Blocking Stroke-Induced Immunodeficiency Increases CNS Antigen-Specific Autoreactivity But Does Not Worsen Functional Outcome after Experimental Stroke

Christine Römer,^{1,2} Odilo Engel,^{1,2} Katarzyna Winek,^{1,2} Sonja Hochmeister,⁷ Tian Zhang,^{1,2} Georg Royl,^{3,8} Juliane Klehmet,^{3,5} Ulrich Dirnagl,^{1,2,3,5,6} Christian Meisel,⁴ and Andreas Meisel^{1,2,3,5}

¹Center for Stroke Research, ²Department for Experimental Neurology, ³Department of Neurology, ⁴Institute of Medical Immunology, and ⁵NeuroCure Clinical Research Center, Charité Universitätsmedizin Berlin, 10098 Berlin, Germany, ⁶German Center for Neurodegenerative Diseases, 10117 Berlin, Germany, ⁷Department of Neurology, Medical University of Graz, 8010 Graz, Austria, and ⁸Department of Neurology, University of Lübeck, 23538 Lübeck, Germany

Stroke-induced immunodepression (SIDS) is an essential cause of poststroke infections. Pharmacological inhibition of SIDS appears promising in preventing life-threatening infections in stroke patients. However, SIDS might represent an adaptive mechanism preventing autoreactive immune responses after stroke. To address this, we used myelin oligodendrocyte glycoprotein (MOG) T-cell receptor transgenic (2D2) mice where >80% of peripheral CD4⁺ T cells express a functional receptor for MOG. We investigated in a murine model of middle cerebral artery occlusion the effect of blocking SIDS by inhibiting body's main stress axes, the sympathetic nervous system (SNS) with propranolol and the hypothalamic-pituitary-adrenal axis (HPA) with mifepristone. Blockade of both stress axes robustly reduced infarct volumes, decreased infection rate, and increased long-term survival of 2D2 and C57BL/6J wild-type mice. Despite these protective effects, blockade of SIDS increased CNS antigen-specific Type1 T helper cell (Th1) responses in the brains of 2D2 mice 14 d after middle cerebral artery occlusion. One month after experimental stroke, 2D2 mice developed signs of polyradiculitis, which were diminished by SIDS blockade. Adoptive transfer of CD4⁺ T cells, isolated from 2D2 mice, into lymphocyte-deficient *Rag-1KO* mice did not reveal differences between SIDS blockade and vehicle treatment in functional long-term outcome after stroke. In conclusion, inhibiting SIDS by pharmacological blockade of body's stress axes increases autoreactive CNS antigen-specific T-cell responses in the brain but does not worsen functional long-term outcome after experimental stroke, even in a mouse model where CNS antigen-specific autoreactive T-cell responses are boosted.

Key words: CNS autoreactivity; immunosuppression; mifepristone; murine stroke; propranolol; stroke-induced immunodeficiency syndrome

Introduction

Despite stroke being one of the major and increasing burdens to global health (Global Burden of Disease Study, 2012), therapeutic interventions in cerebral ischemia continue to be a challenge. Today, thrombolysis with tissue plasminogen activator is the only approved causal treatment but due to a narrow time window of 3 h (4.5 h in European countries), can only be applied in 1%–8.5% of patients (Millan and Davalos, 2006). In the acute course

of the disease, stroke patients often develop complications (Kumar et al., 2010), such as pneumonia (Emsley and Hopkins, 2008), increasing morbidity and mortality (Katzan et al., 2003; Finlayson et al., 2011; Koennecke et al., 2011; Westendorp et al., 2011; Rocco et al., 2013). Susceptibility to infection after stroke is facilitated by systemic immunodepression, described as stroke-induced immunodeficiency syndrome (SIDS) (Prass et al., 2003; Meisel et al., 2005; Chamorro et al., 2012). This crosstalk between the CNS and immune system is mediated by sympathetic nervous system (SNS), hypothalamic-pituitary-adrenal axis (HPA), and vagus nerve (Tracey, 2002; Meisel et al., 2005; Chamorro et al., 2007).

Recently, immunomodulatory therapies targeting SIDS have been identified to prevent harmful poststroke infections (Meisel and Meisel, 2008). However, immunodepression after acute CNS injury may represent an adaptive response limiting tissue damage and preventing detrimental immune responses against CNS antigens (Meisel and Meisel, 2011). Thus, stimulating immune system after stroke might boost inflammation in the ischemic brain, worsening functional outcome.

CNS trauma has been associated with increased autoreactivity for >80 years (Rivers et al., 1933). Disruption of the blood–brain

Received March 13, 2015; accepted April 3, 2015.

Author contributions: C.R., O.E., K.W., S.H., J.K., U.D., C.M., and A.M. designed research; C.R., O.E., K.W., S.H., and T.Z. performed research; G.R. and J.K. contributed unpublished reagents/analytic tools; C.R., K.W., T.Z., G.R., and A.M. analyzed data; C.R. and A.M. wrote the paper.

C.R. received NeuroCure Cluster of Excellence scholarship. The work was supported by the German Research Foundation (Exc 257; SFB TR43), the Federal Ministry of Education and Research (01 EO 08 01), and the European Community's Seventh Framework Programme (FP7/2007–2013; Grant Agreement 201024). We thank Dr. Rudolf Volkmer for providing MOG peptide; and Yvonne Amonet, Sabine Kolodziej, and Marco Foddis for expert technical assistance.

The authors declare no competing financial interests.

Correspondence should be addressed to Dr. Andreas Meisel, Department for Experimental Neurology (CC15), Charité Universitätsmedizin Berlin, Charitéplatz 1, 10117 Berlin, Germany. E-mail: andreas.meisel@charite.de.

DOI:10.1523/JNEUROSCI.1532-14.2015

Copyright © 2015 the authors 0270-6474/15/357777-18\$15.00/0

barrier and damage to brain parenchyma during stroke deliberates myelin and exposes brain antigens to peripheral immune cells (Wang et al., 1992; Kuchroo et al., 2002). The autonomic nervous system mitigates inflammation by reducing proinflammatory cytokine (IL-12, TNF- α , IFN- γ) production, dampening innate and adaptive immune responses and immune memory (Livnat et al., 1985; Whalen and Bankhurst, 1990; Tuosto et al., 1994; Sanders et al., 1997; Woiciechowsky et al., 1999; Vega et al., 2003; Meisel et al., 2005). In particular, SNS and HPA regulate adaptive immunity and might therefore mitigate detrimental autoimmune responses mediated by Th1 and Th17 cells (Ando et al., 1989; Cua et al., 2003; Langrish et al., 2005).

Only a few pioneering experimental studies have addressed autoreactive immune responses and long-term consequences after stroke (Gee et al., 2008; Gee et al., 2009). One of the likely targets for CNS antigen-specific autoreactivity is myelin oligodendrocyte glycoprotein (MOG) in the outer layer of myelin (Brunner et al., 1989) capable of inducing pathogenic B- and T-cell responses with demyelination (Genain et al., 1996).

Here, we hypothesized that blocking SIDS enhances CNS antigen-specific autoreactivity and worsens functional outcome in a murine model of middle cerebral artery occlusion (MCAo). We used 2D2 mice in which the majority of peripheral CD4⁺ T cells express functional receptor for MOG (Bettelli et al., 2003) and prevented SIDS by blocking SNS and HPA mediated SIDS simultaneously with β -blocker propranolol and anti-glucocorticoid mifepristone (modified from Prass et al., 2003).

Materials and Methods

Animals and housing. Female C57BL/6J (Charles River Laboratories; RRID:IMSR_JAX:000664), 2D2 T-cell receptor (TCR) transgenic mice (strain name: C57BL/6-Tg(Tcr α 2D2,Tcr β 2D2)1Kuch/J; stock number 006912; The Jackson Laboratory; RRID:IMSR_JAX:006912), and gender-mixed *Rag-1*KO mice (strain name: B6.129S7-*Rag1*^{tm1Mom/J}; stock number 002216; The Jackson Laboratory; RRID:IMSR_JAX:002096) were used in the study. Mice were 9–16 weeks old when entering the study. Animal division into groups was randomized, and experiments were performed in a blinded manner. Mice were housed in Charité animal facility with a 12 h light/dark cycle (lights on from 7:00 until 19:00). Cages were lined with chip bedding, enriched with a mouse tunnel and igloo (Plexx BV). Mice had *ad libitum* access to food (standard chow) and water (where study design indicates, water was replaced with an antibiotic solution). All animal experiments were conducted in accordance with the European Community Council Directives 86/609/EEC and German national laws and approved by local authority (Landesamt für Gesundheit und Soziales, Berlin, Germany).

Drug administration. Preventive antibiotic (enrofloxacin; Baytril 2.5%) was administered to minimize interdependencies between infection and outcome parameters (Engel and Meisel, 2010; Hetze et al., 2013). Enrofloxacin was diluted in drinking water (0.35 mg/ml) and provided 24 h before MCAo surgery until 7 d after MCAo. Mice received additional 10 mg/kg of enrofloxacin intraperitoneally once a day for the first 3 d after MCAo. SNS and HPA (SNS/HPA) were blocked with propranolol and mifepristone, respectively (both from Sigma-Aldrich). Original injection scheme, drug preparation, and dosage for single treatments (Prass et al., 2003) were optimized for the current experiment. Mifepristone was dissolved in 100% ethanol (Rotipuran, Carl Roth) at 30 mg/ml on a heated water bath and diluted in sesame oil (Fluca Analytical, Sigma-Aldrich) until final concentration of 3 mg/ml. Mifepristone was injected intraperitoneally (20 mg/kg) 24 and 5 h before MCAo as well as at reperfusion (60 min after MCAo). Propranolol was dissolved in 0.9% NaCl (Fresenius Kabi Deutschland) at 3 mg/ml and administered intraperitoneally (30 mg/kg) at MCAo, 4 and 8 h after MCAo (see Fig. 1A). The vehicle group received diluents without active substance according to the same injection scheme.

Animal model of stroke. MCAo was performed according to the standard operating procedures of the laboratory (Dirnagl, 2012). In brief, anesthesia was induced with 2.5% isoflurane (Forene, Abbott) in 1/2 mixture of O₂/N₂O and maintained at 1.0%–1.5% isoflurane. Silicon-rubber-coated monofilament with a diameter of 0.19 $\pm$ 0.01 mm (Doccol) was introduced into the common carotid artery, advanced along the internal carotid artery to the origin of the MCA, and left there for 60 min until reperfusion. Body temperature was maintained with a heating pad. A drop of 2% lidocaine gel was applied to the wound for pain relief. Success of MCAo was verified applying the modified Bederson score (Bederson et al., 1986). After surgery, animals were allowed to recover in a heated cage before returning to home cages. Soft pellet food was provided postoperatively for 3 d.

Cerebral blood flow (CBF) and diffusion-weighted imaging (DWI) measurement with MRI. As soon as the filament entered the origin of MCA, the head of the mouse was fixed using a stereotactic frame in magnet bore. Anesthesia was maintained at 0.8%–1.0% isoflurane in 1/2 mixture of O₂/N₂O. Body temperature was maintained at physiological range with a heated water jacket. During scan time, rectal temperature, electrocardiogram, and respiration rate were monitored with Small Animal Monitoring and Gating System (model 1025; SA Instruments; RRID:SciRes_000157). ¹H (300 MHz) mouse head surface radiofrequency coil was used as a receiver and a 72 mm linear volume resonator for transmission. Flow-sensitive alternating inversion recovery (FAIR)-MRI and DWI were performed on a 7T Bruker PharmaScan 70/16 magnet using Bruker Paravision 4.0 software (both from Bruker, software RRID:SciRes_000158) as described in detail elsewhere (Leithner et al., 2008). Briefly, pilot images were collected using a Fast Low Angle Shot sequence (TE 5 ms, TR 307.7 ms, FOV 25 mm, a total of 27 slices with a thickness of 600 μ m in three views, 128 $\times$ 128 in plane resolution) to select an area between the olfactory bulb and the cerebellum. FAIR-MRI images were collected with a spin echo planar imaging (TE 42.55 ms, TR 9122.7 ms, FOV 20 mm, imaging slice thickness 2 mm, 64 $\times$ 64 in plane resolution, inversion slab thickness 6 mm, inversion recovery time 15.55 ms, increment of inversion recovery time 800 ms). Together, three slices 2 mm apart were selected; and for each slice, two series of 11 images (alternating slice selective inversion and slice nonselective inversion) were collected and hemispherical CBF calculated (Leithner et al., 2010). FAIR-MRI was followed by DWI using spin echo-echo planar imaging sequence (TE 40.93 ms, TR 3000 ms, FOV 20 mm, imaging slice thickness and interslice distance 1 mm, 128 $\times$ 128 in plane resolution, total of 10 slices and 6 averages). FAIR-MRI and DWI were repeated 3 h after MCAo.

Hemispheric CBF was calculated from FAIR-MRI data as described previously (Leithner et al., 2008; Royl et al., 2009) using custom script routines based on MATLAB (The MathWorks; RRID:nlx_153890). From DWI imaging, the apparent diffusion coefficient (ADC) maps were calculated. Lesions visible in the ADC images were delineated with ImageJ (National Institutes of Health; RRID:nif-0000-30467) and lesion volumes calculated by multiplying with voxel size.

T2-weighted imaging for infarct volume determination. For quantification of ischemic lesion, animals were subjected to MRI 24 h, 48 h, or 7 d after the MCAo surgery. Anesthesia was applied and physiological characteristics monitored as described above. T2-weighted images were acquired with a 7T Bruker PharmaScan 70/16 magnet, 20 mm quadrature volume resonator radiofrequency coil, and Bruker Paravision 4.0 software (Bruker) using Rapid Acquisition with Relaxation Enhancement (RARE) sequence (TE 36 ms, TR 4200 ms, FOV 28 mm, 20 slices with a thickness of 500 μ m and interslice distance 500 μ m, 256 $\times$ 256 in plane resolution). Axial slices covered the distance between the olfactory bulb and the cerebellum. Acquired images were analyzed semiautomatically with Mayo Clinic Analyze software version 5.0 (Biomedical Imaging Resource, Analyze Direct, Analyze Software System; RRID:nif-0000-00263). The difference between hemispheric volumes (excluding the lesion) was divided with the volume of ipsilateral hemisphere and converted into percentage to obtain edema-corrected infarct volumes.

