Supplementary Materials

Supplementary Materials and Methods

Induction of cerebral ischemia

Three different experimental models of focal ischemia were used: (1) an intracortical injection of the powerful vasoconstrictor peptide endothelin-1 (ET-1); (2) a permanent occlusion by cauterization of a distal segment of the middle cerebral artery (MCA); or (3) the standard transient MCA occlusion using an intraluminal suture followed by reperfusion. For ET-1-induced focal cerebral ischemia, rats were anesthetized with a mixture of ketamine and xylazine (100 and 10 mg/kg, i.p.) and placed in a stereotaxic apparatus (David Kopf Instruments, Tujunga, CA). Body temperature was maintained at 37°C during the procedure by using a feedback-controlled heating pad. Eye ointment was applied to prevent corneal dryness. After a midline scalp incision, a small burr hole was drilled under constant saline cooling and 2 μL of ET-1 (1 mg/mL; EMD Millipore, Billerica, MA) or vehicle (sterile saline) was injected using a 10 μL syringe with 35-gauge needle (World Precision Instruments, Sarasota, FL) connected to a micropump (0.2 μL/min injection rate; KdScientific, Holliston, MA). The following stereotaxic coordinates were targeted based on a rat brain atlas (AP 0 mm, ML +4.0 mm, and DV -1.5 mm)[1]. After the injection, the needle was left in place for 5 min to avoid backflow. The burr hole was covered with bone wax and the incision was sutured.

Permanent focal cerebral ischemia was induced by cauterizing a distal branch of the MCA as described previously[2]. Briefly, the animals were placed in the lateral position, and a vertical skin incision was made at the midpoint between the left orbit and the external auditory canal. A small burr hole was made with a micro drill through the outer surface of the skull at the junction between the medial wall and the roof of the inferotemporal fossa. The dura was opened to expose the MCA and the MCA was occluded between the inferior cerebral vein and the lateral olfactory tract by bipolar electrocoagulator (ERBE USA, Atlanta, GA).

Transient focal cerebral ischemia was induced using the intraluminal filament model of MCA occlusion (tMCAO), as described previously [3]. Briefly, animals anesthetized with isoflurane (5% induction and 2% maintenance in a mixture of N_2O/O_2 (70/30%). A 4-0 silicone-coated nylon suture (Doccol, CA) was introduced in the external carotid artery and advanced through the internal carotid artery to occlude the MCA. During 60 min occlusion time, rats were allowed to recover from anesthesia in a controlled heating box. Afterwards, the animals were re-anesthetized with the same combination and the suture was retracted to allow reperfusion. Physiological parameters were monitored with SurgiVet® capnograph (model V90041; Smiths Medical, Waukesha, WI): heart rate was maintained around 350 beats/min and blood oxygen level (SpO_2) was kept >95%. The body core temperature was monitored during surgery with a rectal probe and maintained at 37 ± 0.5°C by a feedback-controlled heating device (model TC-1000; CWE, Ardmore, PA). Laser Doppler flowmetry (Moor Instruments, Wilmington, DE) was used to monitor regional cerebral blood flow (CBF) in the MCA territory. CBF was measured serially at baseline (before ischemia), immediately after insertion of the suture (ischemia), immediately after removal of the occluding suture (reperfusion).

Pharmacological treatments

Rats were randomly assigned to one of eight groups: saline-vehicle, stroke-vehicle, stroke-JZL184, stroke-MJN110, stroke-AM251-vehicle, stroke-AM251-JZL184, stroke-AM630-vehicle,

and stroke-AM630-JZL184. JZL184 (2,5-dioxopyrrolidin-1-yl 4-(bis(4-chlorophenyl)methyl)piperazine-1-carboxylate) (Cayman Chemical; 16 mg/kg) or MJN110 (4-[Bis(1,3-benzodioxol-5-yl)hydroxymethyl]-1-piperidinecarboxylic acid 4-nitrophenyl ester) (Sigma-Aldrich; 20 mg/kg) were initially dissolved in dimethyl sulfoxide, further diluted in sterile saline with 40% polyethylene glycol and 10% Tween-80 and administered i.p. as a post-ischemic treatment (1 h after stroke induction) in the presence or absence of the cannabinoid receptor antagonists. The CB1 receptor antagonist AM251 (Cayman Chemical; 1 mg/kg) and the CB2 receptor antagonist AM630 (Cayman Chemical; 1 mg/kg) were administered i.p. 30 min after stroke. Routes and dose of administration for each compound were chosen based on previous studies[4-6].

