Transient Receptor Potential Melastatin Subfamily Member 2 Cation Channel Regulates Detrimental Immune Cell Invasion in Ischemic Stroke

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Background and Purpose—Brain injury during stroke results in oxidative stress and the release of factors that include extracellular Ca²⁺, hydrogen peroxide, adenosine diphosphate ribose, and nicotinic acid adenine dinucleotide phosphate. These alterations of the extracellular milieu change the activity of transient receptor potential melanatin subfamily member 2 (TRPM2), a nonselective cation channel expressed in the central nervous system and the immune system. Our goal was to evaluate the contribution of TRPM2 to the tissue damage after stroke.

Methods—In accordance with current quality guidelines, we independently characterized Trpm2 in a murine ischemic stroke model in 2 different laboratories.

Results—Gene deficiency of Trpm2 resulted in significantly improved neurological outcome and decreased infarct size. Besides an already known moderate neuroprotective effect of Trpm2 deficiency in vitro, ischemic brain injury by neutrophils and macrophages was particularly reduced in Trpm2-deficient mice. Bone marrow chimeric mice revealed that Trpm2 deficiency in the peripheral immune system is responsible for the protective phenotype. Furthermore, experiments with mixed bone marrow chimeras demonstrated that Trpm2 is essential for the migration of neutrophils and, to a lesser extent, also of macrophages into ischemic hemispheres. Notably, the pharmacological TRPM2 inhibitor, N-(p-amylcinnamoyl)anthranilic acid, was equally protective in the stroke model.

Conclusions—Although a neuroprotective effect of TRPM2 in vitro is well known, we can show for the first time that the detrimental role of TRPM2 in stroke primarily depends on its role in activating peripheral immune cells. Targeting TRPM2 systemically represents a promising therapeutic approach for ischemic stroke. *(Stroke. 2014;45:3395-3402.)*

Key Words: inflammation ■ ion channels ■ neutrophils ■ stroke

Ischemic stroke is the second most common cause of death worldwide. Tissue damage is thought to follow a biphasic course. The initial hypoxic damage is determined by immediate neuronal cell death leading to the formation of the infarct core, whereas secondary infarct growth is considered to be a consequence of systemic and local sterile inflammation.

Ischemia in the central nervous system is characterized by oxidative stress and the release of a manifold of stress mediators, among them adenosine diphosphate ribose (ADPR) that is produced by poly-ADPR polymerase in response to oxidative stress, cyclic ADPR, calcium and nicotinic acid adenine dinucleotide phosphate. Because these factors modulate the open-probability of the calcium-permeable transient receptor potential melanatin subfamily member 2 (TRPM2) cation channel, this channel has been implicated in stroke pathophysiology. The highest expression levels of TRPM2 are found throughout the nervous system, such as neurons and microglial cells, but it can also be detected in a variety of other tissues including cells of the peripheral immune system, such as polymorphonuclear neutrophils and monocytes.
Therefore, TRPM2 might be involved in early ischemic neuronal cell death but also in the subsequent detrimental sterile inflammation.

Involvement of TRPM2 in cerebral ischemic injury has recently been investigated,\textsuperscript{5,6} showing a pathogenetic contribution of TRPM2 to ischemic stroke. However, these analyses focused on the role of TRPM2 in neuronal injury during ischemia. Of note, Trpm2 deficiency in ischemic stroke has been suggested to preferentially protect neurons of male mice because of TRPM2 regulation by androgen signaling.\textsuperscript{5,7,8}

Although in vitro experiments point toward a central role of TRPM2 in neuronal injury,\textsuperscript{8,9} its relative contribution to ischemic tissue injury in vivo by controlling immune cell activation has not been investigated. Notably, TRPM2 signaling controls specific functions in immune cells including production of cytokines and chemokines, chemotaxis of immune cells, and inflammasome activation.\textsuperscript{10–13} Activation of microglia through TRPM2 was observed after \textit{H2}O\textsubscript{2} and ADPR stimulation in vitro\textsuperscript{14} and was associated with nitric oxide synthesis and chemokine production.\textsuperscript{10}

Here we show at 2 different experimental sites that TRPM2 detrimentally contributes to ischemic brain injury after stroke, which primarily depends on its role in activating peripheral immune cells. Although \textit{Trpm2}-deficient neurons are protected against hypoxic stimuli in vitro, TRPM2 regulates neutrophil and macrophage infiltration in vivo that primarily determines its injurious role in stroke. Using separate and mixed bone marrow chimeric mice, we are able to unequivocally demonstrate that the \textit{Trpm2} deficiency on neutrophils and macrophages is key for the outcome after stroke, whereas \textit{Trpm2} deficiency in central nervous system–resident cells does not contribute to stroke outcome.

