Material and methods

Induction of permanent focal cerebral ischemia and volume of the infarct

We performed permanent MCAO in IL10T (n = 6) and WT (n=6) mice. Animals were anaesthetized intraperitoneally with xylazine (20 mg/kg) / ketamine (100 mg/kg) in 0.9% NaCl (100µl/10g body weight), the right temporoparietal region of the head was shaved and a 2-mm incision was made vertically between the orbit and the ear and the skull exposed. Using a high-speed microdrill, a small burr hole of 1 mm^2 was made through the outer surface of the semitranslucent skull under an operating microscope. This was located just above the visibly identified medial cerebral artery at the level of the parietal cerebral artery. Saline was applied to the area throughout the procedure to prevent heat injury. The inner layer of the skull was removed with a fine forceps, the dura and the arachnoid were opened and right permanent MCAO was performed by electrocoagulation (by means of a small-vessel cauterizer) without damaging the brain surface. If the brain surface was visibly damaged or if the middle cerebral artery bled owing to incomplete artery occlusion/coagulation, the animal was not used for the study. The duration of the surgery did not exceed 15 min in any case (de Bilbao *et al*. 2004). Permanent inhibition of cerebral blood flow after the lesion was assessed by visual inspection and by transcranial measurements of CBF that were made using laser Doppler flowmetry Oxford Optronix Ltd (UK) just before and after MCAO. Animals were placed under a sterotactic head frame and then a fine needle probe (MNP110XP, 0.48 mm diameter) was lowered onto the temporal bone surface 0.5 to 1 mm dorsal to the opening giving access to the MCA and wetted with a small amount of physiological saline.

Four days later, the animals were perfused through the ascending aorta with a solution of paraformaldehyde 4% in phosphate-buffered saline (PBS, pH 7.35). Brains were removed and processed for paraffin embedding. Sections (7µm) of the whole infarct area were cut with a microtome and collected on slides pretreated with 3-aminopropyltriethoxy-silane (Sigma, Buchs, Saint Gallen, Switzerland). Sections were counter-stained with cresyl-violet for the histological identification of the nuclear boundaries and peri-infarct areas and mounted in Eukitt. For each animal, quantification of the infarcted area was performed on the cresyl-violet stained sections at five representative levels throughout the rostro-caudal extent of the lesion (A 0.26, -0.22, -0.40, -0.70 and -1.2 -4mm relative to Bregma) (Franklin and Paxinos 1997). The rostro-caudal extent of the infarct was the same in both groups of mice. The infarcted area of each section was calculated by the substraction of healthy tissue areas of the contralateral to the ipsilateral

side of the section in order to compensate for the effect of brain edema (Guegan *et al*. 1998) using a computer-assisted image analyzing system (Software «Morphometry», Samba 2005 TITN, Alcatel). Infarct volumes (mm^3) were calculated for each animal after integration of areas with the distance between each level (de Bilbao *et al*. 2004).

To evaluate whether local alterations in cerebral vasculature anatomy contribute to different susceptibility to injury, an additional series of WT and IL10T mice (n=5 for each group) were killed on days 1 and 4 after ischemia. Cerebral vasculature was studied in non-operated and operated mice after intracardial perfusion of a mixture of equal proportion of gelatinous water (5%) and China ink (Sennelier, France) warmed at 40°C (1 ml). Then brains were removed and immersed for 24 hrs in 4% paraformaldehyde at 4°C (Chen *et al*. 2005). The cerebral vasculature was observed with a Zeiss stereo zoom microscope.

Physiological parameters for MCAO study

Physiological parameters including arterial blood pressure (Kent mouse tail blood pressure system RTBP2000, Kent Scientific Corporation, Torrington, USA), plasma glucose (using Roche Glucotrend Active, Rotkreuz, Switzerland) and hematocrit were measured daily in IL10T and WT mice (n=5) before MCAO and on days 1 and 4 post-injury. Body temperature was measured prior to MCAO, during and following MCAO by a rectal thermometer probe (Ellab DM 852, Roeovre, Denmark). During surgery, mice were placed on a warm mat and rectal temperature was measured. In the operation period rectal temperature was similar in WT (38.0 ± 0.1 °C) and IL10T (38.0 ± 0.1 °C) mice and remained stable after the operation. After the operation, mice were placed in their cage with a heating lamp to maintain body temperature at 38°C up to 3 hours after they woke up and were moving around. Daily body temperature was measured at 10:30 am.

