | 54.9 ± 20.3 | 49.1 ± 11.4 a |
| Latent period of passive avoidance, s | | | | | | | | | | | | |
| rF | | | 49.9 ± 29.0 a | 71.7 ± 29.1 a | 152.3 ± 41.8 | 137.4 ± 45.4 a | | | 43.6 ± 14.3 a | 30.1 ± 9.6 a | 86.9 ± 35.3 a | 126.0 ± 35.1 a |
| rENT | | | 61.0 ± 31.6 a | 276.6 ± 16.5 a,b,c,e | 205.8 ± 47.2 b | 180.8 ±48.0 | | | 54.4 ± 34.6 a | 280.6 ± 19.4 a,b,c,e | 230.4 ± 33.0 b,c | 198.8 ± 46.8 |
| hoNSC | | | 46.4 ± 34.2 a | 59.8 ± 33.2 a,c,e | 234.5 ± 34.4 b | 198.8 ± 44.2 | | | 80.5 ± 45.2 a | 213.0 ± 43.5 b,c,e | 204.9 ± 41.0 b,c | 271.0 ± 27.3 b,c |

Note: Each group consisted of 9 animals, excepting the group of rF with intracerebral implantation (n = 10). $P < 0.05$ as compared with:
a – intact group (Table 1)
b – PHIBD group (Table 1)
c – rF group
d – rENT and hoNSC group
e – Analogous groups in intravenous and subcutaneous routes of administration

In the latter case there was complete restoration of the memory function. It was confirmed by analysing the ratio of rats having preserved the passive-avoidance reflex learnt on week 4 of rats' life (Figure 4).

Efficacy of cell therapy was assessed as a difference in the parameters of functional deficits in groups of implantation of neural SCs (rENT CP, hoNSC CP) and functional deficits of the respective groups of cell control (rF implantations). Such an approach made it possible to exclude probable contribution into the value of the calculated parameter of the efficacy of implantations of neural SC factor of non-specific stimulation of reparative processes in the body following the procedure of implantation. Efficacy of intracerebral administration of cell preparations of rENT and hoNSC to rat pups with PHIBD calculated (as described in 4.6) 8 weeks after implantation of cells by the Rotarod test amounted to 17% and 44%, whereas in subcutaneous administration equalling 39% and 21%, respectively. The "hanging" test demonstrated virtually complete self-restoration of the rat pups' ability without cellular therapy to hold onto the string commencing from the age of 5-6 weeks. Along with it, efficacy of cellular therapy according to this test, calculated for the 4-week-old rat pups amounted to 18% in intracerebral and to 32% in subcutaneous administration of the both preparations.

Efficacy of subcutaneous implantation of cell preparations at the end of the experiment (8 weeks) turned out not only comparable with efficacy of intracerebral administration (Figure 5), but also was slightly higher according to the "Rotarod" tests (rENT – 39 % versus 17 %), "hanging" (32 % vs 18 % for the both CPs) and "passive avoidance" (hoNSC 49 % vs 25 %).

No pronounced differences in efficacy of allogenic cells rENT and xenogenic hoNSC cells were revealed (Table 2) and they were of a variously directed character. Thus, this index by the "Rotarod" test in cerebral implantation in the rENT group amounted to 17 % and 44% in the rENT and hoNSC groups, in implantations under the skin to 39% and 21%, respectively, and by the "passive avoidance" test in cerebral implantation – to 17%