Materials and Methods

Animals and In Vivo Stroke Model

All animal experiments were approved by local animal care committees (Behörde für Lebensmittelsicherheit und Veterinärwesen Hamburg and Regierung von Unterfranken). We conducted the experiments according to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85–23, revised 1996) and performed all procedures in accordance with the ARRIVE guidelines (http://www.nc3rs.org/ARRIVE). Male C57BL/6\textit{Trpm2}–/–\textit{mice} were kindly provided by Dr Y. Mori, Kyoto University, Japan. We randomized all mice and conducted transient middle cerebral artery occlusion (tMCAO) for 1 hour as previously described\textsuperscript{15} using the intraluminal filament method (6-0 nylon) in a blinded fashion. The detailed experimental description can be found in the online-only Data Supplement. We dissolved the TRPM2 channel blocker, N-(p-amylcinnamoyl)anthranilic acid (ACA; Sigma, St Louis, MO), in dimethylsulfoxide and then further diluted it in PBS and injected it intraperitoneally 2 hours after tMCAO in wild-type (WT) littermate controls and \textit{Trpm2}–/– mice at 5 or 25 mg/kg body weight.\textsuperscript{16} We administered PBS-diluted dimethylsulfoxide by intraperitoneal injections in sham-treated mice. Sample size calculation was performed (stroke size from pilot experiments, significance level 0.05, power 90%) and resulted in 9 animals per group to see a difference of 23% in stroke size.

Analysis of Infarct Size by TCC Staining

We analyzed infarct size by harvested brains and cutting them into 1 mm slices (Braintree Scientific, 1 mm) followed by vital staining using 2% (wt/vol) TTC in phosphate buffer. We determined infarct volumes in a blinded fashion using NIH ImageJ software.

Bone Marrow Chimeras

For generation of mixed bone marrow chimeras, we irradiated 10-week-old mice using a cesium-137 gamma irradiator (BIOBEAM 2000, Leipzig, Germany). After 24 hours we reconstituted them with bone marrow cells. The detailed experimental description can be found in the online-only Data Supplement.

Antibodies and Flow Cytometry

We performed flow cytometry for the analysis of cell types as previously described.\textsuperscript{15} The detailed experimental description can be found in the online-only Data Supplement.

Cell Sorting and RNA Isolation

We isolated immune cells according to the protocol for flow cytometry and stained with antibodies (all eBioscience) against CD11b (M170), CD11c (N418), CD45 (30-F11), and Ly6G (1A8). We sorted cells using a BD FACSAria and collected cells in Dulbecco modified Eagle medium with 30% fetal calf serum. We isolated total RNA from cells using QIA-Shredder spin columns and the RNeasy Micro Kit (Qiagen, Hilden, Germany) and transcribed complementary DNA using Maxima First Strand cDNA Synthesis Kit for reverse transcription polymerase chain reaction (Fermentas, Waltham, MA).

Quantitative Real-Time Polymerase Chain Reaction

We obtained real-time PCR primers from Applied Biosystems (Carlsbad, CA): \textit{Actinb} Mm00607939_m1; Ccfl Mm00438359_m1; Ccl2 Mm00441242_m1; Csf1 (granulocyte-macrophage colony-stimulating factor) Mm00438334_m1; Ccl2 Mm00436450_m1; Ilt6 Mm00446190_m1; Il10 Mm00439616_m1; Nos2 (iNOS) Mm00440502_m1; Il1b Mm00434228_m1; Tnf Mm00434258_m1; Trpm2 Mm00630989_m1 and Mm01177249_g1; TATA box binding protein Mm00446971_m1. We purchased probe mixtures from Fermentas (Waltham, MA). The detailed experimental description can be found in the online-only Data Supplement.

Hippocampal Neuron and Microglia Cell Culture and Brain Slice Preparation

We obtained neuronal cell cultures from mouse embryos (embryonic day 18), microglia cell cultures from mice on postnatal days 1 to 5, and acute coronal brain slices from 6- to 10-week-old \textit{Trpm2}-deficient mice and their WT littermates as previously described.\textsuperscript{15,16} The detailed experimental description can be found in the online-only Data Supplement.

Immunohistochemistry

We stained brains after standard immunohistochemistry procedures with antibodies against GFAP (1:200; DAKO, Hamburg, Germany), Iba-1 (1:200; Wako, Osaka, Japan), Ly-6G (1:1000; Biologend, San Diego, CA), NeuN (1:1000; Chemicon, Billerica, MA), cleaved caspase 3 (1:400; CellSignal, Danvers, MA), CD16/32 (1:100; BD Bioscience, Franklin Lakes, NJ), CD206 (1:100; RnDSystems, Minneapolis, MN). The detailed experimental description can be found in the online-only Data Supplement.

Statistical Analysis

Data are reported as mean±SD. Statistical analyses were performed using the appropriate test indicated in the figure legends. Briefly, Student \textit{t} test was used to compare infarct volumes, Mann–Whitney \textit{U} test for the comparison of clinical scores, and 1-way ANOVA for multiple comparisons with Bonferroni post hoc test, after validating the normal distribution of these data sets (Kolmogorov–Smirnov test). \textit{P} values <0.05 were considered statistically significant.
Results

TRPM2 Deficiency Is Protective in Stroke

We assessed infarct size and neurological scores after 1 hour of transient tMCAO. At 2 independent experimental sites (Würzburg and Hamburg), Trpm2−/− mice showed a significantly reduced infarct size and milder disability scores compared with littermates at day 1 (P < 0.0001) and day 3 (P = 0.01) after tMCAO (Figure 1A and 1C). All Trpm2−/− and littermate control mice included in the tMCAO experiment for infarct volume survived until the analysis of the infarct volume. Notably, both genotypes showed the same reduction in regional cerebral blood flow in the tMCAO model assessed by laser Doppler and were not different in physiological parameters before, during, and after tMCAO (Figure 1B; Figure I in the online-only Data Supplement).

