Supplementary Data

Supplementary Methods

Materials

Rabbit anti-nicotinamide adenine dinucleotide phosphate (NADPH) oxidase 1 (Nox1), goat anti-Nox1, rabbit anti-Nox2, rabbit anti-Nox4, rabbit anti-NOX organizer 1 (Noxo1), rabbit anti-NOX activator 1 (Noxa1), rabbit anti-interleukin-1beta (IL-1β), rabbit anti-IL-6, and rabbit anti-tumor necrosis factor alpha (TNF-α) and Nox1 antibody blocking peptide from Santa Cruz biotechnology; rat anti-CD11b from Serotec; and anti-goat immunoglobulin G (IgG), anti-rat IgG, and anti-rabbit IgG antibodies from Jackson ImmunoResearch were used. We obtained an enhanced chemiluminescence kit from Pierce, and Taq polymerase from Roche Applied Science. Vectastain ABC kit and biotinylated anti-rabbit, anti-mouse IgG, or anti-rat IgG were from Vector Laboratories. Trizol reagent, dihydroethidium (DHE), superscript II reverse transcriptase, 10%–20% sodium dodecyl sulfate (SDS) polyacrylamide gel, and 10%–20% tricine gel were purchased from Invitrogen and a Rac1 activation kit was obtained from Cell Biolab. Apocynin, protease inhibitor cocktail (AEBSF, aprotinin, bestatin hydrochloride, E-64-[N-(trans-Epoxysuccinyl)-L-leucine 4-guanidinobutylamide], leupeptin, pepstatin A) were purchased from Sigma-Aldrich. All other chemicals of reagent grade were from Sigma Chemicals or Merck.

Animals and surgery

All procedures were approved by the Animal Experiment Review Board of the Laboratory Animal Research Center of Konkuk University. Males are more susceptible to the cerebrovascular complications of hypertension, such as a stroke and vascular dementia (31). Therefore, chronic cerebral hypoperfusion was induced in male Wistar rats (weighing 200–250 g; 10 weeks age; Samtako BioKorea. Co. Ltd.).

The rats were anesthetized with 70% nitrogen and 30% isoflurane. Through a midline cervical incision, both common carotid arteries were exposed and double ligated with silk sutures (two-vessel occlusion [2VO]). The sham-operated animals (SC) were treated in a similar manner to the operated rats except for the common carotid arteries occlusion. The rectal temperature was monitored and maintained between 37°C ± 0.5°C using a heating pad (Homeothermic blanket system; Harvard Apparatus, Inc.) during the surgical procedure. After the operation, the rats were kept in an animal resource facility with food and water ad libitum. The rats were sacrificed after 1, 2, 4, and 10 weeks ($n = 6$–7/group). In the Nox inhibition study, the animals received an intraperitoneal (IP) injection of 10 mg/kg/day apocynin (Sigma) or vehicle immediately after the sham or 2VO operation, and received the same dosage every day for 1, 4, or 8 weeks ($n = 6$/group). In the Nox1 knockdown study, the animals were subjected to stereotaxic surgery in the hippocampal CA1 subfield for 4 weeks before 2VO or sham operation ($n = 10$/group). Detailed animal allocation and timeline for experiment were described (Supplementary Fig. S6).

To verify Nox1 antibody specificity, eight animals received an ipsilateral injection of 0.02% ascorbic acid, while all other rats received an ipsilateral injection of freshly prepared 2 μl of 6-hydroxydopamine (6-OHDA; Sigma) at the concentration of 7.5 μg/μl containing 0.02% ascorbic acid at two coordinates in the right striatum (coordinate: anteroposterior [AP], +0.7 mm; mediolateral [ML], +2.2 mm and +2.0 mm; dorsoventral [DV], −5.0 mm) (3). The injection rate was 0.5 μl/min, and the syringe was kept in place for an additional 5 min before being retracted slowly. Rats were sacrificed after 5 days. Substantia nigra tissue sections were used in immunochemical analysis.