Magnetic resonance imaging (MRI)

Magnetic resonance imaging (MRI) was performed 24 h and again 7 d after induction of stroke on a 7 T/30 cm AVIII MRI system (Bruker Biospin Inc., Billerica, MA) under inhalation anesthesia as described previously[7]. We used a home-built transmit-only birdcage volume RF coil (11 cm internal diameter) and a home-built receive-only surface coil designed for examining rats. The whole-brain imaging protocol comprised a multi-slice coronal T2-weighted sequence with the following acquisition parameters were as follows: TR/TE = 12000/74 ms, FOV = 25.6 × 25.6 mm^2, matrix = 128 × 128, slice thickness = 1 mm. Infarct volumes were calculated by planimetry of the hyperintense area on T2-weighted MR images using NIH ImageJ software. The extent of brain swelling was determined by calculating the percentage increase of the ipsilateral (V_i)/contralateral (V_c) hemisphere area: % brain swelling = $[(V_i-V_c)/V_c]\times100$.

Assessment of functional outcome

Neurological deficits were scored and quantified on day 1 after MCAO as described previously[8]: 1) postural reflexes (0, no deficit; 1, contralateral forelimb flexion; 2, decreased resistance to lateral push); 2) proprioceptive placement of contralateral forelimb and hindlimb (0, complete immediate; 1, incomplete or delayed (< 2 s); 2, absent); 3) unidirectional circling (0, absent; 2, present). After ET-1-induced stroke, animals were examined by using the adhesive removal test to assess sensorimotor function in the forelimb[9, 10] and the cylinder test to quantify the forelimb asymmetry during spontaneous exploratory activity[11]. For the adhesive removal test, two adhesive tapes (1 × 1 cm) were applied with equal pressure on each forepaw of the animal and measured the time until contact with the mouse (time-to-contact) and the time-to-remove them up to a maximum of 60 sec. Five trials per testing day were performed and the order of placement of the adhesive tape (contralateral or ipsilateral) was alternated between each animal and each trial. The animals were trained daily before surgery to decrease inter-individual differences. For the cylinder test, the animals were placed in a transparent cylinder (20 cm diameter and 30 cm height) and the number of times each forelimb placement on the cylinder wall during exploration was recorded. The percentage of contralateral forelimb use was calculated using the equation contralateral contacts ÷ (contralateral + ipsilateral contacts) × 100. This value was compared to the theoretical value of 50% whereby both forelimbs are used equally.

Histology and immunohistochemistry

Animals were deeply anesthetized and transcardially perfused first with saline and then with fixative solution (4% paraformaldehyde in 0.1 M phosphate buffer). Brains were removed and immersed overnight at 4°C in the same fixative solution, and cryoprotected in 15 and 30% sucrose. Coronal sections (30-μm in thickness) were obtained using a cryostat (CM1850, Leica Microsystems, Wetzlar, Germany). For Nissl staining, sections (distance 180 μm) were immediately mounted onto slides and stained with 0.1% thionin or cresyl violet (Electron Microscopy Sciences, Hatfield, PA). For immunofluorescence staining, free-floating sections were blocked for 1 h in 0.2% Triton X-100 and 5% donkey serum in PBS to prevent unspecific binding. A mouse anti-NeuN antibody (EMD Millipore, Temecula, CA), a rabbit anti-active caspase-3 antibody (BD Biosciences, San Jose, CA), a rabbit anti-ionized calcium-binding adaptor molecule 1 antibody (Iba-1; Wako Chemicals, Richmond, VA), or a mouse anti-glial fibrillary acidic protein antibody (GFAP; Sigma-Aldrich) was applied overnight at 4°C in 1% donkey serum in PBS. Sections were then incubated for 1 h with Alexa Fluor 488 and Alexa Fluor 546-labeled secondary antibodies (ThermoFisher Scientific, Waltham, MA) at dilutions of 1:1000 in 1% donkey serum in PBS. Afterwards, sections were mounted on slides in Immu-Mount (ThermoFisher Scientific) and images were acquired with the LSM 780 confocal microscope (Carl Zeiss, Gottingen, Germany). For quantification, identical brain sections at the level of the primary somatosensory cortex (1.0 mm anterior from bregma; distance 180 μm) were selected and the number of positive cells within the ischemic hemisphere was counted from different animals. For Iba-1 expression, the intensity of fluorescence was calculated from the acquired images at the level of the primary somatosensory cortex (-1.0 mm from bregma). For 2,3,5-triphenyltetrazolium chloride (TTC) staining, brains were quickly removed and cut in 2-mm thick coronal slices using a rat brain matrix (Stoelting). The slices were immersed in 2% TTC (Sigma-Aldrich) in PBS for 20 min at 37°C to visualize the infarcts.