Bronchoalveolar lavage (BAL) and microbiology. Three days after MCAo, mice were anesthetized with an intraperitoneal injection of midazolam (5.0 mg/kg; Roche Pharma AG) and medetomidin (0.5 mg/kg; Orion Pharma) and intubated with a 22 G venous catheter (BD Bio-

Figure 1. Study outline. Stroke-induced immunodeficiency was blocked by inhibition of SNS and HPA using β -blocker propranolol and glucocorticoid receptor antagonist mifepristone. **A**, Scheme illustrating time points of substance administration relative to MCAo surgery. The paradigm was applied in all experiments. **B**, **C**, Paradigm for adaptive cell transfer study. **B**, Female 2D2 donor mice received propranolol and mifepristone according to **A** and underwent MRI for infarct volume measurement. On day 7, mice were sacrificed. Spleen and lymph nodes (cervical, mesenteric, inguinal, and lumbar) were used for separation of untouched CD4⁺ T cells through MACS. **C**, Two million of these cells were transferred intravenously to gender-mixed Rag-1KO recipients 6 h after MCAo. Rag-1KO mice were examined for functional outcome and neurological deficits using CatWalk gait analysis on days 10 and 30, modified EAE score day 7–30, and EMG on day 14 after MCAo. On day 30, animals were euthanized, and their brains and vertebrae containing nerve roots were analyzed for immune cell infiltration and axonal damage. 2D2 donor and Rag-1KO recipient mice received preventively enrofloxacin from 1 d before until 7 d after MCAo. CW, CatWalk gait analysis.

sciences PharMingen) under direct illumination with fiber optic (MacDonald et al., 2009). BAL was collected by flushing with 300 μ l of 0.9% NaCl. After the procedure, anesthesia was antagonized with a subcutaneous injection of flumazenil (0.5 mg/kg; Inresa Arzneimittel) and atipamezolhydrochloride (2.5 mg/kg; Orion Pharma). BAL fluid was serially diluted, plated on Lysogeny broth medium agar plates, and incubated at 37°C, 5% CO₂. Bacterial colonies were counted 24 h later and colony-forming units (CFUs) calculated. Criteria for infection were met when log₁₀ of CFU/ml exceeded 5.

Daily health score. Animal well-being was assessed daily in a detailed fashion throughout the experiment (for scoring criteria, see Hetze et al., 2013). Scores for each parameter (e.g., grooming, eye and nasal discharge, breathing, activity) ranged from 0 to 2, where 0 indicates normal appearance and 2 major deficits. The maximum score possible was thus 16.

Modified experimental autoimmune encephalomyelitis (EAE) score. We observed mice showing gait alterations reminiscent of EAE. Therefore, mice were evaluated for the appearance and severity of these symptoms daily starting 7 d after MCAo until the end of the study on day 30 using modified EAE score (modified from Adelman et al., 1995): 0, no disease; 0.5, partial tail paralysis; 1.0, complete tail paralysis; 1.5, limb weakness without tail paralysis; 2.0, limb weakness with tail paralysis; 2.5, partial limb and tail paralysis; 3.0, complete limb paralysis; 3.5, paraplegia; 4.0, quadriplegia. The gait behavior of mice was first observed on a smooth surface. Mice were then transferred onto metal grid to follow foot faults. Tail tone was assessed by holding the mouse by the scruff of the neck to examine a reflex of spontaneous tail raise.

CatWalk gait analysis. Gait impairments after stroke were assessed using CatWalk (Noldus Information Technology) automated computer-assisted method as described in detail previously (Hetz et al., 2012). Before the acquisition of baseline values, mice were trained on the CatWalk system in three sessions (three runs each) on 3 consecutive days. Duration of compliant runs was 0.5–5.0 s, speed variation <60%. Run interruptions were not accepted. Poststroke acquisitions were performed 10 and 30 d after MCAo. Stroke-sensitive individual paw and gait parameters were selected based on a previously published model (Hetz et al., 2012). Swing speed was normalized to overcome bias from individual run speed.

Electrophysiology. Electrical stimulation of the distal nerve triggers a motor response and a second voltage change, termed the F-wave, as a

fraction of spinal motoneurons backfire to periphery (Fisher, 2007). Here, the mean latencies to F-wave and F-wave chronodispersion (difference between the shortest and longest F-wave latency within one animal) (Fisher, 2007) were measured before and 14 d after MCAo. Anesthesia was induced with 2.5% and maintained at 1.0%–1.5% isoflurane in 1/2 mixture of O₂/N₂O. Body temperature was controlled with a heating pad. Two pairs of needle electrodes were used: (1) reference and stimulation; and (2) reference and recording. Electrical stimulus was applied at the sciatic notch, and corresponding reference electrode was inserted subcutaneously into peritoneal cavity. Acquisition and nerve stimulation parameters were controlled with Medtronic KeyPoint Portable software version 5.11 (Medtronic; RRID:SciRes_000159). Single stimulation lasted 0.2 s at 1.2 mA. Recording electrode was inserted into the muscle innervated by sciatic nerve and corresponding reference into a lower extremity. Surface electrode was used and moistened with 0.9% NaCl to increase the signal-to-noise ratio.

Tissue harvesting. Mice were anesthetized with ketamine (150 mg/kg; Deltaselect) and xylazine (15 mg/kg; Bayer Vital). Spleen and lymph nodes (cervical, mesenteric, inguinal, and lumbar) were removed, and animals were

transcardially perfused with 20 ml of ice-cold 0.9% NaCl per mouse at 80 cm water height.

In experiments designed for immunological analysis (endpoint on day 7 or 14 after MCAo), brains were collected into complete RPMI 1640 medium (Biochrom AG), supplemented with 10% FCS (FCS Gold; PAA Laboratories), 50 U/ml penicillin, 50 μ g/ml streptomycin (Biochrom AG), and 2 mM L-alanyl-L-glutamine (Biochrom AG), and processed immediately.

In experiments where the primary outcome was assessment of neurological outcome (endpoint on day 30), perfusion with 0.9% NaCl was followed by perfusion with 20 ml ice-cold 4% PFA (Sigma-Aldrich) in 0.1 M Sørensen buffer, pH 7.4. Brains and vertebral columns were collected into 4% PFA for 24 h after fixation. Thereafter the brains were incubated for 5 d in 30% sucrose (D(+)-sucrose; Carl Roth) solution in PBS (PAA Laboratories) for cryoprotection and snap frozen using 2-methylbutan (Carl Roth). Vertebral columns were placed into 0.1 M PBS until decalcification. Bone structures were decalcified with a 24 h incubation in Osteosoft (Merck KGaA), and tissue was stored in 0.1 M PBS until paraffin-embedding using routine protocol. Paraffin-embedded tissue blocks were cut on a microtome (thickness 2–3 μ m), and slices were collected for immunohistochemistry.

Cell isolation from brain, spleen, and lymph nodes. Before enzyme-linked immunospot (Elispot) assay, the cerebellum was removed and brain tissue forced through a 70 μ m pore size cell strainer (BD Falcon) into complete RPMI 1640 medium. Mononuclear cells (MNCs) were isolated from the interface of 35% and 70% Easycoll gradient (Biochrom AG). MNCs were stained with Trypan Blue dye (Biochrom AG) and quantified under light microscope.

Spleens and lymph nodes (cervical, mesenteric, inguinal, and lumbar) were forced through a 100 μ m pore size cell strainer (BD Falcon) and washed with complete RPMI 1640 medium (or magnetic-activated cell sorting [MACS] buffer containing 2% FCS in sterile PBS for MACS assay). Before the MACS assay, erythrocytes in spleen were lysed with 30 s incubation with distilled water. Splenocytes and lymphocytes were forced through a 40 μ m cell strainer (BD Falcon) and washed with complete RPMI 1640 medium (or MACS buffer). Splenocytes were quantified using automated CasyTon cell counter and analyzer system (Casy-Technology Innovatis AG, SciRes_000160) and lymphocytes on the Trypan blue method.

Figure 2. Infarct volumes were reduced after blocking SNS and HPA axis using propranolol and mifepristone. Ischemic lesions were smaller after SNS/HPA block in female 2D2 mice 24 h (vehicle, $N = 56$; SNS/HPA block, $N = 52$), 48 h (vehicle, $N = 19$; SNS/HPA block, $N = 19$), and 7 d (vehicle, $N = 8$; SNS/HPA block, $N = 13$) after MCAo (**A**). Infarct volumes were ~ 3 times reduced at each time point (Student's t test; at 24 h, $t_{(106)} = 6.3$, $p < 0.0005$; at 48 h, $t_{(36)} = 4.5$, $p < 0.0005$; at 7 d, $t_{(19)} = 4.7$, $p < 0.0005$). Same effect was observed in C57BL/6J mice at 24 h (vehicle, $N = 25$; SNS/HPA block, $N = 28$), 48 h (vehicle, $N = 4$; SNS/HPA block, $N = 6$), and 7 d (vehicle, $N = 4$; SNS/HPA block, $N = 7$) after MCAo (Mann–Whitney U test; at 24 h, $U = 49.0$, $p < 0.0005$; at 48 h, $U = 0.0$, $p = 0.010$; at 7 d, $U = 3.5$, $p = 0.021$; **B**). Infarct volumes of vehicle-treated C57BL/6J mice differed between 24 h and 7 d after MCAo (Mann–Whitney U test; $U = 10.0$, $p = 0.020$) and of propranolol- and mifepristone-treated mice between 24 h and 48 h after MCAo (Mann–Whitney U test; $U = 18.5$, $p = 0.001$; **B**). T2-weighted images of ischemic lesions 24 h after MCAo in vehicle-treated (**C**) and SNS/HPA block (**D**) group of mice. Lesions in vehicle-treated mice involved cortex, striatum, and hippocampus (**C**), whereas those after SNS/HPA blockade comprised of multiple smaller ischemic foci rarely involving cortex (**D**). Follow-up among surviving C57BL/6J (**E**, **G**) and 2D2 mice (**F**, **H**) 24 h and 7 d after MCAo after vehicle treatment (**E**) and SNS/HPA block treatment (**G**) compared with 2D2 mice (**F**, vehicle treatment; **H**, SNS/HPA block treatment). Infarct volume reduction within the vehicle and SNS/HPA block groups of C57BL/6J and 2D2 mice between 24 h and 7 d after MCAo was similar. * $p < 0.05$. ** $p < 0.01$. *** $p < 0.001$.

MACS assay, FACS, and cell transfer. Lymph nodes (cervical, mesenteric, inguinal, and lumbar) and spleens were collected from naive female 2D2 mice and from treated female 2D2 mice 7 d after the MCAo for an adoptive transfer experiment (see Fig. 1B,C). Cells were harvested as described above and pooled per group (naive, vehicle, SNS/HPA block). Untouched CD4⁺ T cell sort was performed using CD4⁺ T cell isolation kit for mice (Miltenyi Biotec) and LS columns (Miltenyi Biotec) according to the manufacturer's instructions. CD4⁺ T cell purity was verified with FACS using APC-conjugated anti-CD4 monoclonal antibody (RM4-5; BD Biosciences, catalog #553051 RRID:AB_398528). Samples were measured on FACSCalibur flow cytometer (BD Biosciences Pharmingen; RRID:SciRes_000153) and analyzed with FlowJo software version 7.6.5. (Tree Star; RRID:nif-0000-30575). Sorted CD4⁺ T cells were characterized immunologically using FACS. Cells were resuspended in FACS buffer (2% FCS and 15.4 mM sodium azide in PBS) and centrifuged at $252 \times g$ at 4°C for 6 min. Unspecific binding was blocked by incubation with anti-CD16/32 antibody (2.4G2; BD Biosciences, catalog #553141; RRID:AB_394656) at 4°C for 15 min. Cellular surfaces were stained with following anti-mouse antibodies: APC-conjugated CD3 (145-2C11; eBioscience, catalog #17-0031-82; RRID:AB_469315), Alexa-700-conjugated CD4 (RM4-5; eBioscience, catalog #56-0042-82; RRID:AB_494000), APC-Cy7-conjugated CD44 (IM7; BD Biosciences, catalog #560568; RRID:AB_1727481), biotin-conjugated TCR $\alpha\beta$ (RR3-16; BD Biosciences, catalog #553218; RRID:AB_394714), PE-Cy7-conjugated CD25 (PC61.5; eBioscience, catalog #25-0251-82; RRID:AB_469608), Pacific blue-conjugated CD62L (MEL-14; BioLegend, catalog #104424; RRID:AB_493380), PE-conjugated CD127 (SB/199; BD Biosciences, catalog #552543; RRID:AB_394417), and FITC-conjugated

KLRG1 (2F1; eBioscience, catalog #11-5893-82; RRID:AB_1311265). Samples were incubated with antibody mixtures light-protected on ice for 30 min. Cell surfaces were stained in a second step for the detection of $\alpha\beta$ TCR receptor with biotin-binding PerCP-conjugated streptavidin (BD Biosciences Pharmingen, catalog #554064; RRID:AB_2336918). Samples were measured on an LSR II flow cytometer (BD Biosciences Pharmingen; RRID:SciRes_000152) using FACSDiva software version 6.1.3 (BD Biosciences Pharmingen, BD FACSDiva Software; RRID:SciRes_000115). Data were analyzed with FlowJo software version 7.6.5. The following cell populations were distinguished: $\alpha\beta$ -TCR transgenic CD4⁺ T cells ($\alpha\beta$ -TCR⁺ CD4⁺ CD3⁺), effector memory (MOG-specific) T cells [CD62L⁺ CD44⁺ from ($\alpha\beta$ -TCR⁺ CD4⁺ CD3⁺ cell population)], central memory (MOG-specific) T cells [CD62L⁺ CD44⁺ from ($\alpha\beta$ -TCR⁺ CD4⁺ CD3⁺ cell population)], and regulatory (MOG-specific) T cells [CD25⁺ CD127⁺ from ($\alpha\beta$ -TCR⁺ CD4⁺ CD3⁺ cell population)].

Two million untouched CD4⁺ T cells were injected intravenously to corresponding gender-mixed *Rag-1* KO recipients 6 h after MCAo.