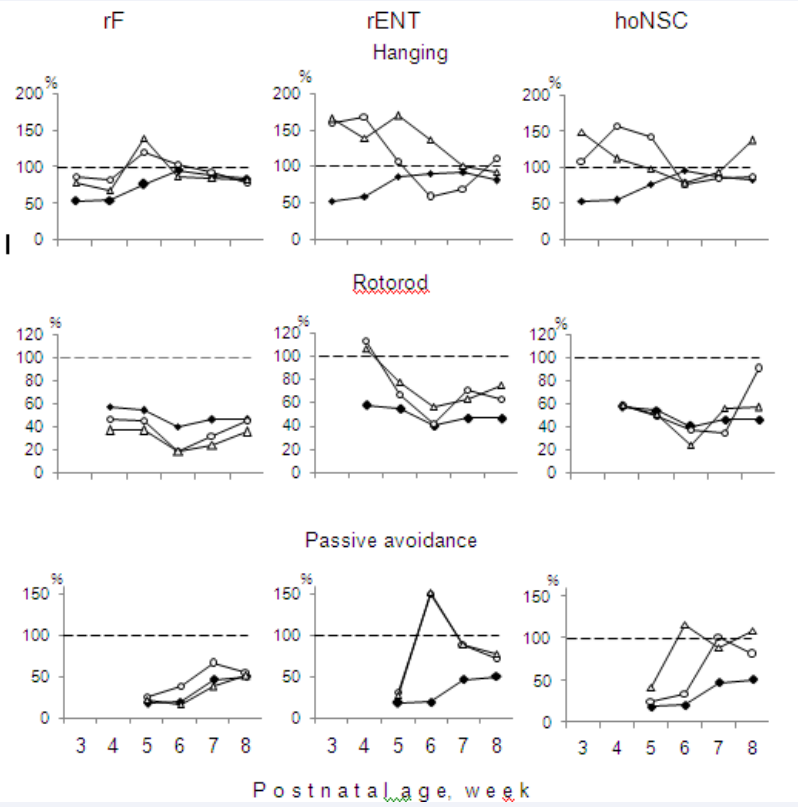


**Figure 3** Relative indices of restoration of the functional state of PHIBD animals after implantation of cell preparations of rat fibroblasts (rF), rat embryonic nervous tissue (rENT) and human olfactory neuronal stem cells (hoNSC). Signs and symbols: --- indices of tests in intact animals, taken as 100 %; –◆– – untreated animals; –O– – intracerebral implantation; –Δ– – subcutaneous implantation. The statistical analysis is shown in Table I.

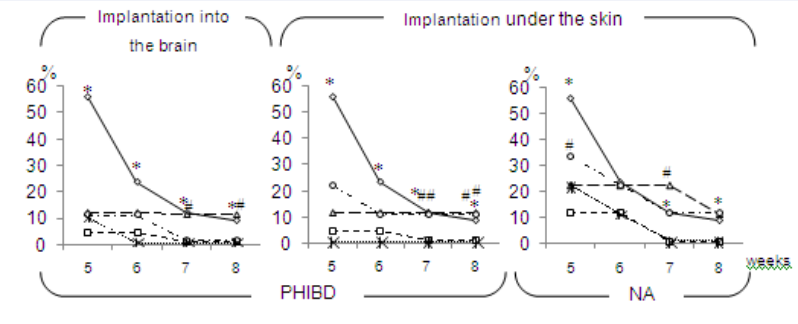


**Figure 4** Ratios of rats (percentage on the y-axis) having preserved the passive avoidance reflex learnt on week 4 of life. Subcutaneous and intracerebral implantations of cell preparations rF (–✱–). rENT (–Δ–). and hoNSC(–O–).. PHIBD (–□–). NA (–□–). –◇– - Intact animals. Note: * $p < 0.05$ when comparing the PHIBD. NA groups with intact rats; # $p < 0.05$ when comparing the rF. rENT and hoNSC groups with the groups of untreated animals rats (PHIBD or NA).

in the rENT group, to 21% in the hoNSC group, in subcutaneous implantation to 39% and 21%, respectively. No differences were revealed according to the "hanging" test (Figure 5).

## Functional state of animals with NA

In a series of the experiments with modelling NA, cell preparations were administered only under the skin. The animals with NA not subjected to cell therapy demonstrated a steady decrease in the memory function ("passive avoidance" test) and their ability to learning training in the Y-shaped maze (Table 3, Figures 4 and 6). Functional deficit by the end of the experiment amounted to 37 % and 64 %, respectively. rF implantation (cell control) did not exert any considerable influence on the parameters studied, while implantation of hoNSC CP and rENT CP resulted in a considerable restoration of the impaired functions (Figures 3 and 5). Thus, deficit of the memory function in the hoNSC CP and rENT CP groups was completely levelled by the end of the experiment. It was confirmed by analysing the ratio of rats having preserved the passive-avoidance reflex learnt on week 4 of rat's life (Figure 4). Deficit of the learning ability in

**Table 3:** Monitoring of the indices (M ± m) of cognitive functions after subcutaneous implantation of cell preparations of rat fibroblasts (rF); rat embryonic nervous tissue (rENT) and human olfactory neuronal stem cells (hoNSC) to rats pups with NA.

| Parameter | Groups of animals | Age, weeks | | | |
|---|---|---|---|---|---|
| | | 5 | 6 | 7 | 8 |
| Latent period of passive avoidance, s | Intact n = 42 | 196.5 | 209.3 | 238.2 | 248.7 |
| | | ± 19.7 | ± 21.0 | ± 16.9 | ± 16.9 |
| | NA n = 12 | 22.3 | 211.4 | 233.4 | 155.6 |
| | | ± 12.1 | ± 52.0 | ± 35.0 | ± 53.0 |
| | | a | | | a |
| | rF CP n = 8 | 142.5 | 114.9 | 143.4 | 143.9 |
| | | ± 52.3 | ± 39.4 | ± 42.8 | ± 42.0 |
| | | | a | a | a |
| | rENT CP n = 9 | 89.6 | 73.6 | 224.1 | 273.9 |
| | | ± 35.5 | ± 31.2 | ± 37.2 | ± 26.1 |
| | | a | a,b,d | | b |
| | hoNSC CP n = 9 | 89.1 | 243.6 | 286.8 | 297.6 |
| | | ± 33.1 | ± 37.4 | ±13.2 | ±2.4 |
| | | a | c, d | a, c | a, b, c |
| Time of finding the non-aversive sleeve in the Y-maze, s | Intact n = 13 | 18 | 23.6 | 16.5 | 24.6 |
| | | ±3.5 | ±4.4 | ±4.4 | ±6.2 |
| | NA | 74.5 | 93.6 | 43.2 | 107.6 |
| | | ±16.3 | ±13.9 | ±4.8 | ±21.6 |
| | | a | | | a |
| | rF CP | 66 | 34 | 27 | 55.1 |
| | | ± 9.5 | ± 13.5 | ± 12.3 | ± 12.5 |
| | | a | | | a |
| | rENT CP | 34 | 21.9 | 21.2 | 19.9 |
| | | ± 6.6 | ± 6.6 | ± 4.4 | ± 4.6 |
| | | b,c,d | | | b, c, d |
| | hoNSC CP | 24.7 | 16 | 23.8 | 34.9 |
| | | ± 2.9 | ± 5.7 | ± 11.5 | ± 8.1 |
| | | b, c, d | b | | d |