Rac1 activation assay

One milligram of protein extracted from hippocampal tissue was incubated with 20 μl of agarose beads containing the p21-binding domain (PBD) of the p21-activated protein kinase 1 (PAK1), an effector of activated Rac, for 1 h at 4°C. The beads were collected by centrifugation and washed twice in the lysis buffer. The beads were resuspended in sample buffer and boiled for 5 min. Proteins were resolved by SDS-PAGE using a 10%–20% Tricine gel, transferred electrophoretically, and visualized using an anti-rat Rac1 antibody followed by enhanced chemiluminescence. For the positive control, the nonhydrolyzable GTP analog GTPgS was used according to the manufacturer's protocol (Cell Biolabs).

In situ *visualization of superoxide*

In situ visualization of superoxide and its derivative oxidant products was performed by hydroethidine histochemistry, as previously described (4). Briefly, sham- or 2VO-operated rats were injected IP with 2 ml of phosphate-buffered saline (PBS) containing 1 μg/μl hydroethidine and 1% dimethysulfoxide. Animals were sacrificed 60 min later, and the brain was removed and frozen on dry ice. Both prefrontal cortex and hippocampal sections (40 μm thick) that had been mounted onto gelatin-coated glass slides were examined for the hydroethidine oxidation product, ethidium, by fluorescence microscopy. A quantitative analysis was performed.

Nissl staining

Nissl staining was performed by incubating the samples in a 0.1% Cresyl violet solution for 5–10 min at room temperature, rinsing quickly in distilled water, dehydration in serially diluted ethanol, and cleaning in xylene. The samples were then mounted sequentially in glass slides using a permanent mounting medium. Mounted slices were evaluated under a light microscope. A quantitative analysis was performed.

Immunohistochemistry of brain tissues

After perfusion with saline and 4% paraformaldehyde in PBS, brains were removed, and the forebrain and midbrain blocks were immersion fixed in 4% paraformaldehyde and cryoprotected in sucrose. Serial coronal sections (40 μm) were cut on a cryostat, collected in a cryopreservative, and stored at −20°C. For immunolabeling studies, the sections

were incubated with a blocking solution (5% horse serum and 0.3% Triton X-100 in PBS, pH 7.5) and then with primary antibodies at 4°C overnight. Finally, the sections were incubated with secondary antibodies in a blocking solution at room temperature for 1 h. The primary antibodies used were rabbit anti-NeuN antibody (1:2000), rabbit anti-Nox1 (1:500), goat anti-Nox1 (1:500), rabbit anti-Nox2 (1:500), rabbit anti-Nox4 (1:500), anti-Noxo1 (1:500), anti-Noxa1 (1:500), rat anti-CD11b (1:500), mouse anti- glial fibrillary acidic protein (GFAP, 1:1000), and mouse anti-8-hydroxy-2′-deoxyguanosine (8-oxo-dG, 1:500). The secondary antibodies were Alexa Fluor 488-conjugated anti-mouse IgG or Alexa Fluor 564-conjugated anti-rabbit IgG (1:200), stained with Topro3 (1:1000 in PBS), and imaged by confocal microscopy (Olympus). A quantitative analysis was performed.

For antibody blocking, Nox1 antibody was preabsorbed with a five-fold high concentration of blocking peptide (Santa Cruz Biotechnology) in 500 μl of PBS at room temperature for 1 h.

Immunohistochemistry of common carotid artery

The vasculature was then flushed with PBS and perfusion fixed by a ventricular injection of 4% paraformaldehyde before the ligated or sham-operated carotid artery was harvested. Five-μm-thick sections were taken from a 100 μm distant area from the ligation site of each artery. Typically, this yielded six sections per artery. For immunolabeling studies, sections were incubated with a blocking solution (5% horse serum and 0.3% Triton X-100 in PBS, pH 7.5) and then with primary antibodies at 4°C overnight. The primary antibodies used were rabbit anti-IL-1β (1:500), rabbit anti-IL-6 (1:500), and rabbit anti-TNF-α (1:500). Sections were incubated with secondary antibodies (Alexa Fluor 488-conjugated anti-rabbit IgG) in a blocking solution at room temperature for 1 h, and then stained with Topro3 (1:1000 in PBS). Images were captured by confocal microscopy (Carl Zeiss Co. Ltd.) and analyzed with ZEN 2009 Light edition software (Carl Zeiss Co. Ltd.). Mean densities of the IL-1β, IL-6, and TNF-α immunoreactivity were quantified by an observer who was blind to the grouping. One region of interest (ROI) of 0.2 mm^2 per one section (six sections per rat) was measured in each ROI and averaged.