Trpm2 Expression in Stroke

We analyzed TRPM2 expression after 24 hours of cerebral ischemia in the tMCAO model and observed a significant upregulation of Trpm2 in whole-brain mRNA (Figure 2A). By immunocytofluorescence we detected Trpm2 expression in MAP-2–positive neurons and CD11b–positive microglia cells in vitro (Figure 2B). In cells of the peripheral immune system, we found Trpm2 to be highly expressed in bone
delineate whether the neuroprotective effect in the WT mice at day 1 after ischemic stroke (Figure 4D). Next, we analyzed whether Trpm2 is regulated in infiltrating macrophages and resident microglia after stroke in vivo or in neuronal in vitro cultures after challenge with oxidative stress or proinflammatory cytokines. Neither macrophages/microglia exhibited a change in Trpm2 mRNA levels 24 hours after tMCAO compared with cells from sham-operated mice (Figure II A in the online-only Data Supplement), nor did neurons show alterations in Trpm2 mRNA levels in vitro (Figure IIC and IID in the online-only Data Supplement).

Neuronal Protection in Trpm2–/– Mice After Ischemia
To address whether TRPM2 may be directly involved in neuronal injury, we analyzed markers of neuronal apoptosis by immunohistochemistry. Indeed, we found a significant decrease \( (P=0.01) \) of apoptotic (cleaved caspase-3-positive) neurons in ischemic hemispheres of Trpm2–/– mice compared with WT mice at day 1 after ischemic stroke (Figure 3A). To delineate whether the neuroprotective effect in the Trpm2–/– mice is because of a direct increase in resistance toward energy shortage, we subjected cortical slice cultures from Trpm2–/– and wild-type littermate control mice were incubated for 6 h either in normal modified artificial cerebrospinal fluid under normoxic conditions or in modified artificial cerebrospinal fluid with reduced glucose concentration (5 mmol/L) and pH (6.4) under anoxic conditions. Neuronal cell death was quantified by immunofluorescence double staining for cleaved caspase 3' and NeuN' (n=3). Scale bar, 50 μm. Data are represented as mean±SD. Statistical analyses were performed by Student t test. \( P \) values ≤0.05 were considered significant.

Trpm2 Deficiency Attenuates Immune Cell Infiltration After Experimental Stroke
Because TRPM2 was shown to fulfill important functions in immune cells, the observed reduction in neuronal injury after tMCAO could also be the result of less secondary injury as a consequence of altered poststroke inflammation. Indeed, 3 days after tMCAO, we observed a significant decrease in neutrophil \( (P<0.0001) \) and macrophage \( (P=0.005) \) infiltration in Trpm2–/– mice compared with littermate control mice in ischemic hemispheres (Figure 4A), whereas infiltration of dendritic cells and CD45+ lymphocytes (Figure 4A) as well as T-cell subsets (NK cells; CD4+, CD8+, and γδ T cells; Figure IIIA in the online-only Data Supplement) did not differ between genotypes. Notably, the observed differences were not because of an alteration of the cell composition in the peripheral blood (Figure IIIB in the online-only Data Supplement). We detected no difference between both genotypes in microglial proliferation, morphological activation, and expression of M1/M2 lineage markers (CD16/32 and CD206) in microglia and macrophages 3 days after tMCAO (Figure 4A–4C; Figures IIIC, IID, and IVD in the online-only Data Supplement). In flow cytometry analysis, differentiation of infiltrating macrophages and microglia was performed by CD45 expression as previously reported. We next analyzed whether Trpm2 deficiency impacts cytokine and chemokine secretion in the ischemic brain. After tMCAO, neither cytokine profiles as measured by intracellular cytokine staining of interleukin-17A (IL-17A) and interferon-γ (lymphocytes; Figure IVA and IVB in the online-only Data Supplement) and tumor necrosis factor-α (macrophages, neutrophils, dendritic cells, and microglia; Figure IVC in the online-only Data Supplement) nor chemokine and cytokine levels in whole-brain mRNA (CXCL-1, CXCL-2, G-CSF, IL-6, CCL-2, IL-10, iNOS, TNF-α, IL-1β) showed significant differences between Trpm2–/– and control animals (Figure 4D).
translate into the rather insensitive measurement of neurological impairment (Figure 5B). To verify that TRPM2 regulates migration of immune cells during stroke, we next created mixed bone marrow chimeric mice. We reconstituted irradiated CD45 congenic C57BL/6J Ly5.1 mice (CD45.1) with equal amounts of bone marrow cells derived from Trpm2–/– CD45.2 and WT CD45.1 mice (Figure 5C). Six weeks after bone marrow transplantation, we analyzed the reconstitution in the peripheral blood by flow cytometry and observed an approximately equal distribution of CD45.1+ and CD45.2+ immune cells in each animal (data not shown). Three days after tMCAO, we detected a significantly reduced infiltration of Trpm2–/– neutrophils in the ischemic hemisphere compared with WT neutrophils (P = 0.002; Figure 5D). Consistent with the finding in Trpm2–/– mice, we also found reduced numbers of Trpm2-deficient macrophages (P = 0.2) albeit to a lesser extent than neutrophils (Figure 5D). By contrast, dendritic cell and lymphocyte migration was not affected by the deficiency of Trpm2 (Figure 5D).