Elispot assay. Elispot assay was performed from brain MNCs and splenocytes of 2D2 mice harvested 14 d after MCAo. Brain MNC Elispot was performed from mice where only the SNS-mediated SIDS was blocked and the SNS- and HPA-mediated SIDSs were both simultaneously blocked. For this, Elispot plates (96-well plates with hydrophobic PVDF membrane, 0.45 μ m pore size; EMD Millipore) were coated overnight with 5 μ g/ml of the following: (1) IFN- γ (for Th1 cells; eBioscience, catalog #14-7313-85; RRID:AB_468472); (2) IL-4 (for Th2 cells; BD Biosciences, catalog #551878; RRID:AB_2336921); or (3) IL-17 (for Th17 cells; eBioscience, catalog #16-7175-85; RRID:AB_763573) specific cap-

Figure 3. CBF, measured by FAIR-MRI, does not differ between vehicle and SNS/HPA block group at filament insertion and 3 h after MCAo. **A**, ADC map lesion sizes were similar in vehicle and SNS/HPA block group immediately after MCAo (vehicle, $N = 8$; SNS/HPA block, $N = 7$) and 3 h after MCAo (vehicle, $N = 8$; SNS/HPA block, $N = 7$). In the vehicle group of mice, lesions on ADC maps decreased within 3 h after MCAo (Wilcoxon test; $Z = -2.3$, $p = 0.008$). **B**, Hemispheric CBF differed in both groups at each time point (Wilcoxon test; immediately after MCAo: vehicle, $Z = -2.4$, $p = 0.016$, SNS/HPA block, $Z = -2.5$, $p = 0.008$; 3 h after MCAo: vehicle, $Z = -2.2$, $p = 0.031$, SNS/HPA block, $Z = -2.5$, $p = 0.008$) but not between the groups of interest [vehicle, 0 h, $N = 7$; 3 h, $N = 6$; SNS/HPA block, 0 h, $N = 8$; 3 h, $N = 8$]. **C**, FAIR-MRI (top row; for CBF evaluation) and ADC images (bottom row) from coronal sections (at 2 mm interval) from the same mouse with an index bar. * $p < 0.05$. ** $p < 0.01$.

ture antibody in sterile PBS. Plates were washed with sterile PBS and blocked with 1% BSA fraction V (Sigma-Aldrich) in PBS for 2 h at room temperature. Wells were filled with 100 μ l of the following: (1) complete HL-1 medium (Lonza) supplemented with 2 mM L-alanyl-L-glutamine, 50 U/ml penicillin, and 50 μ g/ml streptomycin, with 2 μ g/ml CD-3 (145-2C11; eBioscience, catalog #16-0031-86; RRID:AB_468849) and 0.2 μ g/ml CD-28 (37.51; eBioscience, catalog #14-0281-85; RRID:AB_467191); duplicates per sample (positive control); (2) complete HL-1 medium with 0.2 μ g/ml CD-28 and 40 μ g/ml myelin oligodendrocyte glycoprotein peptide (pMOG_{35–55}, amino acid sequence: MEVGWYRSPFSRVVHLYRNGK; a gift from Dr. Rudolf Volkmer; Charité Universitätsmedizin Berlin, Berlin, Germany); triplicates per sample (MOG_{35–55}-peptide stimulation); and (3) complete HL-1 medium with 0.2 μ g/ml CD-28; triplicates per sample (negative control). In brain Elispot, brain MNCs were added at 1.75×10^5 in 50 μ l complete HL-1 medium per well together with 1×10^6 naive C57BL/6J splenocytes

in 50 μ l complete HL-1 medium. In spleen Elispot, 3.75×10^5 (IFN- γ) or 1×10^6 (IL-4 and IL-17) splenocytes were added in 100 μ l complete HL-1 medium per well. Cells were incubated at 37°C, 5% CO₂ for 24 h (IFN- γ plates) or 48 h (IL-4 and IL-17 plates). Plates were washed with PBS and subsequently with PBS supplied with 0.05% Tween 20 (Sigma-Aldrich) before incubation with a biotinylated secondary antibody [IFN- γ (eBioscience, catalog #13-7311-81; RRID:AB_466936); IL-4 (BD Biosciences, catalog #551878; RRID:AB_2336921); or IL-17 (eBioscience, catalog #13-7177-85; RRID:AB_763571)] in a solution of 0.05% Tween 20 and 1.5 mM BSA in PBS at 4°C overnight. Plates were washed with 0.05% Tween 20 in PBS. Secondary antibody binding was visualized with streptavidin-HRP (BD Biosciences Pharmingen) and 50 mM 3-amino-9-ethylcarbazole (Sigma-Aldrich) in 30 mM acetic acid glacial (Merck KGaA), 117 mM sodium acetate (Sigma-Aldrich), and 5 μ M H₂O₂ (Merck KGaA). Formed spots were analyzed semiautomatically using ImmunoSpot software version 4.013 (Cellular Technology; RRID:SciRes_000119).

Multiplex immunoassay and ELISA for quantifying cytokine production from brain and spleen. Fourteen days after MCAo, brain-infiltrating cells and splenocytes were isolated and stimulated as for Elispot assay. Cell culture supernatants were collected and stored at -20°C until measurement. From each sample, 25 μ l supernatant was used for multiplex assay to quantify T cell-specific cytokine (IL-2, IL-4, IL-10, IL-17, and IFN- γ) production. Mouse multiplex kit (Millipore) and Human/Mouse TGF- β 1 ELISA Ready-SET-Go kit (Affymetrix) were used according to the manufacturer's recommendations. Plates were read at 450 nm by a Luminex Instrument (Bio-Plex 200 System and Bio-Plex Manager Software version 6.1; Dartmouth DartLab; RRID:SciEx_11307). For quantification of TGF- β 1 production, samples were acidified with 1 M HCl to activate latent TGF- β 1 to immunoreactive form and then neutralized with 1 M NaOH. Each sample was measured once.

Histological analysis of nerve root damage and inflammation. Paraffin sections (2–3 μ m thick) containing vertebral columns and nerve roots were routinely stained with hematoxylin and eosin for assessing inflammation and Luxol fast blue for assessing demyelination. Adjacent serial sections were used for immunohistochemistry. Antigen retrieval was done by cooking the slides in citrate (pH 6.0) or EDTA buffer (pH 8.5; for V α 3.2 TCR staining) for 1 h (both from Sigma-Aldrich). After cooling down and washing in PBS, slides were incubated overnight with primary antibodies raised against Mac3 (M3/84; host species rat; dilution 1/100; BD Biosciences, catalog #553322; RRID:AB_394780) for macrophages and CD3 (SP7; host species rabbit; dilution 1/500; Lab Vision, catalog #RM-9107-S1; RRID:AB_149924) for T cells. Axonal injury was visualized with β -amyloid precursor protein staining (β -APP; host species mouse; dilution 1/500; Millipore, catalog #MAB5228; RRID:AB_95173). Immunoreactivity to V α 3.2 TCR was detected with biotin-conjugated V α 3.2 TCR antibody (RR3-16; host species rat; dilution 1/100; BD Biosciences, catalog #553218; RRID:AB_394714). All antibodies were diluted in 10% FCS in PBS. Immunopositive structures were detected with biotin-avidin technique using 3,3'-diaminobenzidine-tetrahydrochloride

Antigen retrieval was done by cooking the slides in citrate (pH 6.0) or EDTA buffer (pH 8.5; for V α 3.2 TCR staining) for 1 h (both from Sigma-Aldrich). After cooling down and washing in PBS, slides were incubated overnight with primary antibodies raised against Mac3 (M3/84; host species rat; dilution 1/100; BD Biosciences, catalog #553322; RRID:AB_394780) for macrophages and CD3 (SP7; host species rabbit; dilution 1/500; Lab Vision, catalog #RM-9107-S1; RRID:AB_149924) for T cells. Axonal injury was visualized with β -amyloid precursor protein staining (β -APP; host species mouse; dilution 1/500; Millipore, catalog #MAB5228; RRID:AB_95173). Immunoreactivity to V α 3.2 TCR was detected with biotin-conjugated V α 3.2 TCR antibody (RR3-16; host species rat; dilution 1/100; BD Biosciences, catalog #553218; RRID:AB_394714). All antibodies were diluted in 10% FCS in PBS. Immunopositive structures were detected with biotin-avidin technique using 3,3'-diaminobenzidine-tetrahydrochloride

(Sigma) as chromogen. Control sections were stained in the absence of primary antibody.

The cumulative area of spinal nerve cross sections (mm^2) within the decalcified vertebral columns and immunostained structures (Mac3^+ , CD3^+ , APP^+ , and $\text{V}\alpha 3.2^+$) were quantified under light microscope using an optical grid. All available nerve root cross sections were examined. Data are presented as numbers of immunopositive structures per mm^2 nerve root.

Histological analysis of inflammation and infiltration of CD4^+ T cells into the brain. Snap-frozen brains were cut into 30- μm -thick sections on a sliding microtome. After washing with Tris-buffered saline (TBS), free-floating sections were incubated with 10% normal goat serum (NGS, BIOZOL Diagnostica Vertrieb) and 0.3% Triton X (LABORAT) in TBS for 1 h at room temperature to block unspecific binding. Primary and secondary antibodies were diluted in 1% NGS and 0.3% Triton X in TBS. Sections were incubated with rat anti- CD4 primary antibody (clone RM4-5, BD Biosciences, catalog #550280; RRID: AB_393575) for CD4^+ T cells and rabbit anti-Iba-1 primary antibody (Wako Chemicals, catalog #019-19741; RRID: AB_839504) for microglia and macrophages at 4°C overnight. After thorough washing, sections were incubated at room temperature with AlexaFluor-488-conjugated goat anti-rat (Invitrogen, catalog #A11006 RRID: AB_10561520) and AlexaFluor-568-conjugated goat anti-rabbit (Invitrogen, catalog #A11011 RRID: AB_10584650) secondary antibodies (both from Invitrogen) for 2 h. Nuclei were counterstained with DAPI (Invitrogen). Sections were mounted with anti-fading mounting medium DABCO (Sigma-Aldrich) on gelatinized glass slides. Microphotographs were taken with a confocal microscope (Leica TCS SPE; RRID: SciRes_000154).

Quantification of CD4^+ T cells, activated microglia, and macrophages was performed using a stereology workstation consisting of a fluorescence microscope (Leica DMRE Fluorescence Microscope; RRID: SciRes_000155) and Stereo Investigator software (MBF Bioscience; RRID: SciRes_000114). Hemispheres were outlined, and Meander scan was selected to count all positively stained cells within the ipsilateral hemisphere. Activated microglia/macrophages were distinguished from resident resting microglia based on morphology and localization. Cell densities were calculated per mm^2 ipsilateral hemisphere.

Statistical analysis. Data were analyzed with SPSS Statistics 21.0 software for Windows (IBM Somers; SPSS; RRID: rid_000042). Normality of data distribution was assessed with Kolmogorov–Smirnov test. p values for parametric data were calculated with two-tailed Student's t test or one-way ANOVA for multiple comparisons with Bonferroni correction where indicated. Nonparametric data were analyzed with Kruskal–Wallis (with pairwise Dunn's method were applicable) and Mann–Whitney U test. Dependent samples with nonparametric distribution were analyzed using Wilcoxon signed rank test. Only exact two-tailed p values were accepted for nonparametric tests. χ^2 test was applied to evaluate survival differences between groups. p value < 0.05 was considered significant. Correlation analysis was performed using Spearman's ρ correlation coefficient for nonparametric data and Pearson correlation coefficient for parametric data. Data are mean $\pm$ SD. Health and EAE score are pre-

Figure 4. 2D2 mice from stress axes blockade group show better long-term (30 d) survival and body temperature maintenance after experimental stroke. **A**, Propranolol and mifepristone treatment improved survival until 30 d after MCAO compared with vehicle treatment (**A**; vehicle, $N = 64$; SNS/HPA block, $N = 61$; $\chi^2 = 5.5$, $df = 1$, $p = 0.020$). **B**, General health score was not significantly improved after SNS/HPA block (vehicle, $N = 42$; SNS/HPA block, $N = 45$). **C**, Rectal temperature curves differed between the groups with better maintenance of physiological temperatures in stress axis blockade group on days 3, 7–9, and 12 after MCAO (Student's t test, day 3: vehicle, $N = 48$; SNS/HPA block, $N = 50$; $t_{(96)} = -2.5$, $p = 0.015$; day 7: vehicle, $N = 29$; SNS/HPA block, $N = 42$; $t_{(47)} = -2.0$, $p = 0.048$; day 8: vehicle, $N = 27$; SNS/HPA block, $N = 40$; $t_{(32)} = -2.2$, $p = 0.038$; day 9: vehicle, $N = 26$; SNS/HPA block, $N = 41$; $t_{(65)} = -2.1$, $p = 0.040$; day 12: vehicle, $N = 24$; SNS/HPA block, $N = 40$; $t_{(62)} = -2.5$, $p = 0.014$). **D**, Body weight loss was overall similar in both groups (vehicle, $N = 52$; SNS/HPA block, $N = 52$; day 2: Student's t test, $t_{(102)} = -2.6$, $p = 0.011$). **B**, Data are median $\pm$ range. **C**, **D**, Data are mean $\pm$ SD. * $p < 0.05$.

sented as median $\pm$ range. Survival data are plotted on Kaplan–Meier curve.

Results

Blockade of SNS and HPA reduced infarct volumes

To investigate the effect of SIDS on CNS antigen-specific autoreactivity, we used 2D2 transgenic mice. These mice are sensitive for investigating CNS antigen-specific immune responses because the majority of their CD4^+ T cells carry a functional receptor for MOG (Bettelli et al., 2003). SNS and the HPA axes both affect strongly cellular immunity and function mainly in an additive manner (Elenkov et al., 2000; Tischner and Reichardt, 2007). Therefore, to prevent SIDS efficiently, we blocked the SNS and HPA axis simultaneously using β -receptor blocker propranolol and glucocorticoid receptor antagonist mifepristone (Fig. 1A).