Total RNA extraction and reverse transcription–polymerase chain reaction analysis

Total RNA was extracted from the hippocampal tissue using the Trizol reagent. RT was performed for 40 min at 42°C with 1 μg of total RNA using 1 U/μl of superscript II reverse transcriptase. Oligo (dT) and random primers were used as primers. The samples were then heated at 94°C for 5 min to terminate the reaction. The cDNA obtained from 1 μg of total RNA was used as a template for polymerase chain reaction (PCR) amplification. Oligonucleotide primers were designed based on Genebank entries for rat Nox1 (sense, 5′-TGACAGTGATGTATGCAG CAT-3′; antisense, 5′-CAGCTTGTTGTGTGCACGCTG-3′), rat Nox2 (sense, 5′-ACTCGAAAACTTCTTGGGTCAG-3′; antisense, 5′-TCCTGTGATGCCAGCCAACCGAG-3′), rat Nox4 (sense, 5′-GCCGGCGGTATGGCGCTGTC-3′; antisense, 5′-CCACCATGCAGACACCTGTCAGG-3′), rat Noxa1 (sense, 5′-TCTAGGGGATCAGATACGGGAC-3′; antisense, 5′-CCAAGGAAATCCATGGGCTCCAG-3′), rat Noxo1 (sense, 5′-ACCCAGTATCAGCCCATGCTG -3′; antisense, 5′-ATGGAGCATCAGGAAGCTTGG -3′), and rat glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (sense, 5′-ATCAC CATCTTCCAGGAGCG-3′; antisense, 5′-GATGGCATGGA CTGTGGTCA-3′). PCR mixes contained 10 μl of 2 × PCR buffer, 1.25 m*M* of each dNTP, 10 pmol each of forward and reverse primers, and 2.5 U of Taq polymerase to a final volume of 20 μl. For Nox1, amplification was performed in 32 cycles for 40 s at 95°C, 30 s at 62°C, and 2 min at 72°C, and for GAPDH, this process was performed over 27 cycles for 40 s at 95°C, 30 s at 58°C, and 2 min at 72°C. After the last cycle, all samples were incubated for an additional 7 min at 72°C. PCR fragments were analyzed on 1% agarose gel containing ethidium bromide, and their amounts were normalized against amplified GAPDH. Each primer set specifically recognized only the gene of interest as indicated by the amplification of a single band of the expected size.

Real-time quantitative reverse transcription–PCR

Real-time quantitative reverse transcription–polymerase chain reaction (qRT-PCR) was performed to quantify the expression of IL-1β, IL-6, and TNF-α in the common carotid artery using the AB7500 Real-Time PCR system (Applied Biosystems). Total RNA was isolated from flash-frozen common carotid artery using the RNeasy Mini kit (Qiagen). RT was performed for 40 min at 42°C with 1 μg of total RNA using 1 U/μl of superscript II reverse transcriptase. Oligo (dT) and random primers were used as primers. The samples were then heated at 94°C for 5 min to terminate the reaction. The cDNA obtained from 100 ng of total RNA was used as a template for PCR amplification. SYBR premix EX Taq II (Takara Bio, Inc.) with the manufacturer's recommended 96-well plates (CoreSciences) and films (Thermo Fisher Scientific, Inc.) were used to conduct all gene expression studies. Oligonucleotide primers were designed based on Genebank entries for rat IL-1β (sense, 5′-AAAATGCCTCGTGCTGTCTG-3′; antisense, 5′-CTATG TCCCGACCATTGCTG-3′), rat IL-6 (sense, 5′-ACAAGTCC GGAGAGGAGACT-3′; antisense, 5′-GGATGGTCTTGGTC CTTAGC-3′), rat TNF-α (sense, 5′-TAGCCCACGTCGTAG CAAAC-3′; antisense, 5′-GGAGGCTGACTTTCTCCTGG-3′), and rat GAPDH (sense, 5′-ATCACCATCTTCCAGGA GCG-3′; antisense, 5′-GATGGCATGGACTGTGGTCA-3′).