$p < 0.05$ as compared with the group: a – intact; b – NA; c – rF; d – while comparing the rENT and hoNSC groups.

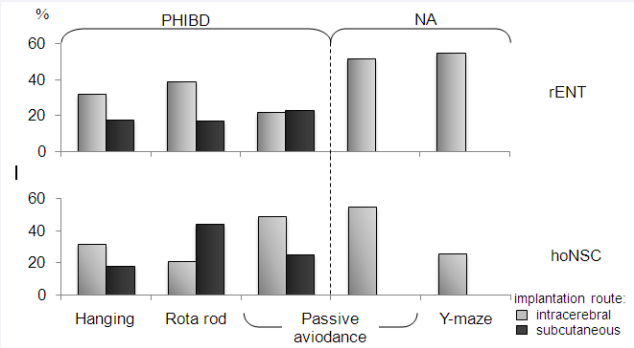


**Figure 5** Efficacy (percentage) of implantation of cell preparations of rENT and NA to rats in acute stage of PHIBD and NA by the condition of cognitive and motor functions at the age of 8 weeks. Efficacy by the hanging test was determined at the age of 4 weeks, since the untreated animals were found to have self-restored the function involved.

rat pups with NA after subcutaneous implantations decreased (absence in the rENT group and 29 % for the hoNSC group). Efficacy of subcutaneous implantations of rENT and hoNSC by learning ability (Y-shaped maze) amounted to 55 % and 26 %, respectively, and while assessing the memory function ("passive avoidance" test) to 52 % and 62 %, respectively (Figure 5).

## DISCUSSION

There exists a number of publications suggesting partial functional restoration in animals with experimental CNS PI following implantation of various-origin SCs both directly into the brain [27-29] and in intravenous, intracardiac [30,31],

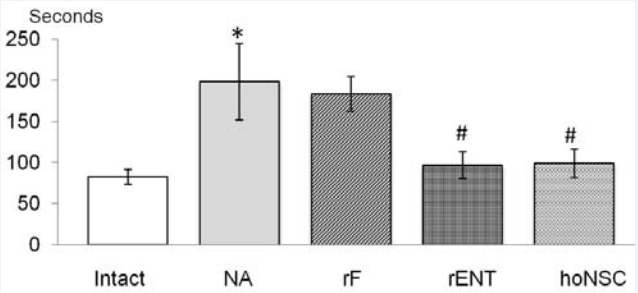


**Figure 6** Effect of implantation of rat fibroblasts rF(■), rat embryonic tissue rENT (■) and human olfactory neural stem cells hoNSC(■) on learning ability of rats with NA(■). On the y-axis – total time of searching nonaversive sleeve (s) in the conditions of altering its special location in the Y-shaped maze in daily trainings of animals from 5th to 8th weeks of life. □– group of intact rats. Note: * - $p < 0.05$ as compared with the intact rats. # - $p < 0.05$ as compared with the NA and rF groups.