**Pharmacological Inhibition of Trpm2 Is Protective in Stroke**

ACA has been reported to effectively inhibit TRPM2-mediated currents at concentrations between 10 and 100 mmol/L. Indeed, although 5 mg/kg body weight ACA was not effective, administration of a single-dose 25 mg/kg body weight ACA 2 hours after stroke induction resulted in a significant reduction (P<0.001) in infarct size and improvement in clinical outcome compared with the injection of vehicle control (Figure 6A–6C). Furthermore, ACA treatment significantly suppressed neutrophil infiltration into the ischemic hemisphere (Figure 6D). Whereas ACA (25 mg/kg) ameliorated clinical outcome in the tMCAO model in WT mice, it caused no additional improvement in Trpm2–/– mice in comparison to vehicle-treated Trpm2–/– mice, indicating that ACA probably exerts its protective properties via targeting TRPM2 (Figure 6A–6C).

**Discussion**

Our study demonstrates a key role for TRPM2 in cerebral ischemia. We show that TRPM2 in neutrophils and macrophages regulates their migratory capacity to ischemic brain thereby secondarily perpetuating brain injury. Trpm2–/– mice are protected from ischemic stroke and show an improved neurological outcome compared with WT mice. Although TRPM2 contributes to neuronal cell death in in vitro conditions, activation of TRPM2 in neurons and microglia seems to have minor effects on the outcome in our murine model of cerebral ischemia. This is implicated by our in vivo experiments with bone marrow chimeric mice that show that TRPM2 directly contributes to the migration of neutrophils and to a lesser extent of macrophages into the ischemic hemispheres and that TRPM2 in these cell types determines tissue damage. Equally, pharmacological inhibition of TRPM2 is able to suppress neutrophil migration and to ameliorate disability after stroke in a clinically relevant setting and therefore commends as treatment strategy in stroke.
Multiple studies have shown that the activation of the immune system has detrimental consequences in stroke. In this context, immunologic signaling cascades, which are involved in neutrophil infiltration and activation, seem to have a major impact on the tissue damage. We and others have shown that disruption of IL-1β or inflammasome signaling as well as neutralization of IL-17 and CXCR2 are protective in stroke. Because TRPM2 is expressed at high levels in neutrophils and has been shown to be involved in the chemotactic response of neutrophils, it represents a particular attractive target. The importance of TRPM2 signaling in brain ischemia is underlined by our finding that Cd38-deficient mice show an attenuated infiltration of immune cells and improved neurological outcome in experimental stroke. The ectoenzyme CD38 is expressed by different leukocyte subsets and generates ADPR and cyclic ADPR from its substrate NAD+, which in turn are able to activate TRPM2.

The detrimental role of TRPM2 in other inflammatory conditions, in which reactive oxygen species acts as a main trigger of inflammation, was reported in a disease model of dextran sulfate sodium–induced colitis. The role of TRPM2 as a sensor for reactive oxygen species with subsequent chemokine production in macrophages was also demonstrated in a model of inflammatory and neuropathic pain. Notably, in the context of ischemia/reperfusion injury of the brain, chemokine production in the ischemic organ seems to be independent of TRPM2, because we could not detect an altered chemokine production in Trpm2−/− mice. Mixed bone marrow chimeras rather suggested a migratory defect for Trpm2−/− neutrophils and possibly macrophages. In support of our finding it has recently been shown in a model of myocardial infarction that neutrophil adhesion, a prerequisite for migration, depends on TRPM2.

Regarding a possible pharmacological inhibition of TRPM2, one has to consider not only local anti-inflammatory properties but also exacerbated systemic immune alterations in patients with stroke. However, short-term selective inhibition of neutrophil migration should be beneficial without aggravating the stroke-induced immune suppression, thereby commending TRPM2 as an attractive target molecule in stroke treatment.

Based on our experiments with bone marrow chimeras, we can attribute the detrimental effects of TRPM2 ion channels mainly to the peripheral immune system. Nevertheless, as seen in our in vitro oxygen glucose deprivation experiments,
N-methyl-\(\text{o}\)-aspartate receptor GluN2A/GluN2B subunits toward a decreased expression of the prosurvival N-methyl-\(\text{o}\)-aspartate receptor GluN2A.

Overall, the current study was performed in a network of experimental laboratories (Hamburg, Muenster, Würzburg) and the key findings were reproduced in independent laboratories. Regarding the current discussion on preclinical phase III stroke trials, our multicenter approach represents a first step to further validate preclinical animal studies with the goal to overcome the translational roadblock.