Quantification of endocannabinoids by liquid chromatography-tandem mass spectrometry

2-AG and AEA levels were quantified from the ipsilateral cortex of SHR after 24 h after JZL184 treatment or vehicle. The brains were rapidly harvested, snap-frozen in dry ice, and stored at -80°C until the time of processing. Tissues were further processed according to methods described previously [12].

Profiling of local cytokine expression

Cortices were taken from the ischemic hemisphere, accurately weighted, snap frozen, and stored at -80°C. Frozen brain samples were homogenized with a glass homogenizer and then centrifuged at 12,000g for 20 min at 4°C. The supernatant was then collected and total protein was determined by the protein assay kit (Bio-Rad, Hercules, CA). Profiling of cytokine expression was performed using the rat cytokine ELISA plate array kit (Signosis, Santa Clara, CA). Briefly, each sample was incubated with ELISA plate, followed by biotinylated antibodies, and further incubated with horseradish peroxidase-conjugated streptavidin and then developed with TMB substrate. Absorbance was read at 450 nm using Infinite F200 Pro (Tecan, Morrisville, NC).

Quantitative real-time PCR

Cortices were dissected from the rat brains and total RNA was prepared with a tissue homogenizer using the RNeasy Lipid Tissue Mini Kit (Qiagen, Valencia, CA). Then, total RNA was reversely transcribed with the TaqMan® Reverse Transcription reagents (Applied

Biosystems, Carlsbad, CA) according to the manufacturer's protocol. Relative gene expression levels of cannabinoid receptor 1 (CB1R) (assay ID: Rn02758689_s1), and cannabinoid receptor 2 (CB2R) (assay ID: Rn01637601_m1), were quantified with the fluorescent TaqMan® technology (Applied Biosystems). Beta-2 microglobulin (B2m) (assay ID: Rn00560865_m1) was used as an endogenous control to normalize the amount of sample. The PCR was performed with equal amounts of cDNA in the QuantStuio 3 Real-Time PCR System (ThermoFisher Scientific) using the TaqMan® Gene Expression Master Mix (Applied Biosystems). The comparative Ct method was used for relative quantification of gene expression.

Statistics

For statistical analysis, the Prism 7 software (GraphPad, La Jolla, CA) was used. All values are expressed as mean values ± SEM. Data were analyzed by unpaired, two-tailed *t* test or by analysis of variance (ANOVA) followed by Bonferroni's multiple comparisons test for *P* values. P values < 0.05 were considered statistically significant.