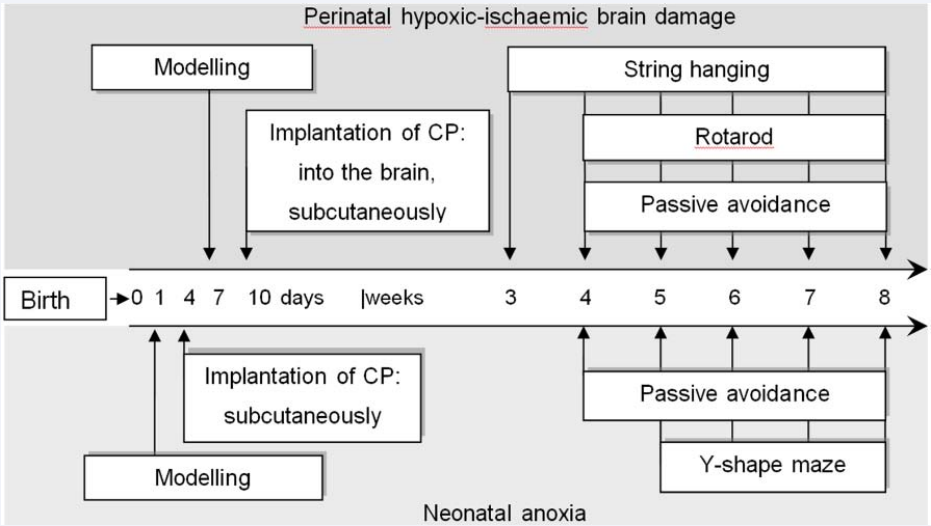


**Figure 7** Scheme of manipulations and functional monitoring in modelling of PHIBD (above) and NA (below).

intraperitoneal [32,33] and intranasal [34], administration of SCs. Even more such positive results were obtained during cellular therapy of experimental stroke in adult rats [2,22], though there are several publications containing data showing no evidence of functional effect after parenteral administration of SCs in experimental lesions of the CNS [35,36]. Regarding the results of subcutaneous implantation of SCs when penetration thereof into the brain is practically impossible or minimal we know of only works by some clinicians reporting improved motor and cognitive functions in patients after administration (subcutaneous) of cellular preparations made of embryonal or foetal human nerve tissue, in a chronic stage of various neurological and mental diseases (hypoxic-ischaemic encephalopathy, Down's syndrome, epilepsy, etc.) [37-39].

In our experiments, we determined that single subcutaneous implantation of xenogenic or allogenic SCs (rENT and hoNSC, respectively) to rat pups with an acute stage of various-severity CNS PI (PHIBD and NA) also resulted in substantial restoration of impaired motor and cognitive functions, similar to that in analogous intracerebral implantations. However, no rENT, hoNSC, nor RF cells ("cellular control") labelled with vital dye CFDA-SE were found in the brain on fluorescent microscopy of cerebral slices 3, 7 and 14 days after their administration under the skin, thought they were visualized in the implantation site (in the subcutaneous fat) and were viable for not less than 28 days. After intracerebral implantation to rat pups the rENT, hoNSC and RF cells also retained their vitality in the cerebral tissue for not less than 28 days. At longer terms of studies we failed to find any structures in the green spectrum characteristic of cell viability due to biodegradation of CFDA-SE [40]. In parallel, intracerebral implants were revealed on Nissl-stained cerebral slices in all rats with PHIBD at the age of 8 weeks, which coincided with the findings of other laboratories [31,41]. Hence, the observed restoration of motor and cognitive dysfunctions in CNS PI animals following subcutaneous administration of the neural SC preparations took place during rat pups' growth, with no implanted SCs appearing in the brain parenchyma. This effect did not depend upon the type of stem cells, either allogenic or xenogenic.

One of the currently important methodological problems of preclinical studies of efficacy of cellular preparations, in our opinion, is the inclusion "cellular control" into the experimental

design in order to compare the outcomes of therapy with SCs to a group of animals receiving an adequate preparation containing cells possessing no stem (progenitor) properties. The available literature contains sporadic studies reporting the inclusion of "cellular-control" groups into the experimental design [42,43].

In our experiments, we used cultured fibroblasts, i. e., rat differentiated cells, serving as "cellular control". At the same time, it has long been known that fibroblasts are capable of expressing a series of growth factors and exert certain reparative effect on the surrounding tissues [44]. Studies showed that these cells might in fact be employed as cellular control, since functional restoration in the groups of rats with CNS PI who were given fibroblast preparations did not differ substantially from that in the animals with CNS PI without implantation of cellular preparations, although in separate tests there was a trend toward a certain decrease in functional deficit (Figure 3).

The data from existing literature concerning efficacy of experimental cell therapy on animal models of lesions and diseases of the CNS are highly contradictory and difficult to interpret and analyse due to differences in the protocols (designs) of the experiments [45]. Efficacy of cell therapy for restoration of the integral functions of the body is typically evaluated by the mean values of the parameters being studied in the groups of treated and untreated animals with a standard experimental lesion of the CNS. Unlike the above-mentioned standard approach, our assessment of efficacy of cell therapy was carried out with due regard for specificity of action of SC preparations (the use of "cellular control") and the levels of the maximally attainable restoration of the functions (comparison with the indices in intact animals taken as 100 %). Our calculations with the use of the data from separate publications demonstrated that 9 days after intracerebral transplantation of neural SC to rat pups in an acute stage of PHIBD, the restoration of motor functions in the Rotarod tests and apomorphine-induced asymmetry amounted to 40 % and 17 %, respectively [46]. When assessing the width of the print of the contralateral forefoot in 3-month-old rats with PHIBD, the efficacy of intraperitoneal transplantation of the mononuclear fraction of the umbilical cord amounted to 14 % [47]. 40 days after intracardiac administration of human mesenchymal stem cells to rat pups with PHIBD efficacy according to the cylinder-test amounted to 33 % and by the Rotarod test to 31 % [31]. Spatial memory 8 weeks after intracerebral implantation of pre-differentiated embryonal SCs to rat pups with PHIBD improved by 56 % as compared with the untreated animals [27].