Western blot procedure for glucose transporters Glut1 and Glut4 and Glucose-6-phophate dehydrogenase enzyme activity

Hemispheres from non-operated WT and IL10T mice were used (n=4 for each groups). Hemispheres were homogenized in solubilization buffer at 4°C (120 mM NaCl, 50 mM Tris [pH 8.0], 20 mM NaF, 1 mM benzamidine, 1 mM EDTA, 1 mM EGTA, 1 mM sodium pyrophosphate, 30 mM 4-nitrophenylphosphate disodium salt hexahydrate, 1% NP-40, and 0.1 M phenylmethylsulfonylfluoride [PMSF]) with a Polytron homogenizer. The homogenate was centrifuged and the supernatant was collected and stored at -20C prior to blotting. Protein content of the supernatants was determined by Bradford (Bio-Rad, Reinbach, Basel, Switzerland). Gel electrophoresis was carried out at 40 micrograms per

sample. Proteins were transferred on to an immobilon-P membrane (Millipore, Zug, Zug, Switzerland). Membranes were then incubated overnight with primary antibody (goat anti-Glut1 (sc1605) (1/200) or goat anti-Glut4 (sc1608) (1/200) from Santa Cruz, CA, US; mouse Anti-tubulin (1/25000), Sigma, Buchs, Saint Gallen, Switzerland). Secondary antibodies Licor anti-mouse for tubulin (1/1000) and Licor anti-goat (1/1000) were used to detect bands (Li-Cor Biosciences, Bad Homburg, Germany). The signals were visualized with the use of the Odyssey Infrared Imaging System (LI-COR Biosciences). Quantification of the signals was performed with the use of Odyssey Application Software 1.2. Protein levels were expressed as the ratio against tubulin (Viswambharan *et al.* 2007).

The enzyme glucose-6-phosphate dehydrogenase (G6PDH) was also studied since it play a key role in glucose metabolism and redox regulation via the pentose phosphate pathway (Spolarics 1998). Hemispheres from non-operated WT and IL10T mice were used (n=4 for each groups). They were homogenized with polytron homogenizer in a solution containing 0.2M Tris buffer (pH7.0) kept on ice. After centrifugation at 8,500 g, the supernatant was collected and kept at -20C prior to assay for G6PDH activity. Fifteen µl of extract containing 1mg/ml of protein as determined by Bradford (Bio-Rad, Reinbach, Basel, Switzerland) was added to 100 µl buffer containing 0.2M Tris (pH7.0**)** and NADP+ (10mg / ml). To start the reaction, 50 µl of G6P (0.3g in 1ml) was added to the previous mentioned solution. The activity was determined by measuring the change in OD at 340 nm during 15 minutes.

Western blot procedure for caspase-3

Ischemic hemispheres from lesioned WT and IL10T mice were used (n=6 for each groups). The equivalent hemisphere was used in control non-ischemic mice. Hemispheres were homogenized in solubilization buffer at 4°C as described above (see Western blot procedure for glucose transporters). Membranes were incubated overnight with primary antibody (rabbit Anti-caspase-3 (sc1605) at 1/1000 from Santa Cruz, CA, US; mouse Anti-tubulin at 1/25000 from Sigma, Buchs Saint Gallen, Switzerland). Secondary antibodies Li-Cor anti-mouse for tubulin (1/1000) and Alexa Fluor anti-rabbit for caspase-3 (1/1000) from Li-Cor Bad Homburg, Germany, were used to detect bands.