Figure 5. Number of brain MNCs and their CNS antigen-specific autoreactive phenotype 14 d after MCAo. **A**, Number of MNCs infiltrating ipsilateral and contralateral hemisphere of the brain in vehicle and SNS/HPA blockade group (three individual experiments; vehicle, $N = 15$; SNS/HPA block, $N = 20$). **B–D**, Elispot experiments. **B**, Higher number of total brain MNCs responded to pMOG_{35–55} stimulation with IFN- γ production (Th1 cells) per equal number of brain MNCs in SNS/HPA block group compared with the vehicle group. **C**, A similar effect was observed in ipsilateral hemisphere MNCs when only the SNS axis of SIDS was blocked with propranolol (two experiments; vehicle group, $N = 5–8$; SNS block, $N = 6$ or 7). **D**, Number of splenocytes reacting to pMOG_{35–55} stimulation with IFN- γ , IL-4, or IL-17 secretion did not differ between the groups. **E, F**, ELISA experiments. TGF- β 1, IL-10, IL-4, IL-17, and IFN- γ secretion (pg/ml) by pMOG_{35–55}-stimulated brain MNCs, ipsilateral hemisphere (**E**) and by pMOG_{35–55}-stimulated splenocytes (**F**).

Dual blockade of stress axes with propranolol and mifepristone resulted in significantly smaller infarct volumes compared with vehicle treatment (Fig. 2A). Approximately three times smaller infarct volumes were sustained for at least 1 week after stroke onset (measurements were performed at 24 h, 48 h, and 7 d after MCAo). Combined treatment effect of propranolol and mifepristone is therefore sustained and not explained by mere delayed infarct volume maturation.

To exclude mouse strain-specific effects, we next investigated the effect of stress axes blockade on infarct volume in wild-type C57BL/6J mice. In accordance with the data from 2D2 mice, SNS/HPA blockade resulted in a robust reduction of infarct volumes (Fig. 2B).

Ischemic lesions in vehicle-treated mice affected striatum, hippocampus, and cortex. Lesions in mice with SNS/HPA blockade involved the cortex only rarely. Another difference between the groups was the uniformly large ischemic lesion in the vehicle group compared with the combination of small focal lesions in mice with SNS/HPA blockade (Fig. 2C,D).

The dynamic changes in ischemic lesion were investigated in surviving wild-type C57BL/6J (Fig. 2E,G) and 2D2 (Fig. 2F,H) mice 24 h and 7 d after MCAo. In surviving animals, the ischemic lesions tended to be smaller in C57BL/6J mice compared with

2D2 mice from both vehicle and SNS/HPA block groups (Fig. 2E–H). However, average reduction in ischemic lesion size within 7 d after MCAo was similar between C57BL/6J ($-4.8 \pm 6.3\%$) and 2D2 ($-4.1 \pm 7.0\%$) mice from vehicle as well as from SNS/HPA block group (C57BL/6J, $-3.1 \pm 1.4\%$; 2D2, $-3.6 \pm 3.4\%$).

Reduction of infarct volume by SIDS blockade was not due to altered CBF

The most straightforward cause for reduced infarct size is increased CBF because it relates inversely with lesion size after cerebral ischemia (Dalkara et al., 1994). To investigate whether the observed infarct volume reduction was due to altered CBF by SNS/HPA blockade, we performed FAIR-MRI combined with DWI in 2D2 and C57BL/6J mice immediately after filament insertion to the MCA and again 3 h after MCAo surgery (Fig. 3). DWI lesions did not differ significantly between the groups (Fig. 3A). During cerebral ischemia sustained by MCAo, a large lesion on ADC map was observed ipsilaterally that shrank 2 h after reperfusion. The size of this lesion did not differ between treatment groups (Fig. 3A). Hemispheric CBF was greatly reduced ipsilaterally to MCAo in all groups but was not affected by stress blockade either 0 h or 3 h after stroke onset (Fig. 3B,C).

Figure 6. EAE-like symptoms were not affected by SNS/HPA block, compared with vehicle treatment. Modified EAE score in vehicle-treated and SNS/HPA blocked 2D2 mice 7–30 d after experimental stroke. 2D2 mice developed EAE-like symptoms in median on day 9 (vehicle group, $N = 21$) and day 14 (SNS/HPA block group, $N = 31$). Data are expressed as median $\pm$ range. * $p < 0.05$.

Differences between the CBF and its dynamics within the 3 h after MCAo were similar in both groups ipsilaterally and contralaterally.

Blocking stress axes improved long-term survival and temperature maintenance of mice

In addition to decreasing infarct volume, propranolol and mifepristone treatment improved survival of 2D2 mice until the end of the observation period 30 d after MCAo (Fig. 4A). Compared with only 52% of mice surviving in the vehicle group, the long-term survival of mice with SNS/HPA blockade was 73%.

Although mice from the SNS/HPA block group did not differ statistically from vehicle-treated mice in terms of general health score (Fig. 4B) and had only minor differences in body weight (Fig. 4D), they were better at maintenance of body temperature (Fig. 4C).

In a *post hoc* analysis of vehicle-treated 2D2 mice, we compared smaller ($<20\%$; $N = 14$) with larger edema-corrected infarct volumes ($>20\%$; $N = 39$). Survival of mice with smaller lesions over the 30 d-follow-up after stroke was better (86%) compared with mice with larger lesions (43%; $\chi^2 = 5.1$, $df = 1$, $p = 0.024$). Health status measured by general health score was better in mice with smaller infarct volumes from day 2 through day 5 after experimental stroke (Student's t test; on day 2, $t_{(35)} = 3.6$, $p = 0.010$; on day 3, $t_{(36)} = 3.2$, $p = 0.030$; on day 4, $t_{(32)} = 3.7$, $p = 0.001$; on day 5, $t_{(28)} = 2.3$, $p = 0.032$), accompanied by better maintenance of body temperature on day 2 to day 4 as well as on day 11 after stroke (Student's t test; on day 2, $t_{(47)} = -4.8$, $p < 0.0005$; on day 3, $t_{(46)} = -2.7$, $p = 0.009$; on day 4, $t_{(39)} = -2.3$, $p = 0.025$; $t_{(22)} = -2.6$, $p = 0.018$). Body weight loss between the groups did not differ significantly (data not shown).

Blocking stress axes promoted recovery from infections

Many hypothermic mice in the vehicle group died 4 d after MCAo (Fig. 4A), reminiscent of severe poststroke infections, such as bacterial pneumonia (Prass et al., 2003; Meisel et al., 2004). To verify this, 2D2 mice (vehicle, $N = 11$, SNS/HPA block,

$N = 14$) were examined by BAL for bacterial burden in lung 3 d after MCAo. The overall infection rate following preventive enrofloxacin treatment was low in both groups. However, mice with SNS/HPA blockade with pulmonary infections (CFU/ml BAL fluid $> \text{LOG}(10) 5$) recovered or survived longer than those from the vehicle group (vehicle, $N = 4$; SNS/HPA block, $N = 5$; $\chi^2 = 7.6$, $df = 1$, $p = 0.006$; data not shown).

Blocking SIDS increased the CNS antigen-specific T-cell response in ischemic brain 14 d after MCAo

Infiltration of CD4^+ T cells into ischemic brain peaks 14 d after MCAo (Stubbe et al., 2013). Hence, we chose this time point to follow immune cell infiltration and CNS antigen-specific T-cell responses to investigate whether inhibition of SIDS by SNS/HPA blockade affects CNS-directed autoreactivity in ischemic brain. The average number of ipsilateral and contralateral hemisphere-infiltrating MNCs 14 d after MCAo was similar in the vehicle and SNS/HPA blockade group of 2D2 mice (Fig. 5A).

To investigate the CNS antigen-specific autoreactivity of brain-infiltrating MNCs, we performed an Elispot assay to quantify the frequency of pMOG_{35–55}-specific T cells and investigate their cytokine profile. SNS/HPA blockade increased the frequency of MNCs responding to pMOG_{35–55} with IFN- γ secretion from 598 per 3.25×10^5 total brain MNCs in vehicle-treated mice to 1122 per 3.25×10^5 of total brain MNCs (Fig. 5B). Despite similar numbers of MNCs infiltrating ischemic brain after MCAo, a higher number of total brain MNCs responded to pMOG_{35–55} stimulation with IFN- γ production in SNS/HPA blockade compared with the vehicle group of 2D2 mice (Fig. 5). These findings support our hypothesis that inhibiting SIDS by SNS/HPA blockade increases CNS antigen-specific Th1 cell response of brain-infiltrating MNCs. When blocking only the SNS but not the HPA axis, the difference in the frequency of MOG-specific autoreactive Th1 cells among ipsilateral hemisphere-infiltrating MNCs became smaller between the SNS block (360 per 1.75×10^5 ipsilateral hemisphere brain MNCs) and the vehicle group (220 per 1.75×10^5 ipsilateral hemisphere brain MNCs) (Fig. 5C), suggesting an additive function of the SNS and the HPA axis in preventing the autoreactive T-cell responses after acute brain injury. We measured multiple cytokines (IL-4, IL-10, IL-17, IFN- γ , TGF- β 1) from *ex vivo* MOG stimulated brain-infiltrating cells by multiplex immunoassay and ELISA. Corroborating our data from Elispot analysis, we observed an upregulation of IFN- γ production in the SNS/HPA blocked group compared with the vehicle group. In addition to autoreactive Th1 responses, we analyzed the production of multiple cytokines, including regulatory cytokines, TGF- β 1, and IL-10, upon MOG stimulation in brain MNCs of 2D2 mice 14 d after MCAo using ELISA. Whereas IL-10 production was reduced in SNS/HPA blockade compared with vehicle groups of mice, TGF- β 1 levels upon MOG stimulation were increased in the SNS/HPA block group of mice (Fig. 5E).

Next, we questioned whether increase in autoreactive T-cell responses after SIDS blockade in stroke animals was confined to the CNS. For this, we harvested splenocytes 14 d after MCAo and quantified the number of IFN- γ (Th1 cell signature cytokine), IL-4 (Th2 cell signature cytokine), and IL-17 (Th17 cell signature cytokine) secreting cells after pMOG_{35–55} stimulation by Elispot assay and regulatory-type cytokine production by ELISA. Blocking SIDS with propranolol and mifepristone did not change the numbers of Th1, Th2, or Th17 effector cytokine-producing cells or the amount of TGF- β 1 and IL-10 secretion by pMOG_{35–55}-stimulated splenocytes significantly (Fig. 5D,F). Ratios between

Table 1. Dynamic changes in gait parameters measured 10 and 30 d after MCAo in 2D2 mice belonging to vehicle (day 10, $N = 21$; day 30, $N = 14$) and SNS/HPA block group (day 10, $N = 29$; day 30, $N = 19$) and their comparison with baseline values ($N = 108$) before MCAo^a

				Vehicle (mean ± SD)		SNS/HPA block (mean ± SD)	
2D2 mice: parameter	Definition	Paw	Baseline	Day 10	Day 30	Day 10	Day 30
Spatial characteristics							
Maximal contact area (mm ²)	Area of a paw print at maximal walking surface contact	RF	26.25 ± 13.82	16.70 ± 7.306 (**p = 0.003)	15.53 ± 6.801 (**p = 0.005)	16.42 ± 5.730 (**p < 0.0005)	18.00 ± 6.306 (*p = 0.025)
		RH	19.82 ± 14.68	12.92 ± 6.435	13.99 ± 8.764	11.79 ± 5.158 (*p = 0.012)	15.51 ± 6.447 (p = 0.042)
		LF	26.89 ± 14.05	19.00 ± 5.348 (*p = 0.026)	18.77 ± 5.564	18.79 ± 6.448 (**p = 0.006)	21.59 ± 6.505
		LH	19.73 ± 14.06	14.51 ± 5.684	17.42 ± 9.744	15.51 ± 8.000	22.00 ± 10.09 (p = 0.024)
Kinetic characteristics							
Run duration (s)	Time for passing the walkway		2.120 ± 0.562	2.798 ± 0.664 (**p < 0.0005)	2.666 ± 0.539 (*p = 0.040)	2.559 ± 0.683 (*p = 0.031)	2.759 ± 0.564 (**p = 0.002)
Normalized swing speed (mm)	Swing speed × run duration	RF	1167 ± 235.4	998.9 ± 212.5	939.4 ± 186.9 (*p = 0.013)	973.0 ± 258.5 (**p = 0.005)	931.1 ± 179.5 (**p = 0.002)
		RH	983.8 ± 242.4	820.2 ± 185.0 (p = 0.050)	804.3 ± 197.9	791.7 ± 194.1 (**p = 0.003)	778.2 ± 223.7 (**p = 0.007)
		LF	1168 ± 233.6	1073 ± 177.2	1001 ± 238.0	1008 ± 228.4 (p = 0.032)	967.4 ± 216.3 (*p = 0.013)
		LH	992.3 ± 235.8	912.7 ± 230.9	906.5 ± 272.1	881.8 ± 255.9	882.0 ± 234.7
Stand (s)	Duration of a paw contact with walking surface	RF	0.137 ± 0.033	0.161 ± 0.048	0.164 ± 0.054	0.156 ± 0.049	0.172 ± 0.039 (*p = 0.037)
		RH	0.115 ± 0.033	0.132 ± 0.039	0.138 ± 0.046	0.131 ± 0.041	0.142 ± 0.041
		LF	0.140 ± 0.031	0.166 ± 0.039	0.170 ± 0.044	0.162 ± 0.043	0.176 ± 0.041 (**p = 0.008)
		LH	0.114 ± 0.032	0.146 ± 0.041 (*p = 0.036)	0.154 ± 0.052	0.140 ± 0.047 (*p = 0.018)	0.163 ± 0.041 (**p < 0.0005)
Comparative statistics							
Regularity index (%)	Regularity of gait		92.01 ± 7.358	87.91 ± 18.94	93.13 ± 4.871	88.34 ± 14.22	93.41 ± 5.221
Base of support (mm)	Distance between paws	RF-LF	7.928 ± 5.334	10.06 ± 5.463	14.19 ± 8.221 (p = 0.042)	12.77 ± 8.814 (p = 0.045)	13.26 ± 7.772
		RH-LH	14.26 ± 9.701	20.16 ± 12.49	28.33 ± 15.53 (*p = 0.014)	26.26 ± 17.40 (**p = 0.005)	27.04 ± 16.87 (*p = 0.012)
Print positions (mm)	Distance of a hindpaw position to previous position of front paw	RF-RH	9.567 ± 3.970	8.856 ± 3.953	8.716 ± 5.010	9.004 ± 3.519	9.221 ± 3.555
		LF-LH	11.38 ± 5.321	9.589 ± 4.481	7.380 ± 4.413	10.42 ± 4.587	8.311 ± 4.095
Stride length (mm)	Distance between successive steps with one paw	RF	63.88 ± 9.181	50.63 ± 17.19 (**p = 0.006)	48.94 ± 15.76 (**p = 0.008)	50.19 ± 17.06 (**p = 0.001)	47.82 ± 14.02 (**p = 0.001)
		RH	60.60 ± 8.631	46.11 ± 18.55 (**p = 0.008)	47.50 ± 17.36	53.62 ± 21.15	43.92 ± 13.39 (**p = 0.002)
		LF	64.25 ± 8.651	48.94 ± 16.65 (**p = 0.001)	50.13 ± 16.35 (*p = 0.012)	51.13 ± 16.95 (**p = 0.001)	47.22 ± 13.09 (**p < 0.0005)
		LH	58.37 ± 10.93	45.78 ± 16.57 (**p = 0.010)	48.35 ± 17.09	46.78 ± 15.37 (**p = 0.008)	45.48 ± 12.75 (**p = 0.011)
Phase dispersions (%)	Contact of a target paw in relation to step cycle of the anchor paw	RF-LH	6.663 ± 7.276	9.099 ± 7.519	1.815 ± 9.420	6.526 ± 6.815	2.952 ± 4.866
		LF-RH	5.535 ± 5.437	7.898 ± 6.963	5.380 ± 6.284	4.049 ± 6.621	7.249 ± 7.746
		LH-RH	42.65 ± 8.210	44.10 ± 10.07	44.96 ± 7.445	42.69 ± 8.282	44.08 ± 7.502
		LF-RF	49.45 ± 3.765	50.25 ± 3.931	51.76 ± 3.799	47.88 ± 7.821	50.94 ± 4.265
		RF-RH	50.66 ± 5.702	49.11 ± 11.09	49.40 ± 6.656	49.90 ± 8.948	48.90 ± 5.527
		LF-LH	48.34 ± 6.634	50.45 ± 4.274	49.27 ± 6.122	48.06 ± 5.767	49.21 ± 6.429