According to the findings of our experiments, efficacy of functional restoration in rat pups with PHIBD after subcutaneous injection of cell preparations was as follows: by the motor functions within the range of 21-39% and by the cognitive functions – 22-49 % in different experimental groups. Mention should be made that cognitive functions restored to a greater extent in rat pups with NA (26-62 %), i. e., with a relatively mild CNS lesion, than in those with PHIBD according to Rice [48] (Figure 5). Thus, therapeutic efficacy of subcutaneous implantations turned out to be not inferior to that of the above-mentioned studies wherein SCs were administered by means of other routes to animals with PHIBD.

In discussing the achieved level of functional restoration, it is important to point out that we, like other investigators [6], administered SCs 3 days after modeling CNS PI. However, taking into account the revealed positive therapeutic effect of subcutaneous implantations of SCs in CNS PI, it seems justified to suppose that earlier subcutaneous (extracerebral) administration of SC in experimental PIs might also be effective. This is in conformity with the literature data on experimental cellular

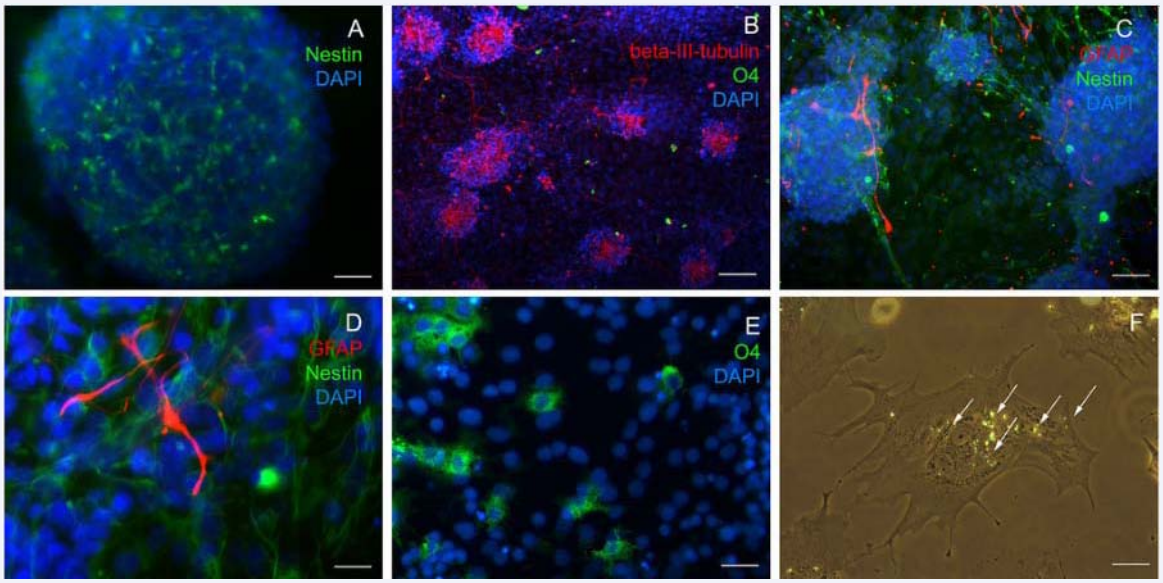


**Figure 8** Immunocytochemical phenotyping of cellular preparations. A, B, C – human olfactory lining neural stem cells. D, E - rat embryonic nerve tissue cells. Immunofluorescent analysis using either anti-human or anti-murine antibodies to the neural progenitor cell marker nestin(A, D), neuroblast marker β-III-tubulin(B), oligodendrocyte marker O4 (B, E), and astrocyte marker GFAP(C, D), with the cellular nuclei counterstained with DAPI. Fluorescence microscopy. F – rat fibroblasts, characteristic absorption of collagenous nanoparticle is indicated with arrows. Fluorescent / dark-field microscopy. Scale bar 100 µm (A, B, C); 20 µm (D, E, F).

therapy of stroke in adult rats with parenteral administration of SCs immediately after inflicting the damage [2].

## CONCLUSION

Hence, it was determined that single subcutaneous implantation of xenogenous or allogenic SCs in an acute period of CNS PI (PHIBD and NA models) to rat pups resulted in considerable restoration being not less effective than that of intracerebral implantation of SCs Reliability of this conclusion has been confirmed not only by mathematical statistics, special calculations of efficacy of cellular therapy, but also by reproducibility of the results in separate series of our experiments on various models of CNS PI.