In conclusion, the present study revealed the role of TRPM2 in postischemic central nervous system inflammation. TRPM2 activation in peripheral immune cells leads to exacerbation of ischemic brain damage, and TRPM2 might therefore display a promising treatment target of the detrimental postischemic inflammatory response after stroke. The general feasibility of this approach has been demonstrated by our experiments with the TRPM2 blocker ACA, which was able to inhibit central nervous system tissue damage in mCAO even when applied 2 hours after the onset of stroke. Therefore, further investigations for specific, safe, and well-tolerable TRPM2 inhibitors are warranted.

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Disclosures

None.

References


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SUPPLEMENTAL MATERIAL.

Supplemental methods:

In vivo stroke model
Male Trpm2\(^{-/-}\) mice on a CD57BL/6J genetic background\(^1\) were kindly provided by Dr. Y. Mori, Kyoto University, Japan. Aged-matched male wildtype (WT) littermates served as controls. We kept mice under pathogen-free conditions and provided access to food and water ad libitum. We anesthetized all mice (20 to 25 g, 12 weeks; TVH, University Medical Center Hamburg-Eppendorf, and Department of Neurology, University of Wuerzburg) using isoflurane 1% to 2% v/v oxygen and buprenorphine 0.03 mg/kg body weight intraperitoneally (i.p.) every 12 hours for 24 hours as analgesia. We monitored mice for heart rate, respiratory rate, oxygen saturation, rectal body temperature, and cerebral blood flow by using transcranial temporal laser doppler. After stroke induction, we repeatedly scored every mouse on a scale from 0–5 (Bederson Score: 0 no deficit, 1 preferential turning, 2 circling, 3 longitudinal rolling, 4 no movement, 5 death) immediately after reawakening and every day until sacrifice. The cerebral blood flow in the area of the MCA showed a reduction of ∼90%, which did not differ between groups. Mice were sacrificed one or three days after reperfusion using isoflurane and decapitation. Only mice with a score greater or equal than one after reawakening were included for stroke size analysis.

Bone marrow chimeras
For generation of mixed bone marrow chimeras we irradiated 10-week-old male CD45 congenic C57BL/6J Ly5.1 mice (CD45.1) by whole-body irradiation (9 Gy; 1 Gy min\(^{-1}\)) using a cesium-137 gamma irradiator (BIOBEAM 2000, Leipzig, Germany). After 24 h we reconstituted them with bone marrow cells derived from tibiae and femurs from Trpm2\(^{-/-}\) (CD45.2) and WT (CD45.1) mice. Every recipient received 1 × 10\(^7\) bone marrow cells intravenously; in mixed bone marrow experiments 5 × 10\(^6\) from each donor. We assessed reconstitution and distribution between CD45.1 and CD45.2 cells by FACS analysis of peripheral blood cells of recipient mice six weeks after grafting. We induced tMCAO in recipient mice six weeks after transplantation. For generation of bone marrow chimeras in Trpm2\(^{-/-}\) or WT controls with either WT or Trpm2\(^{-/-}\) bone marrow respectively 10-week-old male recipients were irradiated, analyzed and stroked as described above.

Antibodies and flow cytometry
Mouse antibodies were as follows (all from eBioscience): CD3 (145-2C11), CD4 (GK1.5), CD8a (53-6.7), TCR-\(\gamma\delta\) (GL-3), NK1.1 (PK136), CD45 (30-F11), CD45.1 (A20), CD45.2 (104), IL-17A (17B7), IFN-\(\gamma\) (XMG1.2), TNF-\(\alpha\) (MP6-XT22), Ly6G (1A8), CD11c (N418), CD11b (M170). For intracellular cytokine staining animals were euthanized and perfused with phosphate-buffered saline. Only ipsilesional hemispheres were dissected, digested for 30 min at 37°C (1 mg/ml collagenase, 0.1 mg/ml DNAse I in DMEM), and pressed through a cell strainer. Cells were incubated with standard erythrocyte lysis buffer on ice and separated from myelin and debris by Percoll gradient (GE Healthcare) centrifugation. T cells were stimulated with phorbol 12 myristate 13-acetate (PMA) (100ng/ml; Sigma) and ionomycin (1\(\mu\)g/ml; Sigma) and in the presence of brefeldin A (3\(\mu\)g/ml; eBioscience) for 4 hours. After staining of surface markers, cells were fixed, permeabilized using (IC) Fixation Buffer in conjunction with Permeabilization Buffer (eBioscience) and stained with anti-cytokine antibodies and the corresponding isotype controls. For intracellular staining of monocytes, neutrophils and microglia, intracellular transport was blocked with brefeldin A (3\(\mu\)g/ml; eBioscience) for 3 hours. After staining of surface markers cells were fixed, permeabilized and stained for intracellular cytokines using (IC) Fixation Buffer.
Buffer in conjunction with Permeabilization Buffer (eBioscience). For absolute quantification, TrueCount tubes (Becton Dickinson) containing fluorescence beads were used according to the manufacturer's protocol and 10% of the cell volume was counted. Data were acquired with a LSR II FACS system (BD Biosciences) and analyzed with FlowJo (TreeStar). Doublets were excluded with FSC-A and FSC-H linearity.