^aGait alterations in 2D2 mice (vehicle and SNS/HPA block groups) 10 and 30 d after MCAo. RF, Right forepaw; RH, right hindpaw; LF, left forepaw; LH, left hindpaw.* p < 0.05; ** p < 0.01; *** p < 0.001 (one-way ANOVA with Bonferroni correction). *Compared with baseline values. *Compared with day 10 SNS/HPA block.

pMOG_{35–55}-specific Th1 and Th2 (vehicle group, 1.97 ± 0.77 ; SNS/HPA blockade group, 2.50 ± 0.88) and Th17 and Th1 cells (vehicle group, 0.24 ± 0.08 ; SNS/HPA blockade group, 0.28 ± 0.19) were similar in both groups.

The total number of splenocytes 14 d after MCAo did not differ significantly between vehicle ($N = 6$, $1.0 \times 10^8 \pm 4.4 \times 10^7$) and the SNS/HPA block group ($N = 9$, $1.4 \times 10^8 \pm 6.1 \times 10^7$). Compared with naive 2D2 splenocytes ($N = 10$, $5.5 \times 10^8 \pm 4.3 \times 10^8$), this number was reduced in both groups (one-way ANOVA, $F_{(2)} = 7.0$: naive vs vehicle: $p = 0.014$; naive vs SNS/HPA block: $p = 0.013$; data not shown).

Blocking SIDS did not augment EAE-like symptoms and locomotor deficits

We next investigated whether increased autoreactive immune responses in ischemic brain after blocking SIDS were associated with worse functional outcome. Mice recover from sensorimotor deficits typically within 1 week after MCAo. Whereas stroke affects immediately and predominantly the contralateral side and in particular the contralateral forepaw, we observed a progressive and ascending paralysis affecting the tail and limbs of both sides symmetrically beginning from 1 week after MCAo. This phenotype observed in 2D2 mice is reminiscent of EAE. Therefore, we applied the modified EAE score starting assessment from day 7 after MCAo. Interestingly, EAE score of 2D2 mice 30 d after MCAo correlated positively with infarct volume 24 h after MCAo among vehicle-treated ($N = 11$, Spearman-Rho correlation coefficient 0.665, $p = 0.026$) but not mifepristone/propranolol-

treated mice ($N = 18$, Spearman ρ correlation coefficient = 0.089, $p = 0.726$). These findings suggest that EAE-like phenotype depends on ischemic lesion size and SIDS. Following experimental stroke, the majority of 2D2 mice from vehicle (87%) and SNS/HPA block groups (91%) developed EAE-like symptoms within the 30 d observation period. Median disease onset in mice after SNS/HPA blockade on day 14 was compared with day 9 in the vehicle group (Fig. 6). Additionally to applying modified EAE score, gait parameters were assessed using the semiautomated CatWalk gait analysis system.

Previously, we have identified sensitive gait parameters typically affected by stroke in male C57BL/6J mice (Hetz et al., 2012). Here, stroke mice from vehicle and SNS/HPA block groups displayed typical alterations in spatial and kinetic characteristics of gait without significant differences between the groups (Table 1).

Together, SIDS blockade boosted CNS antigen-specific immune response in the brain but, compared with vehicle treatment, did not worsen functional long-term outcome measured by modified EAE score or gait analysis.

Blocking SIDS reduced immune cell infiltration into spinal nerve roots

In the long-term course and beyond the initial hemiparesis after experimental stroke, 2D2 mice suffered from paralysis affecting hindpaws and tail. Hence, we hypothesized myelitis caused by CNS antigen-specific immune cell infiltration into the spinal cord. To investigate this phenomenon, we performed immuno-

Figure 7. Representative immunohistochemistry demonstrating infiltration of Mac3⁺ macrophages and CD3⁺ T cells, APP⁺ inflamed axons, and Vα3.2⁺ 2D2 TCR-expressing T cells in the spinal nerve roots of 2D2 mice. Mac3 (A), CD3 (D), APP (G), and Vα3.2 (J) staining of the spinal cord in conjunction with nerve roots at sacral level where most of the cauda equina locates. Areas outlined in A, D, G, and J are zoomed in B, E, H, and K and C, F, I, and L, respectively. C, F, I, L, Arrows indicate immunostained cells (Mac3, CD3, Vα3.2) and axons (APP). There is increased cellular infiltration in single nerve roots, whereas many remain infiltrate-free. Scale bars: A, D, G, J, 200 μm; B, E, H, K, 100 μm; C, F, I, L, 20 μm.

histochemical analysis of the vertebral columns containing nerve roots removed from 2D2 mice 30 d after MCAo. Tissue was stained for macrophages (Mac3⁺), T cells (CD3⁺), inflammatory axons (APP⁺), and MOG-specific T cells (Vα3.2⁺). No immune cells were found in the spinal cord. Surprisingly, immune cells had infiltrated into the spinal nerve roots (Fig. 7). CD3⁺ T cells were typically found in close vicinity of Mac3⁺ macrophages.

Average density (cells/mm²) of Mac3⁺ and CD3⁺ cells was significantly higher in the nerve roots of vehicle-treated mice

(Fig. 8A). The average infiltration of MOG-specific T cells into the spinal nerve roots did not differ significantly between the groups (Fig. 8B), suggesting a considerable recruitment of nontransgenic T cells into the lesions of vehicle-treated mice. APP⁺-inflamed axons appeared at similar density in vehicle and SNS/HPA blockade groups. Spinal nerve roots revealed immunohistochemical signs of polyradiculitis at 14 d after MCAo also in wild-type C57BL/6J mice, however, without significant differences between the vehicle and SNS/HPA block groups (Fig. 8C). Infarct size 24 h after

Figure 8. Blocking SIDS in 2D2, but not in C57BL/6J, mice diminished infiltration of Mac3⁺ and CD3⁺ cells into the spinal nerve roots. **A**, Numbers of Mac3⁺, CD3⁺, and APP⁺ structures per mm² nerve root. Densities of Mac3⁺ (Mann–Whitney U test; $U = 51.0$, $p = 0.031$) and CD3⁺ cells (Mann–Whitney U test; $U = 27.0$, $p = 0.001$) were reduced in the nerve roots of mice from the SNS/HPA block group ($N = 18$) compared with the vehicle-treated mice ($N = 11$) 30 d after experimental stroke. **B**, The number of Va3.2⁺ cells in the nerve roots of 2D2 mice at the same time point did not differ significantly between the groups. **C**, In C57BL/6J mice 14 d after MCAo, the numbers of Mac3⁺, CD3⁺, and APP⁺ structures per mm² spinal nerve root did not differ between the groups. * $p < 0.05$. *** $p < 0.001$.

MCAo did not correlate with any immunohistochemical marker of polyradiculitis, either in 2D2 or in wild-type C57BL/6J mice (data not shown).

SIDS blockade abolished elongation of sciatic nerve F-wave latencies after experimental stroke in 2D2 mice

The phenotype of paralysis accompanied by infiltration of immune cells into the spinal nerve roots was rather flaccid than spastic and thus resembled the clinical characteristics of polyradiculitis. We aimed at characterizing this condition further and measured sciatic nerve conduction velocities to indirectly evaluate damage to myelin sheath around peripheral nerves and neuromuscular function. We measured F-wave latencies and chronodispersion in 2D2 mice upon sciatic nerve stimulation 14 d after MCAo (Fig. 9A, C). Latencies to F-wave were longer in vehicle-treated mice compared with baseline and SNS/HPA block group values (Fig. 9B). F-wave latencies of animals with SNS/HPA blockade remained at baseline, suggesting that SIDS blockade impeded elongated F-wave latencies after experimental stroke in 2D2 mice. Chronodispersion of F-waves did not differ significantly between treatment groups and was in the range of the baseline values (Fig. 9D).

Findings from electrophysiology and immunohistochemistry suggest, that following experimental stroke, 2D2 mice developed a disease phenotype reminiscent of polyradiculitis. Blocking stress axes diminished inflammation of the spinal nerve roots.

Adoptive transfer of 2D2 CD4⁺ T cells to Rag-1KO mice suggests that blockade of SIDS does not further aggravate EAE-like and polyradiculitis-like symptoms or performance on CatWalk gait analysis

The experiments described above demonstrated that inhibiting stress axes to block SIDS reduces infarct volume and boosts CNS antigen-specific autoreactive immune responses in the brain. Because both effects are potentially opposing concerning functional outcome, lack of differences between the vehicle and SNS/HPA

block groups does not necessarily exclude that blockade of SIDS influences long-term outcome of stroke detrimentally. In an attempt to prove whether autoreactive immune response has detrimental effects on long-term outcome after stroke, we needed to exclude the bias introduced by the protective effect of stress blockade with propranolol and mifepristone on infarct volume.

To address this issue, we performed an adoptive transfer experiment using 2D2 mice as donors and Rag-1KO mice lacking functional B and T cells as recipients. Influences of spontaneous poststroke infections on outcome were minimized with preventive antibiotic use. Two million CD4⁺ T cells were harvested from the spleens and lymph nodes of 2D2 donor mice from the vehicle or SNS/HPA block groups 7 d after MCAo and transferred intravenously to Rag-1KO recipients 6 h after MCAo (Fig. 1B, C). For a control, Rag-1KO mice received CD4⁺ T cells from naive 2D2 mice that were not treated, except from antibiotics, and were also not subjected to MCAo. Based on do-

nor, Rag-1KO mice were divided into three groups, transferred with splenocytes and lymphocytes from the naive, vehicle, or SNS/HPA block group of 2D2 mice.

To characterize the cellular composition of lymphocytes for adoptive transfer experiments we analyzed MACS-sorted CD4⁺ T cells isolated from lymph nodes (cervical, mesenteric, inguinal, lumbar) and splenocytes from donor 2D2 naive ($N = 3$), vehicle-treated ($N = 6$), and mifepristone/propranolol-treated mice ($N = 5$) 7 d after MCAo using FACS. The number of MOG-TCR-specific CD4⁺ T cells was similar in all three groups. The vehicle-treated group had less MOG-specific effector memory (CD44⁺ CD62L[−]) and central memory T cells (CD44⁺ CD62L⁺) compared with naive 2D2 mice. Vehicle and SNS/HPA block groups of mice had similar numbers of MOG-specific effector and central memory T cells. The total numbers of effector memory T cells were lower in the vehicle group compared with the SNS/HPA block group of mice. The percentage of regulatory T cells among (MOG-specific) CD4⁺ T cells was not different between the three groups (Fig. 10).

Rag-1KO mice from all three treatment groups developed equally large infarcts measured 24 h after MCAo (Fig. 11A). Therefore, a neuroprotective effect of blocking stress axes with propranolol and mifepristone was apparently not conveyed by adoptively transferred 2D2 CD4⁺ T cells. Time course of body weight and rectal temperature was similar in all groups of Rag-1KO mice (Fig. 11B, C).

EAE-like disease symptoms tended to be less severe in all three transfer groups of Rag-1KO mice compared with 2D2 mice (Figs. 6, 12A) and were developed by 90% of the naive group, 89% of the vehicle group, and 78% of the SNS/HPA block group of reconstituted Rag-1KO mice. Thus, EAE-like symptoms in Rag-1KO mice from the SNS/HPA block group were not aggravated by SIDS blockade in donor 2D2 mice. Consistent with less pronounced neurological deficits in comparison with 2D2 mice, the gait of Rag-1KO recipients after MCAo was not significantly altered, and mice from all three groups performed similarly on CatWalk (data not shown).

These findings were corroborated by electrophysiology where sciatic nerve conduction velocities in recipient *Rag-1KO* mice from all three groups did not differ. Fourteen days after MCAo, F-wave latencies as well as chronodispersion remained at baseline in naive, vehicle, and SNS/HPA block group (Fig. 12*B,C*).

In line with milder EAE-like disease in *Rag-1KO* mice, immunohistochemical analysis of spinal nerve roots revealed ~4 times lower frequencies of CD3⁺ and Mac3⁺ cells and APP⁺ inflamed axons in *Rag-1KO* mice 30 d after MCAo compared with 2D2 mice (Figs. 8, 12*D*). The three groups of *Rag-1KO* mice did not differ regarding the densities of CD3⁺ cells, Mac3⁺ macrophages, and APP⁺ axons in the spinal nerve roots.