The phenomenon of therapeutic effect of SC on subcutaneous administration we have hereby revealed in the experiments on rats with CNS PI cannot be explained by an immediate (direct) effect of the implanted SCs on the brain. The fact that we revealed no SC implanted under the skin in the brain of rats in whom there occurred significant restoration of the impaired integral functions makes it possible to suppose that the mechanisms of the therapeutic action of exogenous SCs include hitherto unknown remote signals exerting a mediated influence on the repair processes in the CNS, probably through acting upon regulatory systems of the body. In particular, mention should be made of the ability of SCs to influence the balance of pro- and anti-inflammatory cytokines [47], and growth factors [49,50]. However, with due regard for the huge amount of the world-accumulated experimental material, a detailed discussion of the above-mentioned trend of studies might be a subject of a special analysis.

## EXPERIMENTAL PROCEDURE

### Animals and experimental groups

The studies were carried out in accordance with the provisions laid down by the Ethics Committee of the State Scientific Centre for Social and Forensic Psychiatry named after V.P. Serbsky and international rules of humane treatment of laboratory animals. The work was performed on both-gender Wistar rat pups kept in standard conditions at the Experimental Biological Clinic of the Centre. The female-to-male ratio in experimental groups was similar (50 % ± 3 %). The scheme of manipulations and functional monitoring is shown in Figure 7.

Severe PHIBD was modelled in 95 rat pups according to the Rice [48], technique. Relatively mild perinatal lesion of the CNS (39 rats) – neonatal anoxia (NA) was induced according to the Speiser [51], technique – a model of the minimal cerebral dysfunction syndrome.

Rat pups with PHIBD were subjected to intracerebral (n = 33) and subcutaneous (n = 28) implantations of cellular preparations (CPs). 26 rats with NA received only subcutaneous injections of CPs. The rest animals with PI (receiving no implantations) constituted the respective control groups (a total of 47 animals). Also studied were intact neonatal rats (n = 42) born and maintained in the same conditions of the vivarium.

The rat pups were kept with their mothers up to 4 weeks of age in individual cages and were then placed by 4-5 animals per cage and were allowed to gain access to food and water ad libitum.

### Modelling technique

**PHIBD**: Surgical manipulations were carried out in aseptic conditions with no antibiotics used. Under halothane inhalation anaesthesia, 7-day-old rats weighing over 10 g were subjected to the procedure of exposing the left common carotid artery cut between two silk ligatures. The skin cut was closed (vicryl *N°* 8-0) to be followed by halothane cessation. The rats awoke rapidly and were placed for 1 hour onto a warmed platform (35C) and then transferred for 120 min into a 3-litre chamber provided with a warmed bottom (35°C) and fed by means of an artificial ventilation machine (Stephan Reanimator F120, Germany) with a humidified warmed mixture consisting of 8 % oxygen and 92 % nitrogen. Once the procedure of "hypoxia" was completed, the rats were returned to the cages to their mothers.

**NA:** 24 hours after being born the rats were placed into a 3-litre warmed-bottom chamber fed with warmed humidified nitrogen at a flow rate of 6 l/min for 25 minutes (n = 14). They were then taken away and placed onto a wormed platform until regular breathing appeared and normal skin colour restored.

### Cell preparations

**CP of rENT:** On day 7-8 of gestation, female rats were deeply anaesthetized with ketamine (200 mg/kg). In sterile conditions, the abdominal cavity was opened, uterine horns removed, embedded in Hanks sterile solution, and embryos extracted under an operation microscope (Olimpus SZ 40). We then isolated the anterior cerebral bladders freed from membranes, with tissue cut into small pieces and placed into a 0.02 % trypsin-EDTA (Gibco) for 2-3 min. followed by adding DMEM cultural medium, pipetted 3 times with Pasteur's pipette with a burnt tip, then drawing off the supernatant. Cell suspension was centrifuged at 1,000 r.p.m. for 4-6 min; cell sediment was resuspended in nutrient medium DMEM supplemented with 10 % foetal calf serum (Gibco). The cells were then cultured for 72 hours.

**hoNSC CP:** Olfactory epithelium tissue was obtained in the clinic "Neurovita" from patients with spinal injury by permission of the Ethics Committee of the Russian State Medical University of the Russian Federation Health Ministry. Under local lidocaine anaesthesia, the fragments of olfactory epithelium sized 10 × 5 mm were dissected from the superior portion of the upper nasal passage. The derived tissue was delivered to the laboratory in Hanks solution with no $Ca^{2+}$ and $Mg^{2+}$ (HBSS) containing a mixture of antibiotics and antimycotics (Gibco, 1:100). The term of delivery did not exceed 2 hours.

After repeat washing in the same solution, the sample was freed from vessels, tissue was comminuted and incubated for 40 min at 36.5°C in 0.25 % trypsin-EDTA solution (Gibco) prepared on 0.01 M PBS with a pH of 7.4. The effect of enzymes was blocked with DMEM (Gibco) containing 3 % foetal calf serum (Gibco), tissue was rinsed 3 times with Hanks solution, and dissociated by repeat pipetting in the nutrient medium. The content of the nutrient medium containing DMEM/F12 (Gibco), 10 % foetal calf serum (Gibco), glutamine 2 mM (Gibco), 0.8 % glucose, a mixture of insulin, transferrin and sodium selenite 1:100 (Gibco), 20 mM

HEPES, growth factors FGF2, 1 ng/ml (Sigma), NGF2, 2 ng/ml (Sigma) (for primary cultures only).

