SUPPLEMENTAL MATERIAL

Animal Surgery

Timed-pregnant rats were purchased from Harlan Laboratories (Indianapolis, IN) and experimental germinal matrix hemorrhage (GMH) was induced in P7 rat pups as previously described.[1] Briefly, rat pups were anesthetized with isoflurane (3 % in a 30/70 oxygen/medical air mixture) and placed prone with their head secured onto a rodent stereotaxic frame. After sterilizing the rodent's scalp, a small midline incision was made to expose bregma. Next, a cranial burr hole was made 1.8 mm rostral and 1.5 mm right lateral from bregma, using a standard dental drill (1 mm). A 26-gauge needle was inserted through the cranial burr hole and stereotactically lowered 2.8 mm into the brain parenchyma. Following that, bacterial collagenase VII-S (0.3 U, Sigma; St. Louis, MO) was infused into the right hemispheric ganglionic eminence, at a rate of 0.25 µl/minute. Back-leakage of collagenase was prevented by keeping the needle in place for 10 minutes after completed infusion. Following that, the needle was withdrawn at a rate of 1 mm/minute, the burr hole was sealed with bone wax, and the scalp sutured. Sham animals were subjected to needle insertion only. Rat pups were returned to their dams after full recovery from the anesthesia.

Behavioral Testing

Neurocognitive deficits and motor coordination were evaluated by Morris water maze, rotarod, and foot fault tests between 21-28 days post-GMH (n=10/group). The mentioned neurofunctional tests have been previously utilized for the evaluation of long-term deficits in rodents subjected to unilateral hemorrhagic brain injury.[1-3] All tests were conducted in a blinded fashion. Learning and memory abilities in rats were assessed via the Morris water maze by measuring (1) the swim

distance for each animal before detecting a slightly submerged platform in a pool of water (diameter: 110 cm) and (2) the time each animal spent searching the target quadrant, after the platform has been removed from the pool (probe trial). An overhead infrared camera linked to a computerized tracking system (Noldus Ethovision, Tacoma, WA) recorded the swim path and time of each animal. The water maze experiments were conducted on day 21 to 25 post-GMH induction, one block per day followed by the probe trial. Motor and coordination function were evaluated on post-operative days 26 to 28. The rotarod apparatus (Columbus Instruments, Columbus, OH), consisting of horizontally rotating cylinders (7 cm in diameter, 9.5 cm in width) rotate either at constant velocity or accelerate 2 RPM every 5 seconds starting at a speed of 5 or 10 RPM. Continuous walking was required to avoid falling; the latency to fall was recorded for each animal by a photobeam circuit. Foot-fault testing was conducted by placing each animal on a horizontally elevated wire-grid (20 cm x 100 cm) and missteps through the grid were recorded over 2 minutes.

Histopathological analysis

Ventricular volume, cortical thickness, white matter loss, and basal ganglia loss were calculated in Nissl stained histological brain sections 28 days post-GMH using NIH Image J software (n=5/group). Brain tissue preparation, staining, and volumetric evaluations were conducted as previously described.[1, 3] Briefly, animals under deep isoflurane anesthesia were euthanized by transcardiac perfusion with PBS and 4% paraformaldehyde. Following that, brains were collected, formalin fixed (in 4% paraformaldehyde for 3 days), dehydrated (in 30 % sucrose for 3 days), and frozen coronal brain slices (10 µl) were obtained, using a cryostat (CM3050S; Leica Microsystems). Nissl stained histological brain sections were evaluated via computer assisted (Image J) hand delineation of cerebral and cerebroventricular structures, based on criteria from

stereologic studies using optical dissector principles.[4] The ventricular volume (mm^3) was calculated as average ventricular area multiplied by the depth of the cerebroventricular system. The cortical thickness was calculated and expressed as ratio of the contralateral brain cortex. Basal ganglia volumes and white matter loss were expressed as % of the sham group. Basal ganglia and white matter volumes were calculated and expressed as % of the sham group by dividing their volumes to the overall average sham volume.

Western blotting

Fibronectin and vitronectin expressions were quantified by Western blot at 28 days post-GMH (n=5/group). Briefly, whole brain samples were processed according to previously published protocols,[5] and equal amounts of protein (50 μg) were separated by SDS-PAGE before being transferred onto nitrocellulose membranes. The latter were then blocked and incubated with the following primary antibodies: anti-fibronectin, anti-vitronectin (1:1000; Abcam) and anti-β-actin (1:4000; Santa Cruz Biotechnology, Santa Cruz, CA). After incubation with the primary and the appropriate secondary antibodies (1:4000; Santa Cruz Biotechnology, Santa Cruz, CA), immunoblots were visualized with the ECL Plus chemiluminescence reagent kit (Amersham Bioscience, Arlington Heights, IL) and bands were quantified using Image J (NIH). Results, expressed as mean±SEM, were normalized to the average values of the sham group.

Supplemental References

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