Recognition Memory Impairments After Subcortical White Matter Stroke in Mice

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Background and Purpose—Small subcortical white matter infarcts are a common stroke subtype often associated with cognitive deficits. The lack of relevant models confined to white matter has limited the investigation of its pathophysiology. Here, we examine tissue and functional outcome after an ischemic lesion within corpus callosum in wild-type (WT) mice and in mice null for a gene, NOTCH3, linked to white matter ischemic injury in patients.

Methods—WT and NOTCH3 knockout mice were subjected to stereotactic microinjections of the potent vasoconstrictor endothelin-1 at the level of periventricular white matter to induce a focal ischemic lesion. Infarct location was confirmed by MRI, and brains were examined for lesion size and histology; behavioral deficits were assessed ≤1 month in WT mice.

Results—Ischemic damage featured an early cerebral blood flow deficit, blood–brain barrier opening, and a lesion largely confined to white matter. At later stages, myelin and axonal degeneration and microglial/macrophage infiltration were found. WT mice displayed prolonged cognitive deficit when tested using a novel object recognition task. NOTCH3 mutants showed larger infarcts and greater cognitive deficit at 7 days post stroke.

Conclusions—Taken together, these data show the usefulness of microinjections of endothelin-1 into periventricular white matter to study focal infarcts and cognitive deficit in WT mice. In short-term studies, stroke outcome was worse in NOTCH3 null mice, consistent with the notion that the lack of the NOTCH3 receptor affects white matter stroke susceptibility. (Stroke. 2014;45:1468-1473.)

Key Words: mild cognitive impairment ◼ models, animal ◼ stroke, lacunar ◼ white matter diseases

The vast majority of experimental stroke research has focused on animal models of large territorial infarction involving both cortical and subcortical grey and white matter. However, 20% to 25% of strokes in humans are small, lacunar-like, involve subcortical white matter tracts, are often recurrent, and may lead to cognitive decline, subcortical dementia, and major disability. Moreover, white matter ischemic lesions are a main pathological manifestation of small vessel diseases, a subset of cerebrovascular alterations leading to stroke and cognitive decline. Only a few experimental models investigating small white matter strokes have been reported.

Recently, Carmichael and collaborators developed a model of selective white matter stroke by injecting the potent vasoconstrictor endothelin-1 into corpus callosum to create a small area of myelin and axonal degeneration. Although reproducibility has been a problem with this model, it generates a lesion confined to white matter and therefore offer advantages to other white matter injury models that in addition cause diffuse brain injury after global cerebral hypoperfusion. After adapting, improving, and extending this new experimental approach, we tested whether subcortical white matter ischemic injury is associated with cognitive impairment in wild-type (WT) mice. Furthermore, we tested whether a NOTCH3 gene deletion negatively affects tissue and functional outcome after selective white matter injury. NOTCH3 mutations are the most common cause of inherited strokes and vascular dementia in young and middle-aged adults. We previously showed that NOTCH3 knockout mice seem especially vulnerable to ischemic injury after middle cerebral artery occlusion, probably because of vascular dysfunction and reduced collateral blood flow. Here, we extended these findings to white matter susceptibility and provided a novel experimental approach to further dissect the relationship between genotype and ischemic phenotype in NOTCH3 mutants.

Materials and Methods

Experiments were conducted according to protocols approved by the Animal Research Committee of Massachusetts General Hospital and the National Institutes of Health Guide for the Care and Use of Laboratory Animals (see online-only Data Supplement).
Animals

NOTCH3 knockout male mice (N3KO; 23–28 g; n=8; genetically engineered as previously described) and sex-, age-, and weight-matched WT (n=34 for method development; n=89 for hypothesis testing) mice (C57BL/J, Charles River Laboratories; background strain) were housed 4 per cage and maintained on a 12/12-hour light/dark cycle and fed ad libitum. Only a limited number of mutants were available for this study. Mice were randomly allocated and coded with tail marks to perform the analysis in a blinded fashion. The number of mice needed for behavioral and infarct size assessments was based on power calculation assuming a group difference of 30% and an SD of 30% to 35%.