Thirty days after MCAo, the transferred CD4⁺ T cells were still present in the brains of *Rag-1KO* mice. They accumulated mainly in the peri-infarct area (Fig. 13*A,D,G,I*), whereas activated Iba-1⁺ microglia/macrophages infiltrated both into infarct and peri-infarct regions (Fig. 13*B,E,H,K*). The relative densities of CD4⁺ T cells and Iba-1⁺ activated microglia/macrophages in the brains of *Rag-1KO* mice 30 d after MCAo did not differ between the three transfer groups (Fig. 13*C*). Upon migrating toward the ischemic core, ramified resting microglia (Fig. 13*F*) had adopted amoeboid-like morphology with thickened processes. In ischemic lesion, their somata appeared irregular or oval (Fig. 13*I*) and cells were observed extravagating through enlarged blood vessels (Fig. 13*L*).

Lymphocyte-deficient *Rag-1KO* mice undergoing MCAo develop EAE-like symptoms following transfer of CD4⁺ T cells from naive, vehicle-treated, and SIDS-blocked 2D2 mice. Dual blocking of SNS and HPA mediated SIDS in donor mice does not further aggravate EAE-like symptoms, performance on CatWalk, or increase the number of CD4⁺ T cell or Iba-1⁺ activated microglia/macrophages in ischemic brains in recipient mice compared with vehicle treatment. Neurological and functional deficits, including EAE-like symptoms, gait deficits, and immune cell infiltration to spinal cord, were lower in *Rag-1KO* mice after adoptive transfer than in 2D2 mice.

Discussion

Stroke induces hyperactivation of stress pathways, including the HPA and the SNS, leading to a rapid and long-lasting systemic suppression of cell-mediated immune responses causing spontaneous systemic bacterial infections within the first days after stroke (for review, see Meisel et al., 2005; Chamorro et al., 2012; Vogelgesang et al., 2014). The biological function of SIDS remains elusive. According to our main hypothesis, SIDS presents an adaptive response preventing autoreactive CNS antigen-specific immune responses after cerebral ischemia (Meisel et al., 2005; Dirnagl et al., 2007). We demonstrate here that preventing SIDS by inhibiting the body's main stress axes SNS and HPA

Figure 9. Increased F-wave latencies upon sciatic nerve stimulation in 2D2 mice after experimental stroke were normalized by blocking SIDS. **A**, Baseline F-wave latency (amplitude in mV vs time in ms). **B**, 2D2 mice from the vehicle group ($N = 24$) had elongated F-wave latencies 14 d after MCAo compared with baseline values (Student's t test, $t_{(149)} = -5.8$, $p < 0.0005$). In contrast, F-wave latencies in the SNS/HPA block group ($N = 37$; Student's t test, $t_{(59)} = 2.5$, $p = 0.016$) remained at baseline ($N = 127$; baseline). Dark gray dotted line indicates median (50th percentile). Gray rectangle represents area between the 25th and 75th percentiles. Light gray rectangle represents range between minimum and maximum values. **C**, Delayed F-wave. **D**, F-wave chronodispersion remained at baseline in both groups of 2D2 mice.

increases delayed MOG-specific autoreactive CD4⁺ T-cell responses in the brains of MOG TCR transgenic 2D2 mice but does not worsen functional long-term outcome after experimental stroke.

After cerebral ischemia, the disruption of the blood–brain barrier allows leukocytes, including neutrophils, macrophages, and lymphocytes, to enter the damaged brain tissue and brain antigens to enter peripheral circulation. This process may expose brain epitopes, which are normally “invisible” to the immune system and thus promote priming and activation of lymphocytes reactive to the CNS antigens after stroke (Becker et al., 2005). Patients with cerebral ischemia were shown to have elevated antibody titers as well as increased numbers of circulating T cells specific for CNS antigens compared with age-matched healthy controls, indicating that inflammatory processes in the damaged CNS tissue may indeed induce self-directed immune responses (Bornstein et al., 2001; Dambinova et al., 2003). Depletion of circulating T-cell populations, which is a key mechanism of SIDS (Prass et al., 2003), reduces ischemic brain damage in experimental models of stroke (Kleinschnitz et al., 2010). While increasing the number of CNS antigen-specific Th1 cells in the brain, SNS/HPA blockade reduced levels of IL-10, which is mainly produced by regulatory T cells. TGF- β 1, a further regulatory type cytokine,

Figure 10. Immunological characterization of CD4⁺ T cells obtained from cervical, mesenteric, inguinal, and lumbar lymph nodes and spleens of donor 2D2 mice (naive, $N = 3$, or vehicle-treated, $N = 6$; and mifepristone-/propranolol-treated mice, $N = 5$, 7 d after MCAo for intravenous transfer into *Rag-1KO* recipient mice. **A**, The number of MOG-specific CD4⁺ T cells was similar in all three groups of donor mice. **B**, Vehicle group of donor mice had less MOG-specific effector (Kruskal–Wallis with Mann–Whitney U test, $U = 1.000$, $p = 0.048$) and central memory T cells (Kruskal–Wallis with Mann–Whitney U test, $U = 0.000$, $p = 0.024$) compared with naive but not SNS/HPA block group of 2D2 donor mice. Cumulative number of effector memory T cells was lower in vehicle compared with SNS/HPA block group of mice (Kruskal–Wallis with Mann–Whitney U test, $U = 3.000$, $p = 0.030$). * $p < 0.05$. **C**, The percentage of regulatory T cells among (MOG-specific) CD4⁺ T cells was not affected by the group of donor 2D2 mice.

Figure 11. Infarct volumes, measured 24 h after MCAo (**A**), body temperature (**B**), and body weight (**C**) of *Rag-1KO* mice, which were adoptively transferred 2×10^6 CD4⁺ T cells from naive ($N = 10$), vehicle-treated ($N = 13$), or SNS/HPA-blocked 2D2 donor mice ($N = 13$), did not differ significantly.

was increased in the SNS/HPA blockade compared with the vehicle group. However, TGF- β 1 is not only produced by regulatory T cells but predominantly by activated microglia/macrophages (Doyle et al., 2010). Thus, SIDS might prevent potential harmful activation of T cells, including those reactive to CNS antigens, after brain injury.

Others have demonstrated that T cells specific to myelin antigens reduce secondary neurodegeneration and promote functional recovery after CNS injury (Moalem et al., 1999; Hauben et al., 2000; Lewitus et al., 2006), indicating that autoimmune responses may be protective under certain conditions. Interestingly, induction of oral tolerance to myelin basic protein (Becker et al., 1997) or MOG (Frenkel et al., 2003) via nasal administration was found to reduce infarct size and improve functional

recovery. In contrast, sensitization with myelin antigens worsens the outcome in experimental models of cerebral ischemia (Becker et al., 1997). The mechanisms that govern protective versus nonprotective autoreactive T-cell responses after CNS injury are not understood but may depend on antigen specificity and phenotype of effector T cells, autoregulatory mechanisms including activation of regulatory T cells, genetic background, and on other factors that determine the strength and timing of autoreactive T-cell activation (Becker et al., 1997; Moalem et al., 1999; Jones et al., 2002, 2005; Frenkel et al., 2003; Lewitus et al., 2006).

Infections may stimulate antigen-presenting cells leading to the activation of autoreactive T cells by otherwise innocuous antigens (Kissler et al., 2001; Hofstetter et al., 2003; Becker et al., 2011; Becker, 2012). In our experiments, we aimed to investigate direct effects of SIDS on CNS antigen-specific autoreactive immune responses. For the following reasons, we treated all mice independently of group assignment with the fluoroquinolone antibiotic enrofloxacin in an infection-preventive manner. Spontaneous poststroke pneumonia is known to affect functional long-term outcome in experimental stroke (Hetze et al., 2013). In addition, the risk of poststroke infections increases with infarct size (Hug et al., 2009), and mice from the SNS/HPA blockade group had significantly smaller infarcts than mice from the vehicle group. Bacterial components, such as lipopolysaccharide, might boost CNS antigen-specific autoreactive immune responses in experimental as well as human stroke (Becker et al., 2005, 2011). Moreover, we have previously

demonstrated that blocking SNS not only blocks stroke-induced immunodepression but also prevents poststroke infections (Prass et al., 2003, 2006). Finally, we have identified spontaneous poststroke infections as a contributing factor for CNS antigen-specific autoreactive immune responses in the mouse MCAo model (Klehm et al., unpublished). Thus, poststroke infection is an interfering factor for investigations on CNS antigen-specific autoreactive immune responses in stroke. Therefore, we performed all experiments under preventive antibiotic treatment with enrofloxacin. We cannot exclude direct immunomodulatory effects by enrofloxacin. However, these effects would rather underestimate the observed CNS antigen-specific autoreactive immune responses because fluoroquinolones might exert anti-inflammatory effects (Dalhoff and Shalit, 2003), and our results suggest that coping with infections is more efficient when SIDS is blocked.

In line with previous studies on laboratory rodents (Vandeputte et al., 2010; Hetze et al., 2012; Li et al., 2013), mice displayed typical stroke-induced alterations in gait: asymmetry, increased stand duration and base of support, shorter stride lengths, reduced maximal contact area, and normalized swing speed. Additionally, with a median onset 9–14 d after stroke, 2D2 mice developed an EAE-like phenotype. MOG-specific T cells, in particular CD4⁺ Th1 and Th17 cells, can mediate EAE (Linington et al., 1993; Genain et al., 1996; Stromnes et al., 2008; Bettini et al., 2009; Jäger et al., 2009). Neurological deficits caused directly by stroke typically subside within the first week after MCAo in mice. However, we observed thereafter that 2D2 mice developed a disease phenotype with symptoms reminiscent of EAE. Whereas stroke affects predominantly the performance of forepaw contralaterally to the ischemic lesion, EAE-like symptoms are characterized by progressive and ascending weakness/paralysis of the tail and limbs. The EAE-like phenotype occurred within 1 and 3 weeks after MCAo. Whereas this phenotype has been consistently observed in 2D2 mice, only a few wild-type mice develop mild EAE-like symptoms (data not shown).

The functional deficit in 2D2 mice manifesting clinically with flaccid rather than spastic paralysis was reminiscent of polyradiculitis, which was supported electrophysiologically with elongated F-wave latencies, suggesting demyelination, and histologically with infiltration of immune cells into the spinal nerve roots. This finding might be counterintuitive because most stroke patients suffer from a functional deficit, which is well explained by the corresponding contralateral ischemic brain lesion. However, it correlates with human studies demonstrating pathological ulnar F-wave variables of severely affected unconscious stroke patients (Chroni et al., 2006, 2007). In contrast to our primary hypothesis

Figure 12. Following intravenous transfer of 2×10^6 CD4⁺ T cells from naive ($N = 10$), vehicle-treated ($N = 13$), or SNS/HPA-blocked 2D2 donor mice ($N = 13$), *Rag-1KO* mice developed milder EAE-like disease symptoms (**A**; naive, $N = 10$; vehicle, $N = 9$; SNS/HPA block, $N = 9$; data are median $\pm$ range) compared with 2D2 mice. F-wave latencies (**B**; naive, $N = 20$; vehicle, $N = 16$; SNS/HPA block, $N = 14$) and F-wave chronodispersion upon sciatic nerve stimulation (**C**) remained at baseline ($N = 19$). Immunological infiltrates, composed of Mac3⁺ and CD3⁺ cells, APP⁺ inflamed axons in the spinal nerve roots (**D**; naive, $N = 9$; vehicle, $N = 8$; SNS/HPA block, $N = 6$) were ~ 4 times less frequent than in 2D2 mice.

that SIDS prevents autoreactive immune responses, the polyradiculitis-like phenotype was rather diminished by blocking SIDS in 2D2 mice. These findings are difficult to interpret because SNS/HPA blockade also reduced infarct size and might reduce infection rate. However, our data suggest that neither lesion size nor poststroke infection accounts for the protective effect of SNS/HPA blockade against polyradiculitis. Moreover, adoptive transfer of 2D2 CD4⁺ T cells to *Rag-1KO* mice suggests that blockade of SIDS does not further deteriorate functional outcome after experimental stroke, including the polyradiculitis-like phenotype. This phenomenon needs to be addressed in further studies.

An unexpected finding of our study was the significantly infarct volume reducing effect of SNS/HPA blockade, which might be responsible for the better survival and temperature maintenance. Both stress systems typically potentiate each other in their effects (McEwen et al., 1987; Malbon and Hadcock, 1988; Mak et al., 1995). Because propranolol and mifepristone carry immuno-

Figure 13. $CD4^+$ T cells and $Iba-1^+$ macrophages were found in Rag-1KO mice brains 30 d after MCAo and adoptive cell transfer from naive and treated 2D2 donors. The latter underwent MCAo. Images from $CD4$ (green) and $Iba-1$ (red) stained brain sections were taken from three regions indicated in **A**, **B**, with solid black line outlining ischemic core: Region 1, contralateral hemisphere; Region 2, nonischemic region adjacent to infarct in ipsilateral hemisphere; and Region 3, peri-infarct region in ipsilateral hemisphere. **D–F**, Thick arrow indicates the typical morphology of resting microglia with thin processes. **G–I**, Thick arrow indicates the morphology of activated microglia with an amoeboid-like shape, large cell body and thick processes. **J–L**, Immune cell infiltration was highest at and within ischemic core. **C**, Densities of $CD4^+$ and $Iba-1^+$ cells per mm^2 Rag-1 KO mouse brain ipsilateral hemisphere in SNS/HPA block ($N = 4$), naive ($N = 8$), and vehicle group ($N = 8$) 30 d after cell transfer and MCAo. VL, Vessel lumen. Scale bar, 20 μm .

modulatory properties, the smaller infarct volumes might be due to effects on early infiltrating immune cells and brain resident cells. Furthermore, different subsets of lymphocytes can exert detrimental as well as protective functions in cerebral ischemia (Yilmaz et al., 2006; Hurn et al., 2007; Liesz et al., 2009, 2011; Shichita et al., 2009; Kleinschnitz et al., 2010, 2013). However, because stress blockade prevented the development of large infarcts very early after stroke onset, immune-mediated mechanisms as primary sources for this are unlikely. Mechanistically, we excluded delayed infarct maturation, which has been observed in experimental stroke when studying mechanisms of inflammation and apoptosis (Du et al., 1996; Becker et al., 1997; Frenkel et al., 2003). CBF has been shown to inversely correlate with infarct volume (Dalkara et al., 1994). Propranolol might affect CBF (Edvinsson et al., 1979; Ley et al., 2009). CBF, measured by FAIR-MRI, was not altered by SNS/HPA blockade. However, we cannot exclude that mild but prolonged CBF elevations, which are below the detection threshold of FAIR-MRI (Leithner et al., 2008), contribute to the neuroprotective mechanism of SNS/HPA blockade. Further possibilities might include induction of Heat shock protein 72 by β_2 -adrenoreceptor antagonist (Han et al., 2009) and antioxidant properties of mifepristone (Parthasarathy et al., 1994; Roberts et al., 1996; Behl et al., 1997; Antonawich et al., 1999; McCullers et al., 2002). Our data suggest that both mifepristone and propranolol are required to elicit neuroprotection in the SNS/HPA blockade group.