Supplementary References

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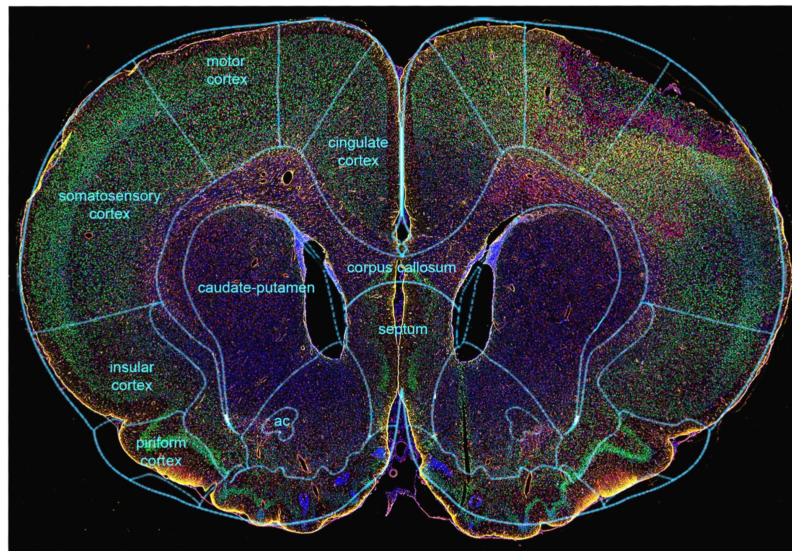
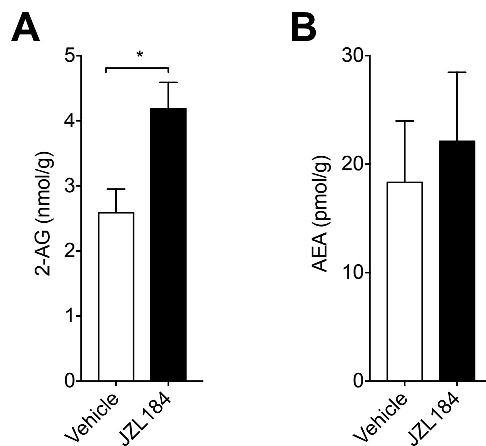
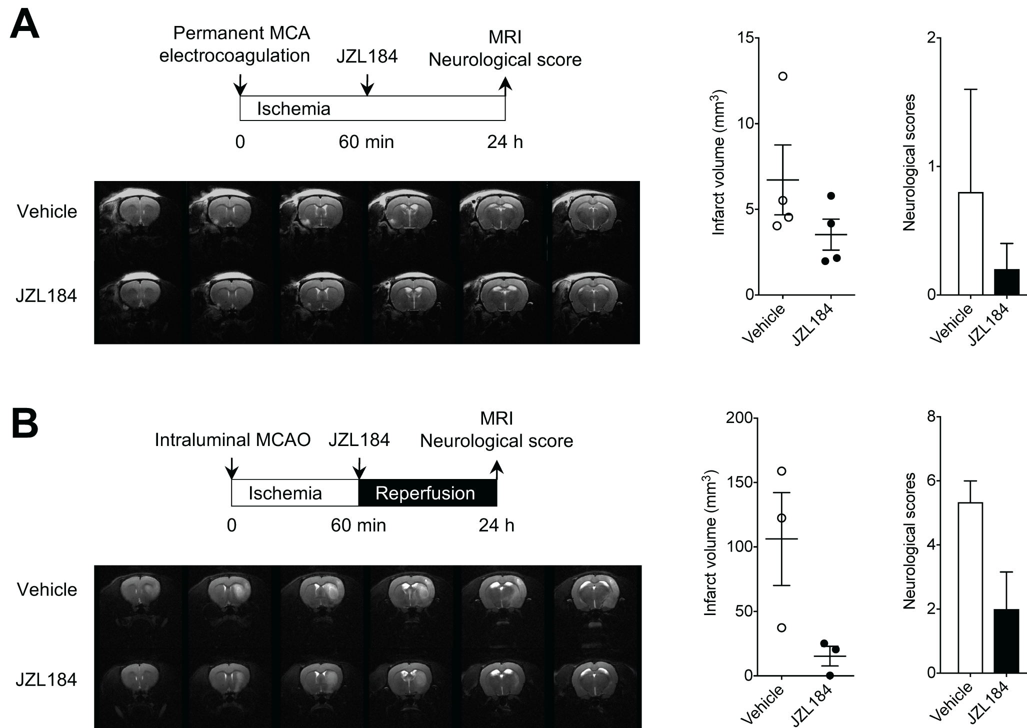
Supplementary Figures



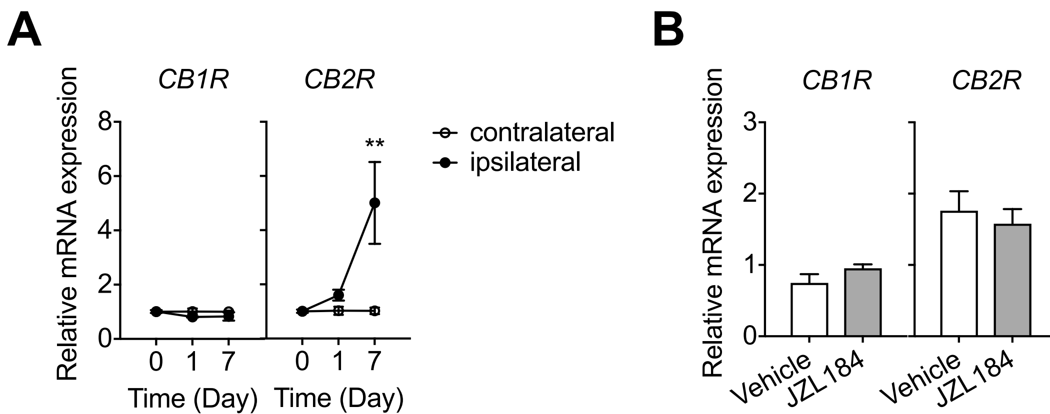
Supplementary Figure I. Representative histological marker expression 72 h after ET-1-induced focal ischemia. Immunofluorescent labeling of neurons (NeuN, green), astrocytes (GFAP, yellow), microglia (Iba-1, red), and nuclei (DAPI, blue).