Endothelin-1 Stroke Model

Subcortical white matter stroke was produced using a recently published method, with slight modifications. Isoflurane anesthetized mice (4% for induction, 1%–1.5% for maintenance, in a 70:30 N2O:O2 mixture) were placed on a stereotactic frame (Stoeling), the head secured with blunted earbars and the body temperature controlled (37°C) using a feedback-regulated homeothermic blanket (FHC). Eye ointment was applied to prevent corneal dryness. After a midline scalp incision, a small (1×1 mm) burr hole was drilled under constant saline cooling, carefully keeping the dura intact, and 2×100-nL microinjections of vehicle (sterile saline, control group) or endothelin-1 (0.3 mg/mL, American Peptide, cat. 88-1-10, Lot. Z05068T1) were performed using a glass micropipette (tip=25 μm) connected to a pressure system (Picospritzer, General Valve). To target the periventricular white matter at the level of corpus callosum, we adopted the following stereotactic coordinates: antero-posterior +1.00, +0.60 mm; medio-lateral −0.20 mm; dorso-ventral −2.20 mm; and 36° angle. After each injection, the pipette was left in place for an additional 5 minutes to avoid backflow. At the end of the procedure, mice were placed in a 28°C incubator for 2 hours before returning to the home cage.

Assessment of Cerebral Blood Flow and Blood–Brain Barrier Permeability

Regional cerebral blood flow (n=5 control, 5 stroke) was analyzed using the [14C]-iodoantipyrine ([14C]-IAP) method as previously described. Blood–brain barrier (n=5 control, 5 stroke) permeability was assessed using the Evans blue technique.

Magnetic Resonance Imaging

Mice (n=5) were imaged 2 days after stroke. A change in magnetization transfer ratio was used to assess white matter damage because it highly correlates with myelin content.

Neurological Assessments

Mice were tested for sensorimotor and cognitive performances at 7 (n=18 control, 27 stroke WT, and 8 stroke N3KO) and 28 days (n=10 control, 9 stroke) after either vehicle or endothelin-1 injection. Cylindrical (forelimb exploration), grid-walk (footfaults), open field (locomotor activity), water maze and Y maze (spatial memory), and novel object recognition (NOR, recognition memory) tests were performed as described in the online-only Data Supplement.

Histology and Immunofluorescence

At designated time points, mice were cardiopressured with saline solution and the brains harvested and snap-frozen at −45°C isopentane. Brains were cryosectioned (20-μm-thick slices, 60-μm interval) and stained with luxol fast blue (myelin), hematoxylin and eosin and cresyl violet (neural and infiltrated cells, microbleeds), neurofilament 200 (NF200, axons), CD68 (activated microglia/macrophages), glial fibrillary acidic protein (reactive astrocytes), and Hoechst 33342 (nuclei). Images were acquired using a Nikon Super Coolscan 9000 ED Scanner and a Nikon TE-2000 microscope with epifluorescence illumination, and then analyzed using ImageJ software (NIH).

Infarct Size Measurement

Infarct size was assessed by measuring the volume of demyelinated tissue (luxol fast blue) and was measured using ImageJ. Each section was outlined and the area of injury calculated. The sum of the areas was integrated to obtain injury volume. The volume of corpus callosum/external capsule was also measured and compared among groups to assess possible volumetric differences caused by edema/swelling.

Statistics

Data are expressed as mean±SD unless otherwise stated. For comparisons between 2 groups, statistical significance was determined using an unpaired Student t test or nonparametric Mann–Whitney test using Prism 5 software (GraphPad Software, San Diego, CA). A P value of <0.05 was considered significant.

Results

Endothelin-1 Microinjections Reduce Blood Flow and Open the Blood–Brain Barrier Into White Matter

There was no mortality after stroke when assessed ≤1 month, and no differences in body temperature were detected between groups during and 2 hours after surgery.

The [14C]-IAP labeling, an index of cerebral blood flow, was significantly reduced at the level of the corpus callosum 4 hours after endothelin-1 microinjection (Figure 1A). Some reduction in flow extended superiorly to grey matter and along the needle tract. Perfusion deficits were not found in vehicle-injected animals (Figure 1B). Extravasation of Evans blue was visible by fluorescent microscopy at the same time point and seemed restricted to subcortical white matter (Figure 1A in the online-only Data Supplement).

Endothelin-Induced Ischemia Triggers Neuropathological Alterations in the White Matter

Two days after stroke, myelin degeneration was already visible by a change in magnetization transfer ratio (Figure 2) and by hematoxylin and eosin and luxol fast blue staining (Figure 1B in the online-only Data Supplement). Because lesion size was highly variable at this time point, we used 1 week after stroke.