A limitation of our study is that we have measured CNS antigen-specific autoreactivity only at one time point after experimental stroke. Although the Elispot is considered a very sensitive method, this assay requires a high number of brain-infiltrating T cells to reliably measure CNS antigen-specific T-cell responses. Therefore, we performed Elispot experiments only at that time point where the number of brain-infiltrating T cells was sufficiently high to receive robust data. In the MCAo model, T-cell infiltration into the brain parenchyma is highest at day 14 and day 30 but relatively low at day 7 after stroke onset (Stubbe et al., 2013). Moreover, we considered this time window to be sufficient for MOG-specific $CD4^+$ T cells to get primed (Janeway, 1989) and to differentiate into effector/memory T cells (Jäger et al., 2009). Another limitation of our study is that the main findings are based on investigations in 2D2 mice. Because the majority of their $CD4^+$ T cells carry a functional receptor for MOG, finding on CNS antigen-specific autoreactivity is most likely an overestimation of the situation in wild-type mice. We observed, even in wild-type C57BL/6J mice, Th1 responses against MOG in brain infiltrating lymphocytes 14 d after MCAo. However, the frequencies were much lower compared with 2D2 mice and only observed in animals with accompanying poststroke infections (Klehmet et al., unpublished).

The concept of SIDS has promoted the development of immunomodulatory treatment approaches for stroke (Shichita et al., 2009, 2012; Wong et al., 2011; Chamorro et al., 2012; Kamel

and Iadecola, 2012). Modulating SIDS, however, might be a double-edged-sword approach reducing the risk for infection at cost of boosting detrimental autoimmune response against CNS antigens and worsening functional long-term outcome (Meisel and Meisel, 2011). Here we provide evidence that reversing SIDS by blocking body's stress axes increases autoreactive CNS antigen-specific immune responses in the brain but does not impair long-term functional outcome in our murine model of experimental stroke compared with vehicle treatment.

References

- Adelmann M, Wood J, Benzel I, Fiori P, Lassmann H, Matthieu JM, Gardinier MV, Dornmair K, Linington C (1995) The N-terminal domain of the myelin oligodendrocyte glycoprotein (MOG) induces acute demyelinating experimental autoimmune encephalomyelitis in the Lewis rat. *J Neuroimmunol* 63:17–27. [CrossRef Medline](#)
- Ando DG, Clayton J, Kono D, Urban JL, Sercarz EE (1989) Encephalitogenic T-cells in the B10.PL model of experimental allergic encephalomyelitis (EAE) are of the Th-1 lymphokine subtype. *Cell Immunol* 124:132–143. [CrossRef Medline](#)
- Antonawich FJ, Miller G, Rigsby DC, Davis JN (1999) Regulation of ischemic cell death by glucocorticoids and adrenocorticotrophic hormone. *Neuroscience* 88:319–325. [CrossRef Medline](#)
- Becker KJ (2012) Activation of immune responses to brain antigens after stroke. *J Neurochem* 123 [Suppl 2]:148–155.
- Becker KJ, McCarron RM, Ruetzler C, Laban O, Sternberg E, Flanders KC, Hallenbeck JM (1997) Immunologic tolerance to myelin basic protein decreases stroke size after transient focal cerebral ischemia. *Proc Natl Acad Sci U S A* 94:10873–10878. [CrossRef Medline](#)
- Becker KJ, Kindrick DL, Lester MP, Shea C, Ye ZC (2005) Sensitization to brain antigens after stroke is augmented by lipopolysaccharide. *J Cereb Blood Flow Metab* 25:1634–1644. [CrossRef Medline](#)
- Becker KJ, Kalil AJ, Tanzi P, Zierath DK, Savos AV, Gee JM, Hadwin J, Carter KT, Shibata D, Cain KC (2011) Autoimmune responses to the brain after stroke are associated with worse outcome. *Stroke* 42:2763–2769. [CrossRef Medline](#)
- Bederson JB, Pitts LH, Tsuji M, Nishimura MC, Davis RL, Bartkowski H (1986) Rat middle cerebral-artery occlusion: evaluation of the model and development of a neurologic examination. *Stroke* 17:472–476. [CrossRef Medline](#)
- Behl C, Trapp T, Skutella T, Holsboer F (1997) Protection against oxidative stress-induced neuronal cell death: a novel role for RU486. *Eur J Neurosci* 9:912–920. [CrossRef Medline](#)
- Bettelli E, Pagany M, Weiner HL, Linington C, Sobel RA, Kuchroo VK (2003) Myelin oligodendrocyte glycoprotein-specific T cell receptor transgenic mice develop spontaneous autoimmune optic neuritis. *J Exp Med* 197:1073–1081. [CrossRef Medline](#)
- Bettini M, Rosenthal K, Evavold BD (2009) Pathogenic MOG-reactive CD8⁺ T cells require MOG-reactive CD4⁺ T cells for sustained CNS inflammation during chronic EAE. *J Neuroimmunol* 213:60–68. [CrossRef Medline](#)
- Bornstein NM, Aronovich B, Korczyn AD, Shavit S, Michaelson DM, Chapman J (2001) Antibodies to brain antigens following stroke. *Neurology* 56:529–530. [CrossRef Medline](#)
- Brunner C, Lassmann H, Waehneldt TV, Matthieu JM, Linington C (1989) Differential ultrastructural-localization of myelin basic-protein, myelin oligodendroglial glycoprotein, and 2',3'-cyclic nucleotide 3'-phosphodiesterase in the CNS of adult-rats. *J Neurochem* 52:296–304. [CrossRef Medline](#)
- Chamorro A, Urra X, Planas AM (2007) Infection after acute ischemic stroke: a manifestation of brain-induced immunodepression. *Stroke* 38:1097–1103. [CrossRef Medline](#)
- Chamorro Á, Meisel A, Planas AM, Urra X, van de Beek D, Velkamp R (2012) The immunology of acute stroke. *Nat Rev Neurol* 8:401–410. [CrossRef Medline](#)
- Chroni E, Katsoulas G, Argyriou AA, Sakellaropoulos GC, Polychronopoulos P, Nikiforidis G (2006) Level of consciousness as a conditioning factor of F wave generation in stroke patients. *Clin Neurophysiol* 117:315–319. [CrossRef Medline](#)
- Chroni E, Argyriou AA, Katsoulas G, Polychronopoulos P (2007) Ulnar F wave generation assessed within 3 days after the onset of stroke in patients with relatively preserved level of consciousness. *Clin Neurol Neurosurg* 109:27–31. [CrossRef Medline](#)
- Cua DJ, Sherlock J, Chen Y, Murphy CA, Joyce B, Seymour B, Lucian L, To W, Kwan S, Churakova T, Zurawski S, Wiekowski M, Lira SA, Gorman D, Kastelein RA, Sedgwick JD (2003) Interleukin-23 rather than interleukin-12 is the critical cytokine for autoimmune inflammation of the brain. *Nature* 421:744–748. [CrossRef Medline](#)
- Dalhoff A, Shalit I (2003) Immunomodulatory effects of quinolones. *Lancet Infect Dis* 3:359–371. [CrossRef Medline](#)
- Dalkara T, Morikawa E, Panahian N, Moskowitz MA (1994) Blood flow-dependent functional recovery in a rat model of focal cerebral ischemia. *Am J Physiol* 267:H678–H683. [Medline](#)
- Dambinova SA, Khounteev GA, Izykenova GA, Zavolokov IG, Ilyukhina AY, Skoromets AA (2003) Blood test detecting autoantibodies to N-methyl-D-aspartate neuroreceptors for evaluation of patients with transient ischemic attack and stroke. *Clin Chem* 49:1752–1762. [CrossRef Medline](#)
- Dirnagl U, Members of the MCAO-SOP Group (2012) Standard operating procedures (SOP) in experimental stroke research: SOP for middle cerebral artery occlusion in the mouse. *Nat Protoc*.
- Dirnagl U, Klehmet J, Braun JS, Harms H, Meisel C, Ziemssen T, Prass K, Meisel A (2007) Stroke-induced immunodepression: experimental evidence and clinical relevance. *Stroke* 38:770–773. [CrossRef Medline](#)
- Doyle KP, Cekanaviciute E, Mamer LE, Buckwalter MS (2010) TGFβ signaling in the brain increases with aging and signals to astrocytes and innate immune cells in the weeks after stroke. *J Neuroinflammation* 7:62. [CrossRef Medline](#)
- Du C, Hu R, Csernansky CA, Hsu CY, Choi DW (1996) Very delayed infarction after mild focal cerebral ischemia: a role for apoptosis? *J Cereb Blood Flow Metab* 16:195–201. [CrossRef Medline](#)
- Edvinsson L, Lacombe P, Owman C, Reynier-Rebuffel AM, Seylaz J (1979) Quantitative changes in regional cerebral blood-flow of rats induced by alpha-adrenergic and beta-adrenergic stimulants. *Acta Physiol Scand* 107:289–296. [CrossRef Medline](#)
- Elenkov IJ, Wilder RL, Chrousos GP, Vizi ES (2000) The sympathetic nerve, an integrative interface between two supersystems: the brain and the immune system. *Pharmacol Rev* 52:595–638. [Medline](#)
- Emsley HC, Hopkins SJ (2008) Acute ischaemic stroke and infection: recent and emerging concepts. *Lancet Neurol* 7:341–353. [CrossRef Medline](#)
- Engel O, Meisel A (2010) Models of infection before and after stroke: investigating new targets. *Infect Disord Drug Targets* 10:98–104. [CrossRef Medline](#)
- Finlayson O, Kapral M, Hall R, Asllani E, Selchen D, Saposnik G (2011) Risk factors, inpatient care, and outcomes of pneumonia after ischemic stroke. *Neurology* 77:1338–1345. [CrossRef Medline](#)
- Fisher MA (2007) F-waves-physiology and clinical uses. *Sci World J* 7:144–160. [CrossRef Medline](#)
- Frenkel D, Huang Z, Maron R, Koldzic DN, Hancock WW, Moskowitz MA, Weiner HL (2003) Nasal vaccination with myelin oligodendrocyte glycoprotein reduces stroke size by inducing IL-10-producing CD4(+) T cells. *J Immunol* 171:6549–6555. [CrossRef Medline](#)
- Global Burden of Disease Study (2012) Global Burden of Disease Study 2010. *Lancet* 380:2053–2260.
- Gee JM, Kalil A, Thullbery M, Becker KJ (2008) Induction of immunologic tolerance to myelin basic protein prevents central nervous system autoimmunity and improves outcome after stroke. *Stroke* 39:1575–1582. [CrossRef Medline](#)
- Gee JM, Zierath D, Hadwin J, Savos A, Kalil A, Thullbery M, Becker KJ (2009) Long term immunologic consequences of experimental stroke and mucosal tolerance. *Exp Transl Stroke Med* 1:3. [CrossRef Medline](#)
- Genain CP, Abel K, Belmar N, Villinger F, Rosenberg DP, Linington C, Raine CS, Hauser SL (1996) Late complications of immune deviation therapy in a nonhuman primate. *Science* 274:2054–2057. [CrossRef Medline](#)
- Han RQ, Ouyang YB, Xu L, Agrawal R, Patterson AJ, Giffard RG (2009) Posts ischemic brain injury is attenuated in mice lacking the beta2-adrenergic receptor. *Anesth Analg* 108:280–287. [CrossRef Medline](#)
- Hauben E, Nevo U, Yoles E, Moalem G, Agranov E, Mor F, Akselrod S, Neeman M, Cohen IR, Schwartz M (2000) Autoimmune T cells as potential neuroprotective therapy for spinal cord injury. *Lancet* 355:286–287. [CrossRef Medline](#)
- Hetze S, Römer C, Teufelhart C, Meisel A, Engel O (2012) Gait analysis as a method for assessing neurological outcome in a mouse model of stroke. *J Neurosci Methods* 206:7–14. [CrossRef Medline](#)