Each qRT-PCR mixture (final volume 20 μl) contained 10 μl of SYBR premix EX Taq II (Takara Bio, Inc.), 0.8 μl of forward and reverse primers (100 n*M* each), reactive oxygen species reference Dye II (Takara Bio, Inc.), and 2 μl of cDNA (100 ng). For IL-1β, IL-6, TNF-α, and GAPDH, amplification was performed in 38 cycles for 30 s at 95°C, 5 s at 95°C, 34 s at 60°C, 15 s at 95°C, 1 min at 60°C, 30 s at 95°C, and 15 s at 60°C GAPDH was used as the internal control in the experiment. To compare the relative abundance of target gene transcripts, the average threshold cycle (Ct) was normalized to that of GAPDH for the samples as follows: $2^{-\Delta Ct}$, where $-\Delta Ct = (C_{t,\ target\ gene} - C_{t,\ GAPDH})$. The fold change for a target gene, as compared with sham control, was calculated as $2^{-\Delta\Delta Ct}$, where $-\Delta\Delta Ct = (C_{t,\ target\ gene} - C_{t,\ GAPDH})_{test\ condition} - (C_{t,\ target\ gene} - C_{t,\ GAPDH})_{sham\ control}$. qRT-PCR was performed twice using three independent tissue pools.

Adeno-associated virus 2-mediated Nox1 knockdown

AAV particles containing either Nox1 shRNA/AAV or scrambled shRNA/AAV (scb shRNA/AAV) (3) were

stereotaxically injected into the hippocampal CA1 subfield for 4 weeks before 2VO or sham operation ($n = 20$/group). Rats were deeply anesthetized (ketamine and xylazine mixture 30 mg/kg, IP) and placed in a rat stereotaxic apparatus. Nox1 shRNA/AAV and scb shRNA/AAV was then injected into both sites in the hippocampal CA1 subfield (coordinate: AP, -3.3 mm; ML, ± 2.0 mm; DV, -3.5 mm). A total of 1×10^{11} genome copy/ml recombinant AAV particles encoding Nox1 shRNA, or scb shRNA diluted in 2 μl ice-cold sterilized PBS were used in every animal. The injection rate was 0.5 μl/min, and the syringe was kept in place for an additional 5 min before being retracted slowly. Rats were subjected to sham or 2VO surgery after 4 weeks ($n = 20$/group, Supplementary Fig. S6). The rats were allocated into four groups: sham-operated rats injected with scramble shRNA AAV particles (ScbRNA control, $n = 10$); sham-operated rats injected with Nox1 shRNA AAV particles (Nox1shRNA control, $n = 10$); 2VO rats injected with scramble shRNA AAV particles (ScbRNA 2VO, $n = 10$); and 2VO rats injected with Nox1 shRNA AAV particles (Nox1 shRNA 2VO, $n = 10$). Ten rats per group were used for the behavioral test, and 4 rats per group were used for the histological study.