![Figure 1. Cerebral blood flow reduction after endothelin-1 microinjection. Representative coronal autographic images ([14C]-iodoantipyrine) showing cerebral blood flow deficit in subcortical white matter after endothelin-1 injection (A, arrowhead), but not after vehicle injection (B).](http://stroke.ahajournals.org/)

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as the main time point for histological assessment. Seven days after endothelin microinjection, focal myelin loss and cellular infiltration were observed at the lesion site by luxol fast blue–cresyl violet and hematoxylin and eosin (Figure 3A and 3D). The majority of animals (>90%) showed a lesion restricted to white matter, sparing both cortical and subcortical grey matter regions. The demyelinated region also showed axonal degeneration as evidenced by the loss of NF200 staining (Figure 3B), indicating tissue damage involving the entire white matter bundle. Because the area of myelin and axon loss seemed congruent, we performed further volumetric analysis using myelin loss as a surrogate for white matter infarction. Inflammatory infiltrate was identified mainly as CD68+-activated microglia/macrophages by immunofluorescence (Figure 3C). Saline microinjections did not cause any of the above changes (Figure II in the online-only Data Supplement).

**White Matter Stroke Is Associated With Long-Term Behavioral Deficit**

Behavioral deficit was assessed at 7 and 28 days after stroke in separate cohorts (Figure 4). WT mice receiving endothelin-1 did not show any evident motor dysfunction or contralateral/ipsilateral asymmetry on the cylinder and grid-walk tests when compared with controls (Figure 4A). Neither did locomotor activity differ between groups (Figure 4B) nor did spatial memory using Morris water maze and Y maze (Figure 4C). By contrast, recognition memory was compromised in NOR (Figure 4D; \( P=0.003 \)), and this persisted for ≥24 weeks after stroke (Figure 4D; \( P=0.0037 \). No difference in total exploration time was found between groups.

**NOTCH3 Mutation Worsens White Matter Stroke Outcome**

There were no obvious developmental differences between WT and N3KO mice at baseline. After endothelin-1 micro-injection, infarct volume was larger in N3KO mice than in WT animals (Figure 5B; \( 0.18\pm0.04 \) versus \( 0.10\pm0.03 \) mm\(^3\), respectively; \( P=0.0002 \). No differences in the volume of corpus callosum/external capsule were detected between groups after endothelin-1 injection, mitigating the potential impact of swelling on infarct size (2.05±0.17 versus 2.02±0.19 mm\(^3\), respectively; \( P>0.05 \). There was minimal or no damage to grey matter adjacent to the injection site. The mutants showed instead greater loss of myelinated fibers into subcortical white matter extending rostro-caudally (Figure 5A) and medio-laterally (Figure IIIA and IIIB in the online-only Data Supplement). When tested at 7 days post stroke, N3KO mice showed a reduced exploratory behavior toward the new object in NOR (Figure 5C; \( P=0.0125 \). No differences in baseline NOR (\( P=0.71 \); Figure IIIC in the online-only Data Supplement), sensorimotor performances (Figure IIID in the online-only Data Supplement), and total exploration time (NOR, 7.75±0.86 versus 7.69±0.75 s, respectively; \( P>0.05 \) were detected among WT and N3KO mice. Analysis of the inflammatory markers, glial fibrillary acidic protein (reactive gliosis), and CD68 (activated microglia/macrophages) did not reveal differences between groups (Figure 6B and 6C; Figure IVA and I VB in the online-only Data Supplement). Microbleeds were not detected when assessed ≤7 days after stroke (Figure 6D; Figure IVC in the online-only Data Supplement).

**Figure 2.** MRI 2 days after white matter stroke. Demonstration of demyelination in a representative mouse by decrease in magnetization transfer ratio (loss of bright signal, dotted lines and arrowhead) 2 days after stroke. D-V indicates dorso-ventral; M-L, medio-lateral; and R-C, rostro-caudal.