**RNA isolation and quantitative real time PCR**

Total RNA from microglia and macrophages derived by cell sorting from ischemic hemispheres was isolated using QIA-Shredder spin columns and the RNeasy Micro Kit (QiAGEN). Complementary DNA was transcribed using Maxima First Strand cDNA Synthesis Kit for RT-qPCR (Fermentas). To isolate RNA from brain tissue, mice were killed with isoflurane at indicated time points after stroke induction or 24 hours after whole body irradiation (9 Gy; 1 Gy min⁻¹). Hemispheres were separated and homogenized in TRIzol Reagent (1 ml per 100 mg tissue), chlorophorm was added, samples were centrifuged at 12000 g for 15 min at 4 °C and the upper aqueous phase was collected. RNA was precipitated by addition of isopropyl alcohol, washed and dissolved in TE-Buffer.

For quantitative real-time PCR we used the following TaqMan Gene Expression Assays (Applied Biosystems): (Actinb Mm00607939_s1; CxCL1 Mm00433859_m1; CCL-2 Mm00441242_m1; CXCL2 Mm00436450_m1; IL-6 Mm00446190_m1; G-CSF Mm00438334_m1; TATA box binding protein Mm00446971_m1). Probe mixtures were purchased from Applied Biosystems and Fermentas (Waltham, MA). The qRT-PCR analysis was performed on a StepOne or 7900HT Fast Real-Time PCR System (Applied Biosystems). The relative gene expression was calculated using the ΔΔCt method, the samples were normalized to the expression of β-actin or TATA box binding protein, and the untreated sample was used as a calibrator.

**Neuronal cell culture**

Neuronal cell cultures were obtained from mice embryos (embryonic day 18) following previously described protocols². Pregnant mice were killed by cervical dislocation, and embryos were removed and transferred into warmed HBSS (Invitrogen). After preparation of hippocampi, tissue was collected in 5 ml of 0.25% trypsin in HBSS. After 5 min of incubation at 37°C, tissue was washed two times with HBSS and dissociated in 1 ml of neuronal medium [10% 10× modified Earl's medium (MEM), 0.2205% sodium bicarbonate, 1 mm sodium pyruvate, 2 mm l-glutamine, 2% B27 supplement (all from Invitrogen), 3.8 mm glucose (Merck), and 1% penicillin/streptomycin (Biochrom)] by triturating with fire polished Pasteur pipettes of decreasing tip diameter. Neurons were diluted in neuronal medium and plated at a density of 60 000 cells/cm² on poly-d-lysine (Sigma)-coated coverslips in four-well plates (Nunc). All cell cultures were incubated at 37.0°C and 5% CO2 and held in culture for up to 5–7 d before experiments. Immunocytochemical staining revealed a purity of about 80% of the neuronal cell culture system. We performed immunocytochemical staining as previously described using antibodies against Trpm2 (1:200, Novus Biologicals, Littleton, CO), MAP2a/b (1:200, Abcam, Milton, England), and CD11b (1:200, Novus Biologicals). For Trpm2 mRNA expression analysis in hippocampal and cortical cell cultures, respective brain regions were prepared from C57Bl/6J mouse embryos at Theiler stage 24 as described previously³. Before RNA isolation the cells were incubated with H₂O₂ (cortex cells; 7 DIC; 200 μM for 1 hour; Sigma-Aldrich) or IL-1β (hippocampal neurons; 10 DIC; 2 ng/mL for 24 hours; NM_010554, BioLegend) or TNF-α (hippocampal neurons; 10 DIC; 10 ng/mL for 24 hours; MP6-XT22, BioLegend).
Microglia cell culture
For preparation of mixed glial cultures, the cerebrum from post-natal days 1 - 5 WT and Trpm2 deficient mice was stripped of meninges. The cortices were mechanically dissociated as described previously (Hucke et al., 2011), seeded into culture flasks in Dulbecco's Modified Eagle's Medium supplemented with 10% fetal calf serum, 50 μM β-mercaptoethanol, 2 mM L-glutamine, 1% non-essential amino acids, 10⁵ U penicillin and 0.1 g/l streptomycin. After 2 weeks, microglial cells were harvested by vigorously tapping the flasks.

Brain slice preparation
We prepared acute coronal brain slices from 6 to 10-week-old Trpm2 deficient mice and their WT littermates as previously described. In brief, we cut coronal sections on a vibratome (Vibratome, Series 1000 Classic) in an ice-cold solution containing 200 mM sucrose, 20 mM PIPES, 2.5 mM KCl, 1.25 mM NaH₂PO₄, 10 mM MgSO₄, 0.5 mM CaCl₂, and 10 mM glucose; we adjusted pH to 7.35 with NaOH. After sectioning, we immediately transferred slices into each well of a 12-well plate filled with modified artificial cerebrospinal fluid (ACSF) containing 125 mM NaCl, 2.5 mM KCl, 1.25 mM NaH₂PO₄, 30 mM HEPES, 2 mM MgSO₄, 2 mM CaCl₂, and 10 mM glucose; we adjusted pH with HCl. We bubbled solutions with a mixture of 95% O₂ and 5% CO₂ for at least 60 min before experimentation. For normal in vitro conditions, we incubated brain slice cultures for 6 hours in normal modified ACSF in an incubator containing an atmosphere of 95% O₂ and 5% CO₂. To induce in vitro ischemic conditions, we incubated brain slice cultures for 6 hours in modified ACSF with reduced pH of 6.4 and lowered glucose concentration of 5 mM in an incubator containing an atmosphere of 95% N₂ and 5% CO₂ during the experiments.

