Supplemental Information for the manuscript titled:

"Dedifferentiated fat cells, as a novel source for cell therapy to target neonatal hypoxic ischemic encephalopathy"

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Hypoxic ischemic insult

Pups were anesthetized by isoflurane inhalation (5% for induction and 2.5% for maintenance of anesthesia). Next, the left common carotid artery was double-ligated and incised between the ligatures. In sham-operated animals, the carotid sheath was opened and the artery was separated from the vagus nerve, but not ligated. The duration of anesthesia and surgery did not exceed 6 min for each pup.

Magnetic resonance imaging (MRI)

Approximately 18–20 h after reperfusion, MRI was executed as a criterion of injury. MR experiments were performed with an MRS 3000/1500 Benchtop System (MR Solutions, Guildford, UK), which was equipped with a 3 T/17 cm cryogen-free superconducting magnet. For the MRI acquisition, the animals were anesthetized using isoflurane/O_2 (5% for induction and 1.5% for maintenance), placed prone on a plastic support, and inserted into a 20-mm diameter quadrature birdcage imaging coil. The pulse and respiratory rate were monitored throughout the examination. Four coronal diffusion-weighted images were obtained with a multislice spin echo sequence using the following settings: repetition time (TR) = 1500 ms, echo time (TE) = 43 ms, field of view (FOV) = 20 mm^2, slice thickness = 1 mm, slice separation gap = 2 mm, and average acquisition time = 10 min. Image analysis was performed using

commercially available software (Easyscan, MR Solutions, Guildford, UK). The injury in our model of neonatal HI has been shown to mainly affect the parietal cortex, hippocampus, thalamus, and striatum.[1] The severity of damage was roughly graded according to the size of the hyperintense region in both the cortex and basal ganglia ipsilateral to the occlusion, and the pups were divided into equivalent control and treatment groups (Fig. 1). Severity of damage on MRI was scored into 1 of 4 grades for each rat: none (no apparent injury), mild (unilateral hyperintensity restricted in parietal cortex), moderate (unilateral hyperintensity occupying the cortex and hippocampus) or severe (unilateral hyperintensity occupying the cortex, hippocampus and extending to the striatum).We observed from preliminary experiments that even pups without evident hyperintensity on P8 would occasionally show evidence of minimal scarring in the regions affected by the HI damage, therefore all animals that underwent MRI were used in the study. Sham operated animals did not undergo MRI imaging.

Immunohistochemistry

Sections were placed in phosphate-buffered saline (PBS) containing 4% donkey serum (Jackson ImmunoResearch Laboratories, Baltimore Pike, PA, USA) and 0.1% Triton X-100 for 1 h at room temperature, before incubating with the primary antibodies overnight at 4°C. Following incubation with the appropriate biotinylated secondary antibodies for 1 h at room temperature, binding was visualized using a Vectastain ABC kit (Vector Laboratories, Burlingame, CA, USA) and by peroxidase detection for 10 min (0.12 mg/ml 3,3′-diaminobenzidine, 0.01% H_2O_2, and 0.04% $NiCl_2$).

For BrdU immunostaining we used the same protocol mentioned above. Sections were deparaffinized and rehydrated, and antigen retrieval was performed by heating for 10 min in 10-mM citrate buffer (pH 5.8). Additional DNA denaturing (2 N HCl, 37°C, 30 min) and rinsing (0.1-M sodium borate, pH 8.5, 10 min) steps [2] were performed before blocking (4% donkey

serum, 0.1% Triton X-100), and finally the sections were incubated overnight with the primary antibody (rat anti-BrdU, 1:1000; Serotec, Oxford, UK) at 4°C. The following day, the sections were rinsed and incubated with biotinylated donkey anti-rat antibody (donkey anti-rat IgG, 1:2000; Jackson Immunoresearch Laboratories, West Grove, PA, USA). The signal was developed as described above using the avidin-biotin complex (Vectastain, ABC) and peroxidase detection method.

Behavioral tests

Rotarod treadmill

Animals were placed on a rotating rod, which accelerated from 4 to 40 rpm over a 5-min period. On the first day, the animals were subjected to a training session and then tested on the following day.

Cylinder test

Each animal was placed in a transparent cylinder and videotaped from above during lateral exploration of the cylinder walls. The forepaw placement of each weight-bearing contact with the wall during a full rear was recorded as right (impaired), left (non-impaired), or both. The animals were tested two times for a minimum of 5 min, or until a total of 10 full rears was performed.

NOR test

The apparatus comprised a square arena where the walls and floor (1 × 1 m) were black. Rats were first submitted to a habituation session, where they were allowed to explore the object-free apparatus for 5 min on three consecutive days. The experimental session comprised two trials: a

sample phase and a “choice” phase interrupted by a defined interval of 24 h. During the sample phase, each animal was placed in the apparatus and exposed to two identical stimuli (two wooden cubes) for 5 min. In the “choice” phase, one of the objects was replaced by a completely different object (in shape, color and material) and exploration was performed for 5 min.

Primary cortical neuron culture

A pregnant female was sacrificed on gestation day 16 and the embryos were obtained. After removing the meninges, the cortices of fetuses were dissected and rinsed three times with dissociation medium, which comprised serum-free DMEM (Sigma-Aldrich, St Louis, MO, USA). Following enzymatic dissociation with 0.05% (w/v) trypsin/0.53-mM EDTA solution (Wako, Tokyo, Japan) for 10 min and mechanical dissociation with a pipette, the cells were centrifuged at 2000 rpm for 3 min and suspended in neurobasal medium (NBM) (Thermo Fischer Scientific Inc.) supplemented with 2% B27, L-glutamine (0.5 mM), glutamate (25 μM), 2-mercaptoethanol (25 μM), 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin b, as the starter medium. Neurons were plated onto poly-L-lysine-coated wells at a density of 10^5cells/well. Neuronal cultures were fed twice a week by removing half the medium and replacing it with fresh culture medium (starter medium without glutamate and 2-mercaptoethanol), where they were maintained in a humidified 5% CO_2 incubator at 37°C until exposure to the experimental conditions.

Oxygen-glucose deprivation

After careful rinsing with 300 μl PBS, OGD was induced by the addition of an aglycemic solution (Glucose-free Neurobasal A Medium, Thermo Fischer Scientific Inc.) to the cultures in an hypoxic atmosphere (1% O_2, 5% CO_2, and 94% N_2) at 37°C for 3 h. Negative control cultures were treated

in an identical manner but they were not exposed to OGD conditions. Instead, they were rinsed only with 300 µl of PBS before receiving regular glucose containing cell culture medium (NBM + B27 + L-glutamine) in a normoxic atmosphere. Termination was achieved by replacing the glucose-free medium with regular cell culture medium.

Enzyme-linked immunosorbent assay (ELISA

Brain tissues from rat pups that underwent the Rice-Vannucci procedure, were injected DFAT cells or vehicle and were decapitated on P9, were divided in ipsilateral/contralateral parietal cortex (including hippocampus) parts and were snap frozen in liquid nitrogen prior to storage at -80°C. The tissue samples were suspended in ice cold homogenization buffer consisting of 100mM Tris/HCl, pH 7.0, 2% bovine serum albumin, 1M NaCl, 4mM EDTA, 2% Triton-X, 0.1% sodium azide and a protease inhibitor cocktail (cOmplete^{TM} Protease Inhibitor Cocktail, Sigma-Aldrich, St Louis, MO, USA). The tissues were homogenized using a Multi-beads shocker (Yasui Kikai, Osaka, Japan).

References

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