**Figure 3.** Neuropathology in white matter after stroke. Focal myelin loss and cellular infiltration seemed restricted to subcortical white matter 7 days after stroke (A, arrowhead). Axonal degeneration at the stroke site was revealed by NF200 immunostaining and fluorescence microscopy (B, arrowhead). CD68+ cell (green) infiltration is shown in C (arrowhead). Nuclei are stained with Hoechst 33342 (blue). Hematoxylin and eosin staining revealed tissue damage and cellular infiltration 7 days after stroke (D, arrowhead). Insets in C and D highlight cellular infiltration. Scale bars, 500 (A), 100 (B), 200 (C and D), and 50 \( \mu m \) ( Insets in G and D). n=8. CC indicates corpus callosum; Co, cortex; and St, striatum.
Discussion

This study shows that stereotactic microinjections of endothelin-1 cause selective and reproducible ischemic damage to periventricular white matter affecting both myelin and axons. Peptide, but not vehicle, injections prompted a drop in regional blood flow surrounding white matter, causing blood–brain barrier opening, focal cellular infiltration, and histological alteration of corpus callosum, with minimal involvement of adjacent grey matter. Detailed behavioral assessments in these animals did not show deficits in sensorimotor, exploratory, and spatial memory after stroke. However, lesioned mice showed persistent recognition memory impairment. Building on these results, we challenged N3KO mice with white matter stroke to test the hypothesis that NOTCH3 gene deletion affects tissue and functional outcome. Although evaluation longer than 1 week was not possible in the N3KO mice, mutants clearly showed significantly greater infarct size and more pronounced cognitive deficit than WT lesioned animals at 7 days. Moreover, tissue and behavioral deficits persisted for many weeks in the WT, suggesting stability of the lesion and its consequences in this model. Hence, our data show that the endothelin-1 stroke model provides a feasible experimental approach to investigate white matter pathology.
after small vessel ischemia and demonstrate that NOTCH3 gene deletion increases white matter ischemia susceptibility.

Modeling white matter stroke is challenging. In the past years several animal models have been proposed, but none entirely reproduces the human pathological phenotype. One of the main limitations is the small white matter content in rodents and the difficulties of restricting the infarct to this small site. Endothelin-1 microinjections can generate small infarcts selectively in white matter and therefore this method offers several advantages specific to brain ischemia. First, endothelin-1 induces a long-term reduction of blood flow in the white matter consistent with ischemic stroke, as already demonstrated in grey matter studies. Second, endothelin-1 injection causes both myelin and axonal loss resembling human white matter strokes, a main advantage over models triggering pure demyelination (eg, lysolecithin injection). Third, given the focal lesion obtained with endothelin microinjection, it seems likely that the observed cognitive deficit is mainly from injury to subcortical white matter tracts, ruling out confounding factors (eg, optic nerve damage) affecting global hypoperfusion models. Moreover, existing models of small vessel diseases (eg, chronic hypoperfusion) are characterized by cumulative vascular impairment leading to tissue and behavioral deficit and reproduce a diffuse, leukoaraiosis-like, white matter damage. Differently, the endothelin model more closely resembles the acute pathophysiology of white matter lacunar stroke, although it does not reproduce the actual pathogenesis (eg, occlusion of a small penetrating artery by complications of atherosclerosis and hypertension).

This animal model, however, was affected by several technical shortcomings that we needed to improve on, namely variability in lesion size and location, plus obstacles relating to peptide formulation, concentration, and administration. Using the modifications described herein (see Methods in the online-only Data Supplement), we found that peptide microinjections could reliably achieve a focal lesion mainly confined to subcortical white matter and with low variance (infarct size, ±30% SD). On these bases, we think that the endothelin-1 model can improve our understanding of white matter stroke pathophysiology and facilitate bench-to-bedside translation. However, in its present iteration, it does not mimic the human condition, which is characterized by a complex clinical scenario including multiple infarcts, cognitive deficit, and frontal hypometabolism. Nonetheless, the present model may be relevant to mild, early-stage, cognitive impairments associated with lacunar, silent, strokes. Indeed, our white matter stroke model triggers episodic-like memory impairment assessed using a novelty-preference task, but not a deficit of spatial navigation assigned to hippocampal dysfunction (see below), often considered an indicator of more severe cognitive decline progressing to dementia.

A major point of this article is that a single white matter lesion can cause a sustained recognition memory deficit, probably because the white matter tracts at the level of the stroke site connect broadly distributed neuronal networks, which coordinate one aspect of cognitive function. A link between white matter lesions, especially at the level of corpus callosum, and impaired cognitive performance has been reported previously. In normal human aging, recognition memory performance depends on the integrity of multiple axonal pathways connecting different brain regions. In multiple sclerosis, reduced fractional anisotropy in white matter tracts correlates with cognitive impairment. Moreover, periventricular white matter lesions are associated with reduced memory speed and performance, and the severity of the lesion can predict the evolution of cognitive decline. In rats, recognition memory is compromised after lesion of the corpus callosum, whereas recognition and spatial memory are impaired after chronic hypoperfusion in mice, along with decreased callosal fiber density. When challenged with chronic hypoperfusion, stroke-prone spontaneously hypertensive rats show diffuse tissue damage (white and grey matter, cortex, and hippocampus) and severe deficit in...
Morris water maze. At variance with these observations, we did not detect impairment in spatial working memory, probably because the anatomic structures (eg, hippocampus) or connections relevant for place navigation tasks were spared because of the small size of the lesion. However, we do not rule out the possibility that the endothelin model can generate deficits in spatial memory by modifying size or location of white matter damage (eg, bilateral injections in corpus callosum, infarct location closer to the hippocampus). Nevertheless, here we provide a proof of concept showing that this model can be valuable to better understand the mechanisms underlying the relationship between white matter injury and cognitive deficits.

It has been shown previously that N3KO mice develop greater behavioral deficits and infarct size after middle cerebral artery occlusion compared with WT. Here, we demonstrate that the loss of function of NOTCH3 receptor is associated with enhanced white matter ischemia susceptibility after 7 days. The larger infarct size found in N3KO mice did not appear related to grey matter microinfarction or differences in white matter swelling or Willis’ circle anomalies; reactive gliosis and activated microglia/macrophage infiltration were comparable between groups. Moreover, small hemorrhages and microbleeds, often associated with human NOTCH3 mutations, were not detected in this model, possibly because the human condition is caused by point mutations and frame shifts rather than by gene deletion. We speculate instead that the larger infarct can be attributed to greater perfusion deficit in response to the ischemic challenge, similar to previous data from grey matter stroke, but this is hard to document with quantitative precision using current autoradiographic techniques because the lesion size is small (=0.1 mm³) and blood flow is normally low in white matter. Probably the greater behavioral deficit in N3KO mice was because of disruption of a larger number of traversing axons in the larger lesion. Future studies will address to what extent human NOTCH3 mutations (eg, R90C, R169C) affect the ischemic phenotype after white matter stroke and whether these mutants show persistent cognitive impairment.

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Disclosures

None.

References

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SUPPLEMENTAL MATERIAL

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SUPPLEMENTAL METHODS

Endothelin-1 white matter stroke model: methodological considerations

The final protocol adopted for this work and described in the Method section of the manuscript has been modified from the original version proposed by Carmichael and collaborators to overcome technical issues related to endothelin-1 solubility and to minimize mechanical damage due to the microinjection.

Endothelin-1 is a peptide commonly synthesized by many vendors as acetate salt, and it is poorly soluble in water but greatly soluble in organic solvents (acetate, ethanol or DMSO 1-5% solutions). In the initial experimental conditions, the peptide was insoluble in aqueous solution at high concentrations (>0.5 mg/mL), even after prolonged sonication. On the other hand, the injection of even low concentration (1%) of organic solvents as vehicles (above) caused tissue damage, and was therefore avoided. We tested different formulations from different vendors in order to identify the product with the best characteristics for our application. American Peptide provided two batches of peptide that were soluble in water at high concentration (>0.3 mg/mL). Both lots produced reproducible lesions when injected at the concentration of 1 mg/mL (T03091) and 0.3 mg/mL (Z05068T1), respectively. We therefore used the peptide Lot # Z05068T1 for our study.

Furthermore, we modified the original injection protocol (3x120 nL) to limit the volume and reduce the number of injection. We tested 3 injection of 100 nL, 3x120 nL, 3x150 nL, 2x200 nL, 2x100 nL, 1x200 nL, 1x500 nL. Two injections of 100 nL, performed at the same depth from the cortical surface (see below) and 400 µm apart (anterior-posterior) gave the most reproducible results.

We adopted the following stereotactic coordinates to induce the white matter lesion: AP +1.00, +0.60 mm; ML -0.20 mm; DV -2.20 mm. The coordinates were calculated zeroing from bregma and the dura mater, and using a 36 degree angle of injection.

The diffusion of the injectate and its restriction to white matter was confirmed by injecting the fluorescent dye Hoechst 33342 (1 mg/mL in saline solution, Sigma) using the stereotactic coordinates and the volume described above.