Immunocytochemistry
Immunocytochemical staining was performed on cultured hippocampal neurons and microglia cells as described. Cells were placed on coverslips coated with poly-L-lysine (Sigma, Germany) and fixed with 4% paraformaldehyde after experimentation. Subsequently, cells were blocked with PBS containing 10% horse serum (PAA Laboratories), 2% bovine serum albumin, and 0.3% Triton X-100 overnight. Next, primary antibodies (rabbit anti-mouse Trpm2, 1:200, Novus Biologicals; mouse anti-mouse MAP2a/b, 1:200, (Abcam); mouse anti-mouse CD11b (1:200, Novus Biologicals) were added and incubated for overnight. Cells were washed with PBS containing 0.3% Triton X-100 and incubated with secondary antibodies (Cy3- or AlexaFluor488-conjugated goat anti-rabbit IgG, 1:800, Dianova; AlexaFluor488- or Cy3-conjugated goat anti-mouse IgG, 1:100; Bioscience) for 1 h, respectively. Counterstaining of cell nuclei was performed using DAPI (0.5 μg/ml, Merck). Finally, cultures were washed and then covered with DABCO (1,4-diazabicyclo-[2,2,2]-octane, Merck). Images were collected by immunofluorescence microscopy (Axiophot, Zeiss). Negative controls without the primary antibody revealed no positive signals (data not shown).

Immunohistochemistry
For histological analysis of mouse brains, animals were perfused with 4% buffered formalin. Brains were embedded in paraffin and 3 μm thick brain sections were stained following standard immunohistochemistry procedures using the Ventana Benchmark XT machine (Ventana, Tucson, Arizona) with antibodies against GFAP (1:200, DAKO) and Iba-1 (1:200, Wako) followed by detection with anti-rabbit histofine Simple Stain MAX PO Universal immuno-peroxidase polymer (Iba-1, Nichirei Biosciences, Tokyo, Japan) or Mouse Stain Kit (GFAP, Nichirei). Sections were chosen in distance of about 2.5 to 23.5 mm from bregma. Three picture of the penumbra region, the ipsilateral periphery of the infarct core and the contralateral hemisphere of each individual were taken and cells were counted from a blinded
experimenter to reduce the risk of counting being biased. Visualization of secondary antibodies was performed using the "Ultra View Universal DAB Detection Kit" from Ventana. For immunostaining of neutrophils, Ly-6G clone 1A8 (1:1000, Biolegend) was used overnight at 4°C following antigen retrieval in 10 mM citrate buffer (pH 6.0). Antibody detection was performed using anti-rat histofine immuno-peroxidase polymer (Nichirei), which was visualized using diaminobenzidine (DAB) (Sigma-Aldrich). Counterstaining was performed with Mayer’s haematoxylin solution.

Immunofluorescence was performed on cryopreserved coronal brain sections of WT and Trpm2 deficient mice from in vitro brain slice experiments (after 6 h) or from identical brain regions (−0.5 mm from bregma) following in vivo stroke experiments (24 h after tMCAO). Ipsi- and contralateral brain hemispheres were analyzed separately. Sections were post-fixated in 4% paraformaldehyde for 10 min and incubated in blocking solution (PBS containing 5% BSA, 1% normal goat serum, and 0.2% Triton X-100). Slices were then incubated simultaneously or consecutively with primary antibodies for 7 hours at 4°C. Afterwards, sections were washed with PBS and incubated with secondary antibodies for 1 hour. Apoptotic neurons were visualized using NeuN/cleaved caspase 3 double-staining (mouse anti-mouse NeuN, 1:1000, (Chemicon) detected with AlexaFluor488-conjugated goat anti-mouse, 1:100, (Bioscience); rabbit anti-mouse cleaved caspase 3, 1:400, (CellSignal) detected with Cy3-conjugated goat anti-rabbit IgG, 1:800, (Dianova)). The M1/M2 differentiation state of microglia and macrophages was visualized using Iba-1/CD16/32 double-staining (rabbit anti-mouse Iba-1, 1:100, (Wako) detected with AlexaFluor488-conjugated donkey anti-rabbit, 1:100, (BD Bioscience); rat anti-mouse CD16/32, 1:100 (BD Bioscience) detected with Cy3-conjugated donkey anti-rat 1:100, (Dianova)) together with Iba-1/CD206 double-staining (rabbit anti-mouse Iba-1, 1:100, (Wako) detected with AlexaFluor488-conjugated donkey anti-rabbit, 1:100, (BD Bioscience); goat anti-mouse CD206, 1:100, (R&D) detected with Cy3-conjugated donkey anti-goat, 1:100, (Dianova)). Counterstaining of cell nuclei was performed using DAPI (0.5 µg/ml, Merck). Finally, cultures were washed and then covered with DABCO (1,4-diazabicyclo-[2,2,2]-octane, Merck). Negative controls without the primary antibody revealed no positive signals (data not shown). Images were collected by immunofluorescence microscopy (Axiophot, Zeiss). Densities of respective cell populations were determined in 10 preselected visual fields per hemisphere within the neocortex including the penumbra region, the ipsilateral periphery of the infarct core and the contralateral hemisphere.
Supplemental Figure I