The obtained cell suspension was centrifuged (for 7 min at 1,200 r.p.m.) and the sediment was resuspended in the same-content nutrient medium. Cell suspensions containing only 90-95% of viable cells were used for further cultivation. Dissociated cells were cultured in 12-well plates on a polylysine-laminated substrate for 14 days. Partial replacement of the nutrient medium was performed 2 times a week. The primary culture after formation of the disposable monolayer was collected by means of a solution of trypsin and EDTA. Following washing in HBSS and centrifuging, the cells were resuspended in the nutrient medium. The cellular suspension was transferred into 25-ml flasks. The free-floating substrate-fixed neurospheres being formed in the cellular monolayer were collected by means of Pasteur's pipettes and dissociated by the described method of enzymatic treatment. The neurospheres' cellular suspension was cultured separately from the substrate-fixed parietal glia cells, fibroblasts and stromal (supporting) cells. The cultures were thus passaged 4 times.

**rF CP:** In sterile conditions, we collected small portions of skin from the neonatal rats, comminuted and placed in a 0.02 % trypsin-EDTA solution for 2-3 min, followed by supplementing the culture medium with DMEM adding antibiotics and antimycotics (Gibco) and placed into 12-well plates. The cells were cultured at 37 °C in a $CO_2$ incubator. Once in 3 days we removed the supernatant with not-fixed cells, replenishing with fresh culture medium. The cells were cultured for 12 days.

**Immunocytochemical analysis and characteristics of cell preparations:** The cell preparations were fixed with 4 % paraformaldehyde in PBS for 30 min at 4°C. After washing in PBS, the cultures were incubated for 24 hours at 4 °C with primary antibodies to β-III-tubulin 1:300 (Chemicon), O4 1:100 (Chemicon), nestin 1:100 (Chemicon), GFAP 1:500 (in-house production). The cultures were then washed with PBS and consequently treated with secondary anti-mouse, anti-human antibodies conjugated with Alexa in a 1:200 dilution (for 2 hours at ambient temperature).

The rat fibroblasts preparation was assessed by the ability to absorb collagen fluorescent nanoparticles (*Invitrogen)* from the solution. The obtained preparations were examined and photographed in the fluorescent microscope Leica DL MB.

The cell preparations prior to implantation contained not less than 95 % of viable cells. According to the findings of immunocytochemical analysis, the preparations of embryonic rat tissue contained 90 % of nestin-positive cells, 50% of beta-III-tubulin-positive cells and 2-3 % of GFAP-positive and O4-positive cells. The preparation of human olfactory neural stem cells contained up to 90% of nestin-positive cells, 30 % of beta-III-tubulin-positive cells, 2-4 % of cells were GFAP-positive, and up to 2 % cells were O4-positive. The preparation of rat fibroblasts was characterized by active absorption of collagen *fluorescent* nanoparticles (Figure 8).

**Preparation of cells to implantation:** On completion of cultivation, the cells were supplemented with a 0.02 % trypsin-EDTA solution for 2-3 min. The obtained cellular suspension was centrifuged, the cellular sediment resuspended in Hanks solution. After calculating the cell count and assessing cell viability by exclusion of *trypan* blue, suspension of each cellular preparation was titrated to 300,000 viable cells in 3μl of the medium and delivered within 5 minutes to the operating room for implantation.

## Implantation of CP

The above-mentioned cell preparations were implanted 3 days after inflicting lesions (modelling of PHIBD and NA).

**Subcutaneous implantation of the cell**: Preparations was performed in a one-stage manner into the interscapular region by means of a microsyringe (Hamilton) provided with a G29 needle.

**Implantation into the brain:** Under inhalation halothane anaesthesia, the rats' heads were fixed in a stereotaxic manipulator (Narishige, Japan). The cellular preparations were administered injected by means of a micropump (Stoelting, USA) at a rate of 1μl/min at the following Bregma coordinates: AP – 2.5 mm, L – 1.8 mm to the right, DV – 1.5 mm from the surface of the dura mater (CA1 zone of the hippocampus). Five minutes after completion of implantation of the preparations, the needle (G29) was withdrawn at a rate of 0.5 mm per minute. The skin incision was then sutured (vicryl, No 8-0). The total duration of the manipulation did not exceed 15 minutes, with no antibiotics used.

## Monitoring of functions

In the rat pups after PHIBD we evaluated the state of the integral motor functions characterizing performance ability, durability endurance (hanging tests from the age of 3 weeks, "Rota rod" from the age of 4 weeks) and the cognitive function (passive avoidance test from the age of 4 weeks). After modelling NA, we evaluated only the cognitive function (passive avoidance test from the age of 4 weeks, training in a Y-shaped maze from the age of 5 weeks). Tests were performed in the morning hours with not more than one test a day for each animal (Supplemental Table).