Magnetic Resonance Imaging

Mice were anesthetized with isoflurane and secured within a cradle designed to interface with a volume transmit RF coil and a four-channel phased-array receiver coil (Bruker Biospin, Billerica MA, USA). All imaging employed an isotropic in-plane resolution of 150 microns with 30 slices of 250-micron thickness that spanned coronal brain sections from olfactory bulb through hippocampus. A fast spin-echo T2-weighted imaging sequence was used to identify anatomy for cross-subject spatial registration. Magnetization Transfer Ratio (MTR) contrast was obtained by an RF preparation pulse, which was 3500 Hz off-resonance relative to the water frequency, that was embedded within a fast gradient-echo sequence. Another identical sequence without the preparation pulse provided a reference signal without magnetization transfer contrast, and temporal averaging (16x) increased the signal to noise ratio of each sequence. At the end of imaging session, mice were euthanized for histological analysis.

In post-processing, data were aligned to the stereotactic coordinates of the Allen Mouse Brain atlas. MTR was calculated for each voxel as the percentage difference of images with and without magnetization transfer contrast.
Neurological assessments

Mice were housed in Massachusetts General Hospital animal facility and allowed to acclimate for 3-7 days before starting the behavioral test. In the same period, mice were handled to reduce the stress and exposed at least once to the experimental setup. The day of the test, mice were carried to the behavioral room and allowed to acclimate for one hour before beginning the experiments. All tests were videotaped and some (Cylinder test and Grid-walk test) analyzed in slow-motion. Extreme care was taken to clean cages, arenas and the testing objects with warm water and 70% ethanol before each trial. All tests were performed in the active/exploratory phase, between 4 and 8 PM. To reduce possible bias introduced by multiple testing on the same animals (e.g., stress, anxiety, reduced or increased exploratory activity), two different cohorts of mice were used to perform behavioral screening test. For the first cohort (8 control and 8 stroke mice), cylinder, grid-walk and novel object recognition tests were performed. For the second cohort (10 control, 11 stroke), open field, Y-maze and water maze tests were performed. An additional cohort was used to assess behavioral deficit at 4 weeks after stroke (9 control, 10 stroke).

In the Cylinder test, a mouse was placed in a 9 cm diameter transparent cylinder, which it rears to explore. The number of times that the animal touched the wall of the cylinder with right, left and both forelimbs was recorded. Only rears in which both forelimbs can be clearly seen were counted. The percentage of ipsilateral and contralateral contacts vs. the total number of wall contacts was then derived and used for statistical comparison between groups.

For the Grid-walk test, each mouse was placed atop an elevated wire grid (1.5x1.5 cm grid opening) and allowed to freely walk for a period of 5 minutes. During this time, the total number of footfaults for each limb vs. the total number of correct steps was counted and a ratio between footfaults and total steps taken was calculated. Percent of footfaults were calculated by the formula \([\text{#footfaults} / (\text{#footfaults} + \text{#correct steps}) \times 100]\).

For the Novel Object Recognition test, the mice were placed individually in a rectangular arena (35 cm x 20 cm) and exposed for 5 minutes (session 1) to two identical objects (orange cylinder, familiar objects). After 1 hour interval, mice were exposed for 5 minutes (session 2) to a familiar object from previous session and a novel object (blue rectangular box). The time spent exploring each object was then calculated and expressed as percentage of the total exploration time during each session. A ratio between the time spent exploring familiar and novel objects during Session 2 was also calculated to derive a discrimination index. Objects were selected in order to be similar but not identical (same dimension, but not same shape or color) and to be equally interesting for the animals (in a pilot experiment, mice spent roughly the same time exploring the different objects when the objects were exposed for the first time simultaneously).

For the Y-Maze Spontaneous Alternation test, each mouse was placed at the end of one arm of a symmetrical Y-shaped maze and allowed to move freely through the maze for a 5-min test period. Alternation was defined as successive entries into the three arms on overlapping triplet sets. The maximum number of possible spontaneous alternations was determined as the total number of arms entered - 2, and the percentage was calculated as the ratio of actual to possible alternations \(\times 100\).

In the Open Field test, mice were placed in an arena (60 cm x 60 cm) and allowed to freely explore the environment for 5 min. Total distance traveled was calculated using the ANY-MAZE tracking system (Stoelting).