- Hetze S, Engel O, Römer C, Mueller S, Dirnagl U, Meisel C, Meisel A (2013) Superiority of preventive antibiotic treatment compared with standard treatment of poststroke pneumonia in experimental stroke: a bed to bench approach. *J Cereb Blood Flow Metab* 33:846–854. [CrossRef Medline](#)
- Hofstetter HH, Sewell DL, Liu F, Sandor M, Forsthuber T, Lehmann PV, Fabry Z (2003) Autoreactive T cells promote post-traumatic healing in the central nervous system. *J Neuroimmunol* 134:25–34. [CrossRef Medline](#)
- Hug A, Dalpke A, Wiczorek N, Giese T, Lorenz A, Auffarth G, Liesz A, Veltkamp R (2009) Infarct volume is a major determiner of post-stroke immune cell function and susceptibility to infection. *Stroke* 40:3226–3232. [CrossRef Medline](#)
- Hurn PD, Subramanian S, Parker SM, Afentoulis ME, Kaler LJ, Vandenbark AA, Offner H (2007) T- and B-cell-deficient mice with experimental stroke have reduced lesion size and inflammation. *J Cereb Blood Flow Metab* 27:1798–1805. [CrossRef Medline](#)
- Jäger A, Dardalhon V, Sobel RA, Bettelli E, Kuchroo VK (2009) Th1, Th17, and Th9 effector cells induce experimental autoimmune encephalomyelitis with different pathological phenotypes. *J Immunol* 183:7169–7177. [CrossRef Medline](#)
- Janeway CA Jr (1989) The priming of helper T cells. *Semin Immunol* 1:13–20. [Medline](#)
- Jones TB, Basso DM, Sodhi A, Pan JZ, Hart RP, MacCallum RC, Lee S, Whitacre CC, Popovich PG (2002) Pathological CNS autoimmune disease triggered by traumatic spinal cord injury: implications for autoimmune vaccine therapy. *J Neurosci* 22:2690–2700. [Medline](#)
- Jones TB, Hart RP, Popovich PG (2005) Molecular control of physiological and pathological T-cell recruitment after mouse spinal cord injury. *J Neurosci* 25:6576–6583. [CrossRef Medline](#)
- Kamel H, Iadecola C (2012) Brain-immune interactions and ischemic stroke: clinical implications. *Arch Neurol* 69:576–581. [CrossRef Medline](#)
- Katzan IL, Cebul RD, Husak SH, Dawson NV, Baker DW (2003) The effect of pneumonia on mortality among patients hospitalized for acute stroke. *Neurology* 60:620–625. [CrossRef Medline](#)
- Kissler S, Anderton SM, Wraith DC (2001) Antigen-presenting cell activation: a link between infection and autoimmunity? *J Autoimmun* 16:303–308. [CrossRef Medline](#)
- Kleinschnitz C, Kraft P, Dreykluft A, Hagedorn I, Göbel K, Schuhmann MK, Langhauser F, Helluy X, Schwarz T, Bittner S, Mayer CT, Brede M, Varallyay C, Pham M, Bendszus M, Jakob P, Magnus T, Meuth SG, Iwakura Y, Zernecke A, et al. (2013) Regulatory T cells are strong promoters of acute ischemic stroke in mice by inducing dysfunction of the cerebral microvasculature. *Blood* 121:679–691. [CrossRef Medline](#)
- Kleinschnitz C, Schwab N, Kraft P, Hagedorn I, Dreykluft A, Schwarz T, Austinat M, Nieswandt B, Wiendl H, Stoll G (2010) Early detrimental T-cell effects in experimental cerebral ischemia are neither related to adaptive immunity nor thrombus formation. *Blood* 115:3835–3842. [CrossRef Medline](#)
- Koennecke HC, Belz W, Berfelde D, Endres M, Fitzek S, Hamilton F, Kreitsch P, Mackert BM, Nabavi DG, Nolte CH, Pöhls W, Schmehl I, Schmitz B, von Brevern M, Walter G, Heuschmann PU, Berlin Stroke Register I (2011) Factors influencing in-hospital mortality and morbidity in patients treated on a stroke unit. *Neurology* 77:965–972. [CrossRef Medline](#)
- Kuchroo VK, Anderson AC, Waldner H, Munder M, Bettelli E, Nicholson LB (2002) T-cell response in experimental autoimmune encephalomyelitis (EAE): role of self and cross-reactive antigens in shaping, tuning, and regulating the autopathogenic T cell repertoire. *Annu Rev Immunol* 20:101–123. [CrossRef Medline](#)
- Kumar S, Selim MH, Caplan LR (2010) Medical complications after stroke. *Lancet Neurol* 9:105–118. [CrossRef Medline](#)
- Langrish CL, Chen Y, Blumenschein WM, Mattson J, Basham B, Sedgwick JD, McClanahan T, Kastelein RA, Cua DJ (2005) IL-23 drives a pathogenic T cell population that induces autoimmune inflammation. *J Exp Med* 201:233–240. [CrossRef Medline](#)
- Leithner C, Gertz K, Schröck H, Priller J, Prass K, Steinbrink J, Villringer A, Endres M, Lindauer U, Dirnagl U, Royle G (2008) A flow sensitive alternating inversion recovery (FAIR)-MRI protocol to measure hemispheric cerebral blood flow in a mouse stroke model. *Exp Neurol* 210:118–127. [CrossRef Medline](#)
- Leithner C, Müller S, Füchtmeier M, Lindauer U, Dirnagl U, Royle G (2010) Determination of the brain-blood partition coefficient for water in mice using MRI. *J Cereb Blood Flow Metab* 30:1821–1824. [CrossRef Medline](#)
- Lewitus GM, Kipnis J, Avidan H, Ben-Nun A, Schwartz M (2006) Neuroprotection induced by mucosal tolerance is epitope-dependent: conflicting effects in different strains. *J Neuroimmunol* 175:31–38. [CrossRef Medline](#)
- Ley EJ, Schemm J, Park R, Schroff S, Dagliyan G, Conti PS, Margulies DR, Salim A (2009) The in vivo effect of propranolol on cerebral perfusion and hypoxia after traumatic brain injury. *J Trauma* 66:154–159; discussion 159–161. [CrossRef Medline](#)
- Li S, Shi Z, Zhang H, Liu X, Chen S, Jin J, Wang Y, Jia W, Li H (2013) Assessing gait impairment after permanent middle cerebral artery occlusion in rats using an automated computer-aided control system. *Behav Brain Res* 250C:174–191. [CrossRef Medline](#)
- Liesz A, Suri-Payer E, Veltkamp C, Doerr H, Sommer C, Rivest S, Giese T, Veltkamp R (2009) Regulatory T cells are key cerebroprotective immunomodulators in acute experimental stroke. *Nat Med* 15:192–199. [CrossRef Medline](#)
- Liesz A, Zhou W, Mracskó É, Karcher S, Bauer H, Schwarting S, Sun L, Bruder D, Stegemann S, Cerwenka A, Sommer C, Dalpke AH, Veltkamp R (2011) Inhibition of lymphocyte trafficking shields the brain against deleterious neuroinflammation after stroke. *Brain* 134:704–720. [CrossRef Medline](#)
- Linington C, Berger T, Perry L, Weerth S, Hinze-Selch D, Zhang Y, Lu HC, Lassmann H, Wekerle H (1993) T cells specific for the myelin oligodendrocyte glycoprotein mediate an unusual autoimmune inflammatory response in the central nervous system. *Eur J Immunol* 23:1364–1372. [CrossRef Medline](#)
- Livnat S, Felten SY, Carlson SL, Bellinger DL, Felten DL (1985) Involvement of peripheral and central catecholamine systems in neural-immune interactions. *J Neuroimmunol* 10:5–30. [CrossRef Medline](#)
- MacDonald KD, Chang HY, Mitzner W (2009) An improved simple method of mouse lung intubation. *J Appl Physiol* 106:984–987. [CrossRef Medline](#)
- Mak JC, Nishikawa M, Shirasaki H, Miyayasu K, Barnes PJ (1995) Protective effects of a glucocorticoid on downregulation of pulmonary beta 2-adrenergic receptors in vivo. *J Clin Invest* 96:99–106. [CrossRef Medline](#)
- Malbon CC, Hadcock JR (1988) Evidence that glucocorticoid response elements in the 5′-noncoding region of the hamster beta 2-adrenergic receptor gene are obligate for glucocorticoid regulation of receptor mRNA levels. *Biochem Biophys Res Commun* 154:676–681. [CrossRef Medline](#)
- McCullers DL, Sullivan PG, Scheff SW, Herman JP (2002) Mifepristone protects CA1 hippocampal neurons following traumatic brain injury in rat. *Neuroscience* 109:219–230. [CrossRef Medline](#)
- McEwen B, Brinton R, Harrelson A, Rostene W (1987) Modulatory interactions between steroid hormones, neurotransmitters and neuropeptides in hippocampus. *Adv Biochem Psychopharmacol* 43:87–102. [Medline](#)
- Meisel A, Meisel C (2008) Stroke-induced immunodepression: consequences, mechanisms and therapeutic implications. *Future Neurol* 3:551–563. [CrossRef](#)
- Meisel C, Meisel A (2011) Suppressing immunosuppression after stroke. *N Engl J Med* 365:2134–2136. [CrossRef Medline](#)
- Meisel C, Prass K, Braun J, Victorov I, Wolf T, Megow D, Halle E, Volk HD, Dirnagl U, Meisel A (2004) Preventive antibacterial treatment improves the general medical and neurological outcome in a mouse model of stroke. *Stroke* 35:2–6. [CrossRef Medline](#)
- Meisel C, Schwab JM, Prass K, Meisel A, Dirnagl U (2005) Central nervous system injury-induced immune deficiency syndrome. *Nat Rev Neurosci* 6:775–786. [CrossRef Medline](#)
- Millan M, Davalos A (2006) The need for new therapies for acute ischaemic stroke. *Cerebrovasc Dis* 22:3–9. [CrossRef](#)
- Moalem G, Leibowitz-Amit R, Yoles E, Mor F, Cohen IR, Schwartz M (1999) Autoimmune T cells protect neurons from secondary degeneration after central nervous system axotomy. *Nat Med* 5:49–55. [CrossRef Medline](#)
- Parthasarathy S, Morales AJ, Murphy AA (1994) Antioxidant: a new role for RU-486 and related compounds. *J Clin Invest* 94:1990–1995. [CrossRef Medline](#)
- Prass K, Meisel C, Höflich C, Braun J, Halle E, Wolf T, Ruscher K, Victorov IV, Priller J, Dirnagl U, Volk HD, Meisel A (2003) Stroke-induced immunodeficiency promotes spontaneous bacterial infections and is mediated by sympathetic activation reversal by poststroke T helper cell type 1-like immunostimulation. *J Exp Med* 198:725–736. [CrossRef Medline](#)

- Prass K, Braun JS, Dirnagl U, Meisel C, Meisel A (2006) Stroke propagates bacterial aspiration to pneumonia in a model of cerebral ischemia. *Stroke* 37:2607–2612. [CrossRef Medline](#)
- Rivers TM, Sprunt DH, Berry GP (1933) Observations on attempts to produce acute disseminated encephalomyelitis in monkeys. *J Exp Med* 58:39–53. [CrossRef Medline](#)
- Roberts CP, Murphy AA, Santanam N, Parthasarathy S (1996) The effect of RU 486 and related compounds on cultured macrophage differentiation and function. *Am J Obstet Gynecol* 175:248–257; discussion 257–259. [CrossRef Medline](#)
- Rocco A, Fam G, Sykora M, Diedler J, Nagel S, Ringleb P (2013) Poststroke infections are an independent risk factor for poor functional outcome after three-months in thrombolysed stroke patients. *Int J Stroke* 8:639–644. [CrossRef Medline](#)
- Royl G, Balkaya M, Lehmann S, Lehnardt S, Stohlmann K, Lindauer U, Endres M, Dirnagl U, Meisel A (2009) Effects of the PDE5-inhibitor vardenafil in a mouse stroke model. *Brain Res* 1265:148–157. [CrossRef Medline](#)
- Sanders VM, Baker RA, Ramer-Quinn DS, Kasprovicz DJ, Fuchs BA, Street NE (1997) Differential expression of the beta2-adrenergic receptor by Th1 and Th2 clones: implications for cytokine production and B cell help. *J Immunol* 158:4200–4210. [Medline](#)
- Shichita T, Sugiyama Y, Ooboshi H, Sugimori H, Nakagawa R, Takada I, Iwaki T, Okada Y, Iida M, Cua DJ, Iwakura Y, Yoshimura A (2009) Pivotal role of cerebral interleukin-17-producing gamma δ T cells in the delayed phase of ischemic brain injury. *Nat Med* 15:946–950. [CrossRef Medline](#)
- Shichita T, Ago T, Kamouchi M, Kitazono T, Yoshimura A, Ooboshi H (2012) Novel therapeutic strategies targeting innate immune responses and early inflammation after stroke. *J Neurochem* 123 [Suppl 2]:29–38.
- Stromnes IM, Cerretti LM, Liggitt D, Harris RA, Gorman JM (2008) Differential regulation of central nervous system autoimmunity by T(H)1 and T(H)17 cells. *Nat Med* 14:337–342. [CrossRef Medline](#)
- Stubbe T, Ebner F, Richter D, Engel O, Randolph Engel O, Klehmet J, Royl G, Meisel A, Nitsch R, Meisel C, Brandt C (2013) Regulatory T cells accumulate and proliferate in the ischemic hemisphere for up to 30 days after MCAO. *J Cereb Blood Flow Metab* 33:37–47. [CrossRef Medline](#)
- Tischner D, Reichardt HM (2007) Glucocorticoids in the control of neuroinflammation. *Mol Cell Endocrinol* 275:62–70. [CrossRef Medline](#)
- Tracey KJ (2002) The inflammatory reflex. *Nature* 420:853–859. [CrossRef Medline](#)
- Tuosto L, Cundari E, Gilardini Montani MS, Piccolella E (1994) Analysis of susceptibility of mature human T lymphocytes to dexamethasone-induced apoptosis. *Eur J Immunol* 24:1061–1065. [CrossRef Medline](#)
- Vandeputte C, Taymans JM, Casteels C, Coun F, Ni Y, Van Laere K, Baekelandt V (2010) Automated quantitative gait analysis in animal models of movement disorders. *BMC Neurosci* 11:92. [CrossRef Medline](#)
- Vega JL, Ganea D, Jonakait GM (2003) Acute down-regulation of antibody production following spinal cord injury: role of systemic catecholamines. *J Neuropathol Exp Neurol* 62:848–854. [Medline](#)
- Vogelgesang A, Becker KJ, Dressel A (2014) Immunological consequences of ischemic stroke. *Acta Neurol Scand* 129:1–12. [CrossRef Medline](#)
- Wang FI, Hinton DR, Gilmore W, Trousdale MD, Fleming JO (1992) Sequential infection of glial cells by the murine hepatitis virus JHM strain (MHV-4) leads to a characteristic distribution of demyelination. *Lab Invest* 66:744–754. [Medline](#)
- Westendorp WF, Nederkoorn PJ, Vermeij JD, Dijkgraaf MG, van de Beek D (2011) Post-stroke infection: a systematic review and meta-analysis. *BMC Neurol* 11:110. [CrossRef Medline](#)
- Whalen MM, Bankhurst AD (1990) Effects of beta-adrenergic receptor activation, cholera toxin and forskolin on human natural killer cell function. *Biochem J* 272:327–331. [Medline](#)
- Woiciechowsky C, Schoning B, Lanksch WR, Volk HD, Docke WD (1999) Mechanisms of brain-mediated systemic anti-inflammatory syndrome causing immunodepression. *J Mol Med (Berl)* 77:769–780. [CrossRef Medline](#)
- Wong CH, Jenne CN, Lee WY, Léger C, Kubes P (2011) Functional innervation of hepatic iNKT cells is immunosuppressive following stroke. *Science* 334:101–105. [CrossRef Medline](#)
- Yilmaz G, Arumugam TV, Stokes KY, Granger DN (2006) Role of T lymphocytes and interferon-gamma in ischemic stroke. *Circulation* 113:2105–2112. [CrossRef Medline](#)