**"Rotarod":** The rats were placed into the automated apparatus Rota rod (Neurobotics, Russia) onto the drum 70 mm in diameter rotating at a rate of 7 r.p.m., situated at a height of 80 cm from a container with sawdust. After falling the rats were again put on the rotating drum until they remained on it for not less than 1 minute. The animals were then returned into their home cages and after 20 minutes were placed on the drum rotating at a speed of 21 r.p.m., registering the time duration (s) of the animals' remaining on the drum.

**"Hanging" (holding onto a horizontal string):** The animals were brought with their forelimb towards to a 3-mm thick string strained at a height of 80 mm from the container with sawdust. The rats clung to the string and hanged on it until they dropped. The time (s) from clinging to the string till detachment there from was registered. If the time of retention exceeded 300 s the test was completed.

**Passive avoidance:** The rats from 4 weeks age were daily placed into a 300 400 × 400-mm plastic chamber onto a 40 × 120 mm platform elevated from one side by 40 mm above the floor

equipped with an electric mesh (Neurobotics, Russia). The animals after some time descended from the platform onto the electric mesh and received an aversive reinforcing stimulus (electric shocks 0.5 imp $min^{-1}$, 2 mA). They then avoided pain irritation by jumping onto the platform to be immediately returned to the home cages. The thus trained rats were weekly tested being placed onto the chamber's platform, measuring latent time (s) of avoidance, i. e., time from being placed till the rat's descent onto the floor. If the rat remained on the platform for 300 seconds, the experimentalist returned it to the home cage. We assessed the ratio of such rats in the study groups. When this time was less than 300 seconds the animal was subjected to learning as described above.

**Y-shaped maze:** Rats are known to possess a hereditary instinct to avoid illuminated spaces. In the Y-shaped maze with each of three sleeves sized 150 × 250 × 300 mm and a floor-fixed electric mesh, the rats during each weekly session with the help of electric shocks (0.5 Hz, 2 mA) were taught not to move from the illuminated starting sleeve to the dark sleeve but to go to the third (finishing) sleeve which was illuminated but with no electric stimulation. We registered time (sec.) from the moment of being placed into the maze's starting sleeve till complete movement of the animal to the illuminated finishing sleeve.

## Calculation of functional deficit and efficacy of cell therapy

The following calculations at the end of the experiment (rats' age 8 weeks) was performed. Functional efficacy (E) was reckoned by the following formula: $E_f$ = Dc – Dt, where Dc is deficit of the function in the cell-control group, and Dt is deficit of the function in the group with implantation of cells, which were calculated according to the formula: Dt = 100% – (Xt/Xi × 100 %); Dc = 100 % – (Xc/Xi × 100 %), where Xt stands for the mean values of the group of intact animals (taken as 100%). If the relative values of the parameters of separate functions (Xt/Xi × 100%) exceeded those in intact animals (i.e., were more than 100 %), we considered that there is no deficit for the function concerned (Dt = 0 %).

## Morphological studies

Once the functional monitoring completed on day 8 of life, the PHIBD animals were deeply narcotized (ketamine 200 mg/kg), perfused through the ascending aorta with 4 % paraformaldehyde in PBS, followed by extracting the brain and preparing 40-µm-thick coronal sections at the level of the septum (AP -0.2 mm form the Bregma) on a freezing microtome. The slices were stained according to the Nissl technique. The obtained preparations were photographed on the microscope Leica DM LB. The ImageJ 1.43 software was used to calculate the area of the parenchyma, subtracting the area of the ventricles (in $mm^2$) from the area of the ipsilateral hemisphere.

## Studying viability of the implanted cell preparations

In a separate series of experiments, the cellular preparations were preliminarily stained with vital dye CFDA-SE (Invitrogen) according to the manufacturer's instructions, followed by intracerebral (PHIBD) or subcutaneous (PHIBD and NA) implantation as described above. At days 3, 7, 14 and 20 after intracerebral implantation according to the above-mentioned method we prepared serial slices of the brain. After subcutaneous implantation of cell preparations at the same terms, besides preparing slices of the brain we excised portions of the skin, placing them into a 4-% solution of paraformaldehyde in PBS for 24 hours at 4°C. Then the fixed skin fragments were by means of needles fixed vertically in 12-well plates and supplemented with saturated agarose solution warmed to 60°C followed by freezing the preparations at -20C for 1 hour. The obtained blocks were used to prepare 50-to-60-µm-thick serial slices to be then monitored on glass slides and closed with covering glasses. The slices were studied using a fluorescent microscope (Leica DM LB).

## Statistical analysis

The average values of the indices were assessed by means of the Student's "*t*"-test with Fisher's adjustment for multiple comparisons. If the distribution of the samples differed from the normal one, we used the nonparametric method Mann-Witney method. To compare the relative indices we used the $\chi^2$ method and Fisher's exact test. Differences were regarded as statistically significant with at $P < 0.05$.

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