For the Water Maze test, mice were placed in a white pool (83 cm in diameter, 60 cm deep) filled with lukewarm water to a depth of 29 cm. Highly visible cues were positioned on the walls of the tank and around the room. A clear Plexiglas goal platform 10 cm in diameter was positioned 0.5 cm below the surface of the water ~15 cm from the southwest wall. Each mouse
was subjected to a series of not more than 2 trials per day. For each trial, mice were randomized to one of four starting locations (north, south, east, and west) and placed in the pool facing the wall. The maximum time allowed to find the platform was 60 seconds. For probe trial, mice were placed in the tank opposite the target quadrant and the time spent in the target quadrant was quantified. Time to reach the platform (latency) was quantified using the ANY–MAZE tracking system (Stoelting).

In preliminary experiments, additional tests were performed including 5–points neuroscore and Hanging wire test, but they didn’t reveal any difference among groups and were excluded from subsequent testing.

Cerebral blood flow and blood brain barrier permeability assessments
For qualitative cerebral blood flow (CBF) assessment\(^9\), mice were infused with \([^{14}\text{C}]-\text{iodoantipyrine}\) (5 \(\mu\text{Ci}\) in 100 \(\mu\text{L}\)) through the femoral vein during 45 seconds via an injection pump (Stoelting). Animals were decapitated and brains harvested and frozen in isopentane in less than 1 minute to avoid artifacts. 20 \(\mu\text{m}\) coronal cryosections were thaw–mounted and then immediately dried using a blow–dryer and exposed to Kodak SBB-5 autoradiographic film for 48 hours in an X-ray cassette along with a series of calibrated \([^{14}\text{C}]-\text{polymer standards}\) (Amersham). Films were then acquired using a scanner (resolution 300 dpi) and saved as TIFF files.

For Blood Brain Barrier (BBB) permeability assessment\(^10\), mice were injected through the lateral tail vein with 4mL/kg of a 2% Evans blue (Sigma) solution. After 4 hours animals were cardioperfused with saline solution, and the brains harvested and snap-frozen in -45°C isopentane. 20 \(\mu\text{m}\) coronal sections were then counter-stained (Hoechst 33342), and imaged with an epifluorescence microscope. Since choroid plexus capillaries are fenestrated and allow the diffusion of small molecules, the presence of fluorescent signal in the choroid plexus was considered as indicator of successful Evans blue injection.

Experimental conditions adopted for Immunofluorescence
Immunofluorescence experiments were conducted using the staining conditions reported below:

- Rabbit anti-NF200 (1:200, Sigma, cat# N4142) was incubated for 60 min at room temperature, in 3% horse serum, 0.3% Triton X100, PBS 0.01M, pH 7.4.
- Rat anti-CD68 (1:200, Adb Serotec, cat# MCA1957) was incubated for 60 min at room temperature, in 3% horse serum, 0.3% Triton X100, PBS 0.01M, pH 7.4.
- Mouse anti-GFAP Cy3-conjugated (1:400, Sigma, cat # C9205) was incubated for 60 min at room temperature, in 3% horse serum, 0.3% Triton X100, PBS 0.01M, pH 7.4.
- Donkey anti-rat secondary antibody-Cy2 conjugated (1:200, Jackson ImmunoResearch, cat# 712-226-153) was incubated for 60 min at room temperature, in PBS 0.01M, pH 7.4.
- Donkey anti-rabbit secondary antibody-FITC conjugated (1:200, Jackson ImmunoResearch, cat# 711-095-152) was incubated for 60 min at room temperature, in PBS 0.01M, pH 7.4.
- Hoechst 33342 (1:1000, Sigma, cat# 14533) was incubated for 10 min at room temperature, in PBS 0.01M, pH 7.4.
Assessment of post–stroke inflammation and microbleeds