Behavior test

The Morris water maze (MWM) test was employed to evaluate the learning and memory of rats (2). The MWM procedure was based on a principle, where if the animals were placed in a large pool of water, they would attempt to escape from the water by finding an escape platform. The MWM consisted of a large circular pool (150 cm in diameter, 45 cm in height), filled to a depth of 30 cm with water that was maintained at 28°C ± 1°C. The water was made opaque using a nontoxic white-colored dye. The tank was divided into four equal quadrants using two threads that had been fixed at right angles to each other on the rim of the pool. A submerged platform (10 cm^2) painted in white was placed inside the target quadrants of this pool, 1 cm below the surface of the water. The position of the platform was not changed throughout the training session. Four consecutive sessions, each consisting of five trials for 2 days (alternating two or three trials per day), were conducted over eight consecutive days. The hidden platform was always placed in the southeast quadrant of the pool. The rat was gently placed in the water of the pool between quadrants, facing the wall of the pool, and the drop location was changed for each trial. The rats were then allowed 90 s to locate the submerged platform and were allowed to stay on the platform for another 30 s. If the rat failed to find the platform within 90 s, it was guided gently onto the platform and allowed to remain there for 30 s. The inter-trial interval was 1 min. Performance accuracy was evaluated based on the analysis of the search error and the time latency data of all trials. The search error measurements were based on a computation of the average distance from the platform during the trial. The distance between the rat and the platform was sampled 10 times/s during each trial, and these distances were averaged in 1-s bins. The cumulative search error is the sum of these 1-s averages of the proximity measurements, where the specific platform location and start location were corrected by subtracting the proximity score that would be produced by a perfect performance in the trial. A probe trial was conducted at 1 min after every 10th training trial. The entire training procedure included two probe trials for each rat, during which the rats swam with the platform retracted to the bottom of the pool for 30 s. After recording the swimming path, the platform was raised to its normal position for completion of the trial. The time spent swimming in the target quadrant of the retracted platform was used as a parameter for the retention of spatial memory. One week after the completion of the MWM task with a hidden platform, all rats were assessed for cued behavior using a visible platform that was raised above the surface of the water. The location of the visible platform varied from trial to trial in a single session of six training trials.

Novel object location and recognition behavior test

Thirteen weeks after sham operation or 2VO, a novel object location and recognition behavior test was performed ($n = 8$ per each group). The test was carried out by modification of the method described in a previous study (1, 2, 7).

The rats were placed in a $40 \times 40 \times 40$ cm (height) sized box and allowed to equilibrate to the testing area 10 min per day for 3 days. After 24 h, two objects were placed in the testing area and then, the rat was allowed to explore the two objects in the testing area for 5 min before being returned to the cage. After a 3 h-interval, one of the objects was either relocated or replaced with a new object and the rats were allowed to explore the testing area once again for 2 min. The intertrial interval between these two tests was 24 h. Exploring the novel object was defined if the center of the rat's head was oriented within 45° of the object and within 4 cm of it. Climbing over or sitting on an object was not included A video camera was positioned over the arena, and exploratory behaviors were videotaped for later analysis. Exploratory time spent for novel objects was recorded, and the discrimination ratio was computed as [(time spent exploring novel object or location − time spent exploring familiar object or location)/total time spent exploring both objects] in a blind manner.

Olfactory discrimination test

Thirty-two Wistar rats (260–350 g) were singly housed in standard plastic rodent cages and maintained on a 12-h light/dark cycle. All testing was conducted in the light proportion of the light/dark cycle. Each rat was initially food deprived to ~85%–90% of their free-feeding weight, but was allowed free access to water throughout testing. The test was carried out by modification of the method described in a previous study (5, 6). The test apparatus consisted of a box that was 48 cm long and 25.5 cm wide with four 30.0 cm high transparent acryl walls. One removable sliding door, also constructed of opaque black acrylic board, was placed at 24 cm from one end of the box. The door divided the box into two separate compartments: a 24×25.5 cm start chamber and a 24×25.5 cm choice chamber. The floor of the maze consisted of acryl that was painted white. The far wall of the choice chamber contained two ceramic food dishes, which were 8 cm in diameter and 4.5 cm in depth. These ceramic dishes were used to stabilize digging cups during testing. For shaping, each animal was trained on the forced-choice discrimination procedure as follows. At the beginning of each trial, a rat was placed in the start chamber, with the divider