Supplementary Figure II. JZL184 produced significant increases in 2-AG levels. Levels of 2-AG (A) and AEA (B) measured in the brain of SHR 24h following ischemic stroke. SHR were treated with JZL184 (16 mg/kg, i.p.) or vehicle 60 min following stroke induction. LC-MS/MS was used to quantify 2-AG and AEA for each sample. Data are expressed as mean ± SEM (n = 3). $^{*}P < 0.05$ versus vehicle, unpaired t-test.



Supplementary Figure III. Lack of response of WKY to treatment with the MAGL inhibitor JZL184 after two different models of ischemic stroke. **A**, Basic experimental scheme of a permanent focal ischemia model consisting of electrocoagulation of a distal branch of the MCA. JZL184 (16 mg/kg) or vehicle was administered i.p. 1 h after induction of ischemia. Infarct volume and neurological scores were evaluated 24 h after stroke. Representative T2-weighted MR images show only a small ischemic lesion in the cortex of both vehicle- and JZL184-treated WKY. Differences in infarct volume and neurological deficits between vehicle- and JZL184-treated WKY were not statistically significant. Data are expressed as mean ± SEM (n = 4/group). **B**, Basic experimental scheme of tMCAO. The MCA was occluded for 60 min, after which the intraluminal suture was removed to allow reperfusion. JZL184 (16 mg/kg, i.p.) or vehicle was administered immediately upon reperfusion and infarct volume and neurological scores were evaluated 24 h after stroke. Differences in infarct volume and neurological deficits between vehicle- and JZL184-treated WKY were not statistically significant. Data are expressed as mean ± SEM (n = 4 and 5/group).



Supplementary Figure IV. CB1R and CB2R expression after ET-1-induced stroke. (A) Time-dependent changes of CB1R and CB2R mRNA expression in the ischemic cortex. mRNA expression in the ischemic cortex (ipsilateral) was normalized to the non-ischemic cortex (contralateral). (B) Effects of the JZL184 on CB1R and CB2R mRNA expression 24 h after stroke (n = 3). Data are expressed as mean ± SEM. $^{**}P < 0.01$ versus contralateral. Two-way ANOVA followed by Bonferroni's multiple comparisons test.

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Table I. Checklist of Methodological and Reporting Aspects for Articles Submitted to *Stroke* Involving Preclinical Experimentation

Methodological and Reporting Aspects	Description of Procedures
Experimental groups and study timeline	☑ The experimental group(s) have been clearly defined in the article, including number of animals in each experimental arm of the study. ☑ An account of the control group is provided, and number of animals in the control group has been reported. If no controls were used, the rationale has been stated. ☑ An overall study timeline is provided.
Inclusion and exclusion criteria	☑ A priori inclusion and exclusion criteria for tested animals were defined and have been reported in the article.
Randomization	☑ Animals were randomly assigned to the experimental groups. If the work being submitted does not contain multiple experimental groups, or if random assignment was not used, adequate explanations have been provided. ☑ Type and methods of randomization have been described. ☑ Methods used for allocation concealment have been reported.
Blinding	☑ Blinding procedures have been described with regard to masking of group/treatment assignment from the experimenter. The rationale for nonblinding of the experimenter has been provided, if such was not feasible. ☑ Blinding procedures have been described with regard to masking of group assignment during outcome assessment.
Sample size and power calculations	☑ Formal sample size and power calculations were conducted based on a priori determined outcome(s) and treatment effect, and the data have been reported. A formal size assessment was not conducted and a rationale has been provided.
Data reporting and statistical methods	☑ Number of animals in each group: randomized, tested, lost to follow-up, or died have been reported. If the experimentation involves repeated measurements, the number of animals assessed at each time point is provided, for all experimental groups. ☑ Baseline data on assessed outcome(s) for all experimental groups have been reported. ☑ Details on important adverse events and death of animals during the course of experimentation have been provided, for all experimental arms. ☑ Statistical methods used have been reported. ☑ Numeric data on outcomes have been provided in text, or in a tabular format with the main article or as supplementary tables, in addition to the figures.
Experimental details, ethics, and funding statements	☑ Details on experimentation including stroke model, formulation and dosage of therapeutic agent, site and route of administration, use of anesthesia and analgesia, temperature control during experimentation, and postprocedural monitoring have been described. ☐ Different sex animals have been used. If not, the reason/justification is provided. ☑ Statements on approval by ethics boards and ethical conduct of studies have been provided. ☑ Statements on funding and conflicts of interests have been provided.

Only male rats were used in this study due to potential effects of sex hormones. Future studies should be conducted in female rodents subjected to ischemic stroke.