To assess reactive gliosis and activated microglia/macrophages, two coronal levels located within the brain area showing the largest white matter infarct (distance from bregma: +1.10 and +0.74) were identified using Franklin & Paxinos mouse brain atlas\textsuperscript{11}. Within each coronal level, 4 regions of interest (ROIs) were selected (40X fields, 0.07 mm\textsuperscript{2}) to obtain a representative sample of infarct core, peri–infarct area, and adjacent cortex and striatum (see Fig. 6A). All the images were acquired using the same magnification, exposure time and gain. Image analysis was conducted using ImageJ software. For semiquantitative analysis of reactive gliosis, GFAP immunoreactivity\textsuperscript{12} was measured by normalizing the fluorescence intensity of each field of view by the relative background signal using the formula: integrated density of the field of view – area of the field of view * mean grey values of background region (mean of 3 measurements). Results represent the mean of measurements at +1.10 and +0.74 coronal levels and are expressed as arbitrary units. To quantify activated microglia/macrophages\textsuperscript{13}, merged CD68 (green) and Hoechst 33342 (nuclei, blue) images were first thresholded (color threshold) and then the cells positive for both markers were counted automatically using the “analyze particles” algorithm of ImageJ. Automated counting results were confirmed by manual counting. Results represent the average of measurements at both coronal levels and are expressed as CD68+ cells per field of view. Differences among the groups were analyzed using unpaired Student’s t–test.

To assess microbleeds load\textsuperscript{14,15}, ~20 coronal sections per animal (~ +2.00 to -1.00 from Bregma) were stained with H&E and examined under brightfield illumination using 10X and 20X magnification. Gross examination didn’t reveal the presence of microbleeds in cortex, corpus callosum and striatum of any animal. Additionally, a total of 3 fields of view (10X, infarct site, cortex, striatum) were selected at two coronal levels (distance from bregma: +1.10 and +0.74) to perform a quantitative assessment. Microbleeds load was quantified in four different regions (core, peri–infarct, cortex and striatum) and expressed as total number of microbleeds per mouse. Since no microbleeds were detected in both groups, statistical analysis was not performed.
Supplementary Figure I – Blood brain barrier opening and brain histopathology after endothelin–1 injection

(A) Representative (n=5 control, 5 stroke) microphotograph showing Evans blue extravasation (red) restricted to the corpus callosum four hours after endothelin microinjection (left panel), but not in controls (center panel). Evans Blue fluorescence at the level of the choroid plexus (arrow), which lacks an intact BBB, was noticeable in both groups (right panel). Nuclei are stained with Hoechst 33342 (blu). Scale Bar: 100 (left and center panels), 200 µm (right panel). Co: cortex; CC: corpus callosum; St: striatum.

(B) Representative LFB-H&E stained section showing tissue damage two days after endothelin–1 microinjection (n=5, left panel). Dashed lines highlight the edge of the ischemic lesion. Higher magnification is shown in right panel. The asterisk is placed at the same anatomical level in both images. The injury is mainly restricted to white matter. Of note, inflammatory infiltration is not present at the infarct site yet. Scale Bars: 200 (left panel), 100 µm (right panel).
Supplementary Figure II – Neuropathological assessment in control mice

No histological damage is detected in control group (n=8). LFB–CV (A) and NF200 (B) staining reveals no myelin or axonal degeneration seven days after stroke. There is no cellular infiltration in the white matter as shown by CD68 (C) and H&E (D) staining. Nuclei are stained with Hoechst 33342 (blue) in C. Scale Bars: 500 (A), 100 (B), 200 μm (C, D). Co: cortex; CC: corpus callosum; St: striatum.
Supplementary Figure III – Infarct and behavioral performance in WT and N3KO mice

Representative (n=8 control, 8 stroke) LFB–stained sections showing tissue outcome in WT (A) and N3KO (B) seven days after white matter stroke. The black arrows indicate the lesion site, and the dashed lines highlight the edges of the infarct. At baseline assessment, NOR didn’t reveal any difference among the groups (C). After stroke, N3KO mice did not show any sensorimotor abnormalities performing cylinder and grid–walk tests (D). Scale Bars: 1 mm.
Supplementary Figure IV – Assessment of post–stroke inflammation and microbleeds in WT and N3KO mice
Representative microphotographs showing reactive gliosis (GFAP, A) and activated microglia/macrophages (CD68, B) in infarct core, peri–infarct region, cortex and striatum of WT (n=8) and N3KO (n=8) mice seven days after stroke. Nuclei are stained with Hoechst 33342 (blue) in B. H&E staining for a representative N3KO mouse (n=6 N3KO, 6 WT) showing the absence of microbleeds at the infarct site (black arrow), peri–infarct region (adjacent white and grey matter), cortex and striatum (C). Reactive gliosis, activated microglia/macrophages and microbleeds assessments did not reveal any difference among the experimental groups. Scale Bars: 50 µm (A, B), 200 µm (C). Co: cortex; CC: corpus callosum; St: striatum.
SUPPLEMENTAL REFERENCES


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