<table>
<thead>
<tr>
<th>Physiological Measurements</th>
<th>WT</th>
<th>Trpm2&lt;sup&gt;−/−&lt;/sup&gt;</th>
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<tr>
<td>Before Isoflurane treatment</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heart rate beats/min</td>
<td>484.7 ± 21.68 N=3</td>
<td>513.3 ± 20.85 N=3</td>
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<td>SpO2, %</td>
<td>94.33 ± 1.856 N=3</td>
<td>93.67 ± 0.8819 N=3</td>
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<tr>
<td>Meas RR (Respiration rate), bpm</td>
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<td>Temperature</td>
<td>36.76 ± 0.2338 N=3</td>
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<td>During Isoflurane treatment</td>
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<td>Heart rate beats/min</td>
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<td>434.3 ± 19.22 N=3</td>
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<td>71.00 ± 4.583 N=3</td>
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<td>Temperature</td>
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<td>30 mins after Isoflurane treatment</td>
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<td>Heart rate beats/min</td>
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<td>511.0 ± 29.19 N=3</td>
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<tr>
<td>SpO2, %</td>
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<td>Meas RR (Respiration rate), bpm</td>
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<td>Temperature</td>
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<td>36.40 ± 0.4041 N=3</td>
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Suppl. Fig. I. Physiological measurements before, during and after isoflurane treatment did not differ between genotypes (n=3). Data are shown as mean values ± SD. Statistical analyses were performed by Student’s t-test. P-values ≤ 0.05 were considered significant.
Suppl. Fig. II. Trpm2 gene expression is not regulated in CNS invading macrophages and microglia during stroke and in challenged neurons in vitro. (A) Relative mRNA expression of Trpm2 in macrophages and microglia from stroked and sham operated brains. RNA was obtained from macrophages and microglia previously purified by cell sorting from ischemic hemispheres 3 days after tMCAO (n = 6 per group). (B) Relative mRNA expression of Trpm2 in irradiated cortices (n = 4) compared to non-irradiated controls (n = 3). (C) Relative mRNA expression of Trpm2 in cultured cortical neurons 1 hour after exposure to H2O2 compared to untreated control cells (n = 3). (D) Relative mRNA expression of Trpm2 in cultured hippocampal neurons after exposure to IL-1β or TNF-α compared to untreated control cells (n = 3). Data are shown as mean values ± SD. Statistical analyses were performed by Student’s t-test. P-values ≤ 0.05 were considered significant.
Suppl. Fig. III. Frequency and distribution of immune cells in ischemic hemispheres and the blood did not differ between genotypes. (A) Frequency of CD4+, CD8+, γδ T lymphocytes and NK cells in ischemic hemispheres at day three after tMCAO. T cell subsets and NK cells were stained for CD45, CD3, NK1.1, CD4, CD8, γδ T cell receptor. Data were obtained by flow cytometry analysis of CNS-infiltrating cells. (B) Percentages of neutrophils, macrophages (MΦ) and DC of blood myeloid cells from Trpm2−/− and WT littermate control mice analyzed by flow cytometry 3 days following tMCAO. Neutrophils were identified as Ly6G+, CD11b+; macrophages as Ly6G−, CD11b+, CD11c−; DC as Ly6G−, CD11b+, CD11c+. The graphs show means ± SD of 9-12 animals per group analyzed three days after tMCAO in three independent experiments. (C) Absolute numbers of microglia (CD11b+, CD45intermediate) in hemispheres of Trpm2−/− mice and WT littermate controls one day after sham surgery (n = 3). (D) Histopathological stainings and cell counts in the periphery of the infarct and contralateral hemisphere for macrophages/microglia (Iba-1) in WT and Trpm2−/− mice three days after tMCAO. Scale bar = 50 μm. Data are shown as mean values ± SD. Statistical analyses were performed by Student’s t-test. P-values ≤ 0.05 were considered significant.
Suppl. Fig. IV. Stroke related cytokine expression is not changed by TRPM2. Flow cytometry analysis of IL-17A (A) and IFN-γ (B) produced by NK cells, CD4+, CD8+ and γδ T cells. The graphs show means ± SD of 9-12 animals per group analyzed three days after tMCAO in three independent experiments. (C) Frequency of TNF-α producing neutrophils, macrophages (Mφ), DC and microglia. Brain macrophages were identified as CD45high, CD11b+, CD11c− and distinguished from microglia by the higher expression of CD45. The graphs show means ± SD of 9-12 animals per group analyzed three days after tMCAO in three independent experiments. (D) Coronary brain sections from identical brain regions obtained from Trpm2−/− and littermate control mice 24 h after tMCAO were analysed for CD16/32+ and CD206+ Iba-1+ macrophages/microglia respectively within the neocortex of ipsilateral (representative images) ischemic lesions (n = 3 mice per group). Scale bar = 50 μm. Statistical analyses were performed by Student’s t-test. P-values ≤ 0.05 were considered significant.
Supplemental References:


