SUPPLEMENTAL MATERIAL

Operative Procedure

Time pregnant Sprague Dawley rats were purchased from Harlan Laboratories (Indianapolis, IN); and P7 rat pups of both genders were subjected to germinal matrix hemorrhage (GMH) induction. General anesthesia was achieved with isoflurane (3% in 30/70% Oxygen/Medical Air). The anesthetized rat pup was placed prone with its head secured onto a stereotaxic frame. Next, the scalp was sterilized with betadine and a small midline incision was made to expose the skull sutures and bregma. The following coordinates were measured relative to bregma: 1.8 mm rostral and 1.5 mm lateral to the right. At these coordinates a small cranial burr hole was made, using a standard dental drill (1 mm), through which a 26-gauge needle was lowered 2.8 mm into the brain parenchyma. Next, clostridial collagenase VII-S (0.3 U, Sigma, St. Louis, MO) was infused through the needle at a rate of 0.25 μl/minute. The needle was left in place for 10 minutes after completed collagenase infusion, to prevent its back-leakage along the needle tract. The needle was then slowly withdrawn, the burr hole was sealed with bone wax, and the skin was sutured closed. All animals were allowed to fully recover on a 37°C warm heating blanket before being returned to their dams. Sham procedure consisted of needle insertion only.

Western Blotting

Whole brain samples were collected at 24 hours after surgery, and processed according to previously published protocols. Briefly, individual protein concentrations of each specimen were determined via DC protein assays (Bio-Rad, Hercules, CA). Equal amounts of protein (50 μg per sample) were separated by SDS-PAGE and transferred onto nitrocellulose membranes, which then were blocked and incubated with the following antibodies: anti-sphingosine-kinase-1 (1:250, Abgent, San Diego, CA), and anti-sphingosine-kinase-2 (1:250, Lifespan Biosciences, Seattle, WA). The following primary antibodies were obtained from Cell Signaling Technology (Denver, MA): anti-phosphorylated-Akt (Ser473, 1:1000), anti-Akt (1:1000), and anti-cleaved-caspase-3 (1:1000). Anti-β-actin and all appropriate secondary antibodies were purchased from Santa Cruz Biotechnology (1:500, Santa Cruz, CA). After incubation with the primary and secondary antibodies, immunoblots were visualized with the ECL Plus chemiluminescence reagent kit (Amersham Bioscience, Arlington Heights, IL). Images were semi-quantitatively analyzed, using Image J (NIH) and results were expressed as mean±SEM normalized to sham.

Long-term Behavioral Testing

Rodent motor and cognitive functions were evaluated between day 21 and 28 after surgery, as previously described. Foot-fault testing assessed the number of limb missteps through the openings of a horizontally elevated wire-grid (20 cm x 40 cm), onto which rats were placed for 2 minutes. The total number of foot faults was counted for each animal.
The rotarod test assessed motor and coordination function in animals subjected to GMH or sham surgery. The apparatus (Columbus Instruments, Columbus, OH) consists of a horizontally rotating cylinder (7 cm in diameter, 9.5 cm in width) that was programmed to maintain a constant velocity or to accelerate 2 rpm every 5 seconds, thus requiring continuous walking in order to avoid falling. The latency to fall was recorded by a photobeam circuit for each animal.

Cognitive function and memory ability was assessed in rodents via the Morris water maze, as previously described. This test required each animal to localize a slightly submerged platform in a round pool (diameter: 110 cm) filled with water. Following the cued trails (visible platform), the platform was submerged for 10 successive trials and then removed from the pool (probe trial). The time each animal spent in the target quadrant (containing the previous location of the platform) was recorded for 60 seconds by an overhead camera, and analyzed via a computerized tracking system (Noldus Ethovision, Tacoma, WA).

**Histopathological analysis**

At day 28 after surgery rats were terminally anesthetized with isoflurane (>5%) and transcardially perfused with ice-cold PBS and 10% paraformaldehyde. Next, brains were removed and post-fixed in paraformaldehyde (at 4°C for 3 days) and dehydrated in 30% sucrose for the same amount of time. Frozen coronal brain sections of 10 µm were cut every 600 µm on a cryostat (CM3050S, Leica Microsystems). All sections were stained with cresyl violet and morphometric analysis was conducted via computer-assisted (Image J, NIH) hand delineation of the cerebroventricular system. The borders of the cerebroventricular system were based on the criteria derived from stereologic studies using optical dissector principles. Ventricular volume was calculated as average ventricular area multiplied by the depth (µm) between first and last brain slice, showing cerebroventricular structures.
Supplemental References