Lipid peroxidation in brain homogenates

We performed measures of malondialdehydes (MDA), an indicator of endogenous lipid peroxidation, on days 1 and 4 after MCAO in WT and IL10T mice (n=5 for each group). Mice were anesthetized and perfused intracardially with a NaCl ($9^0/_0$) solution. After perfusion, brains were

removed and washed 3 times in ice-cold Tris-HCl 20 mM buffer (pH 7.4). Tissues from the ischemic hemispheres of operated animals and equivalent hemisphere of control non-operated animals were homogenized to give a 30% solution (ie 300 mg of tissue in 1 ml of buffer) in 20 mM Tris buffer pH 7.4. To measure lipid peroxidation, the Biotech LPO-586 colorimetric assay kit and its protocol (Oxis, Portland, USA) was used. In order to prevent sample oxidation 0.5 mM butylated hydroxytoluene (BHT, Sigma – Buchs, Saint Gallen, Switzerland) was added to the tissue homogenates. Subsequently samples were centrifuged at 4°C to remove cellular debris. Aliquots were then taken for determination of MDA using the methanesulfonic acid solvent procedure (de Bilbao *et al*. 2004). Optical density readings were made at 586 nm using a microplate reader (DynaTech MR5000) and corrections were made for sample and reagent blanks. Concentrations were then determined using a standard curve. Non-operated mice were used as controls (n=5 for each group).

Brain superoxide dismutase activity one day after MCAO

We investigated SOD activity in the ischemic hemispheres of IL10T and WT mice subjected to ischemia (one day post-MCAO) (n=6 mice per group) using a biochemical assay (Ewing and Janero 1995). The equivalent hemisphere was used in control non-operated animals. Hemispheres were homogenised in phosphate buffered saline and frozen immediately in liquid nitrogen. Aliquots of tissue supernatants were processed as previously described (Ewing and Janero 1995). Briefly, tissue aliquots were added (25 µl) to a 125 µl solution containing 50 mM phosphate buffer (pH 7.4, 0.1 mM EDTA, 50 µM NBT, 78 µM NADH). To start the reaction, 25 µl of 3.3 µM PMS phenazine methosulfate (final concentration) was added and the absorbance at 560 nm was measured continuously using a microplate reader (DynaTech MR5000). The readings were made every minute for 10 minutes. The first 5 minutes were used to determine the rate of superoxide production by the samples.

Brain glutathione levels

Ischemic hemispheres of operated animals and the equivalent hemisphere for control non-operated animals were homogenised in phosphate buffer (50 mM, pH 7.4). Total GSH levels were measured using a method based on the formation of a chromophoric product resulting from the reaction of 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB, Sigma, Buchs, Saint Gallen, Switzerland) and GSH (Sigma, Buchs, Saint Gallen), (de Bilbao *et al.* 2004). The absorbance was immediately measured at 412 nm. Glutathione contents were calculated by using a calibration curve established with standard samples. Brain GSH levels were measured 1

and 4 days after the lesion (n=4 for each group). Non-operated mice were used as controls (n=4 for each group).

Determination of brain cytokine levels

In order to determine if IL10T had altered cytokine production in response to MCAO treatment, we measured TNFα, IFNγ, IL1β, IL10 and NGF in the brain ischemic hemispheres of WT and IL10T mice subjected to ischemia (n=4 for each group). The equivalent hemisphere was used in control non-operated animals. Brain hemispheres of WT and IL10T mice were collected one day after the operation. 100 mg of tissue were homogenized with 600 µl of 1% CHAPS (3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate) in RPMI-1640 medium without Phenol red (R7509, Gibco) with a polytron homogenizer (Nakane *et al*., 1992). The supernatant was collected and frozen at -20 C (Arsenijevic *et al.* 2006). TNFα, IFNγ, IL1β, IL10 and NGF were measured using immunoassay kits (Amersham (GE Healthcare, Otelingen, Zurich, Switzerland) for the four former and Promega, Dubendorf, Zurich, Switzerland for NGF) as described previously (Arsenijevic *et al.* 2006). Non-operated mice were used as controls (n=4 for each group).

Enzyme immunoassay for neuropeptide Y (NPY), orexin-A and melanin-concentrating hormone (MCH)

Hypothalami were dissected out in WT and IL10T mice one day after MCAO (n=5) and were treated as previously described (Gallmann *et al*. 2006). Tissues homogenates were left on ice for 1 h. Brain tissue proteins were extracted using 1% (w/v) 3-((3-cholamidopropyl) dimethylammonio)-1-propanesulfate (CHAPS, Pierce Biotechnology, Rockford IL, USA) (for method see cytokine extraction section). Extracts were stored at -80°C until peptide assays. Orexin-A, MCH and NPY were quantified by enzyme immunoassay (PhoenixPeptide, Belmont, California USA) (Gallmann *et al*. 2006). Non-operated mice were used as controls (n=5).

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