between the two chambers closed. Two ceramic pots were then placed in the test chamber, used to present the odorant, and concealed the reward (chocolate rice cereal; Post, Seoul, Korea). The dishes were evenly filled with 50 cm^3 of bedding, after which 50 μl of diluted odorant was applied to the top of the bedding in the center of the pot. Another 50 cm^3 of bedding was then added to bury the odorant within the bedding. For shaping, butylacetate was used for an odorant that was chosen as the rewarded odorant; this remained consistent for any given rat but varied among different rats. One pot was scented with the rewarded odorant and contained a reward, whereas the other dish was non scented and contained no reward. When the divider was removed, the rat entered the test chamber and was permitted to dig into both dishes until it retrieved the reward. Shaping continued by placing a reward on top of the bedding and by allowing the animal to find the reward. Across subsequent shaping trial presentations, the food reward was buried, partially at first and then deeper in the bedding, until the rat dug in the bedding even when the reward was not visible. Once an animal consistently dug in the bedding, the rat was shaped to dig in a cup in the choice chamber of the test apparatus. During training, subjects received fifteen 5 min trials a day. The intertrial interval was 5 min. If a rat did not succeed in shaping during fifteen 5 min trails, that rat was excluded from the olfactory discrimination test. The next day, a discrimination task was conducted to assess the ability of sham-operated rats injected with scramble shRNA AAV particles ($n = 10$), 2VO-operated rats injected with scramble shRNA AAV particles ($n = 10$), and 2VO-operated rats injected with Nox1 shRNA AAV particles ($n = 10$) to perceptually discriminate between two simultaneously presented odors. Each rat was given 15 trials 1 day. For the preference task, two odors (L-limonene and R-limonene) similar to those used in the task were selected. One odor was designated as the rewarded odor, and the other was designated as the nonrewarded odor. On each trial, two digging cups were simultaneously presented to the rat. The cup containing bedding mixed with the rewarded odor contained a reward, but the cup with the nonrewarded odor contained no reward. When the divider was removed, the rat entered the test chamber and was permitted to dig into both dishes for 2 min and if they failed to find reward food, the reward was put on top of the bowl. The task consisted of 15 trials. The intertrial interval was 5 min. Every fifth trial was tested for the probe trial, which was to just present the reward odorant without a reward. The rat succeeded in finding the reward pot and dropped the reward food into the pot. The measured parameter was the latency to find the reward pot. Data were presented as the total time spent digging.

Motor function

Major neurological deficits that could physically limit swimming ability such as forelimb flexion or unilateral circling were evaluated before every trial. Further, swimming speed was monitored during every trial.

Supplementary References

1. Bell RD, Winkler EA, Sagare AP, Singh I, LaRue B, Deane R, and Zlokovic BV. Pericytes control key neurovascular functions and neuronal phenotype in the adult brain and during brain aging. *Neuron* 68: 409–427, 2010.
2. Choi BR, Lee SR, Han JS, Woo SK, Kim KM, Choi DH, Kwon KJ, Han SH, Shin CY, Lee J, Chung CS, Lee SR, and Kim HY. Synergistic memory impairment through the interaction of chronic cerebral hypoperfusion and amlyloid toxicity in a rat model. *Stroke* 42: 2595–2604, 2011.
3. Choi DH, Cristovao AC, Guhathakurta S, Lee J, Joh TH, Beal MF, and Kim YS. NADPH oxidase 1-mediated oxidative stress leads to dopamine neuron death in Parkinson's disease. *Antioxid Redox Signal* 16: 1033–1045, 2012.
4. Kim YS, Choi DH, Block ML, Lorenzl S, Yang L, Kim YJ, Sugama S, Cho BP, Hwang O, Browne SE, Kim SY, Hong JS, Beal MF, and Joh TH. A pivotal role of matrix metalloproteinase-3 activity in dopaminergic neuronal degeneration via microglial activation. *FASEB J* 21: 179–187, 2007.
5. Linster C, Johnson BA, Yue E, Morse A, Xu Z, Hingco EE, Choi Y, Choi M, Messiha A, and Leon M. Perceptual correlates of neural representations evoked by odorant enantiomers. *J Neurosci* 21: 9837–9843, 2001.
6. Martin C, Beshel J, and Kay LM. An olfacto-hippocampal network is dynamically involved in odor-discrimination learning. *J Neurophysiol* 98: 2196–2205, 2007.
7. Winters BD and Bussey TJ. Transient inactivation of perirhinal cortex disrupts encoding, retrieval, and consolidation of object recognition memory. *J Neurosci* 25: 52–61, 2005.