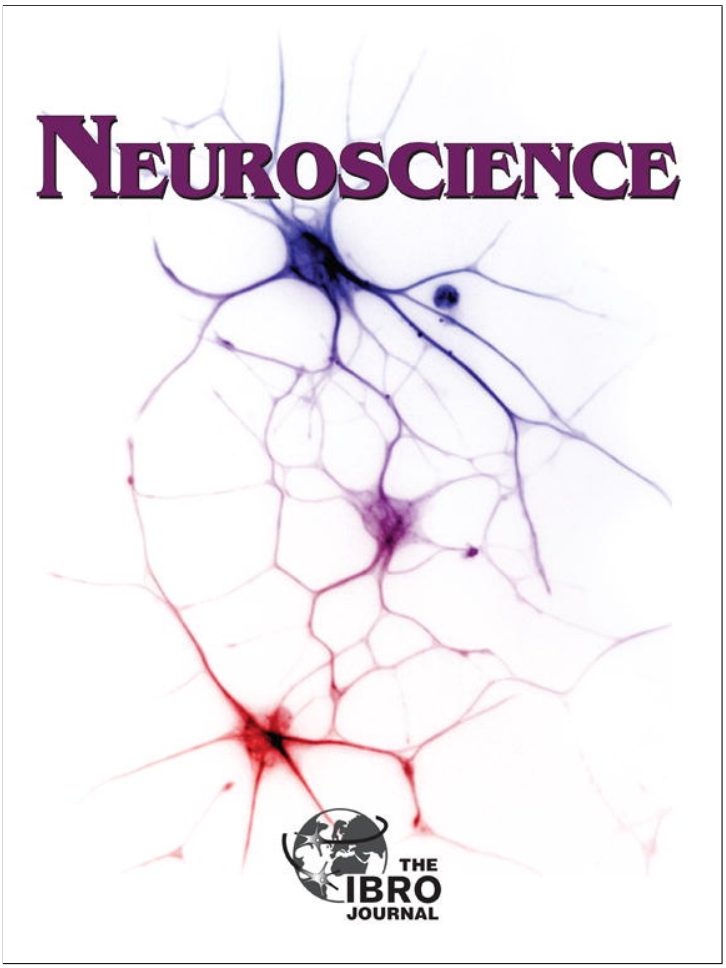
NEUROSCIENCE
THE
IBRO
JOURNAL

Neuroscience 242 (2013) 1–10

NEUROPROTECTIVE EFFECT OF CEFTRIAXONE ON THE PENUMBRA IN A RAT VENOUS ISCHEMIA MODEL

T. INUI,[a,b] B. ALESSANDRI,[a] A. HEIMANN,[a] F. NISHIMURA,[b] K. FRAUENKNECHT,[c] C. SOMMER[c] AND O. KEMPSKI[a]*

[a] *Institute for Neurosurgical Pathophysiology, University Medical Center of the Johannes Gutenberg-University Mainz, Mainz 55131, Germany*

[b] *Department of Neurosurgery, Nara Medical University, Nara, Japan*

[c] *Department of Neuropathology, University Medical Center of the Johannes Gutenberg-University Mainz, Mainz 55131, Germany*

Abstract—*Objective:* Glutamate transporter-1 (GLT-1) maintains low concentrations of extracellular glutamate by removing glutamate from the extracellular space. It is controversial, however, whether upregulation of GLT-1 is neuroprotective under all ischemic/hypoxic conditions. Recently, a neuroprotective effect of preconditioning with a β-lactam antibiotic ceftriaxone (CTX) that increases expression of GLT-1 has been reported in animal models of focal ischemia. On the other hand, it is said that CTX does not play a neuroprotective role in an *in vitro* study. Thus, we examined the effect of CTX on ischemic injury in a rat model of two-vein occlusion (2VO). This model mimics venous ischemia during, e.g. tumor surgery, a clinical situation that is best suitable for pretreatment with CTX.

***Methods:* CTX (100 mg/kg, 200 mg/kg per day) or vehicle (0.9% NaCl) was intraperitoneally injected into Wistar rats for 5 days before venous ischemia ($n = 57$). Then, animals were prepared for occlusion of two adjacent cortical veins (2VO) by photothrombosis with rose bengal that was followed by KCl-induced cortical spreading depression (CSD). Infarct volume was evaluated with hematoxylin and eosin (H&E) staining 2 days after venous occlusion. [^{3}H]MK-801, [^{3}H]AMPA and [^{3}H]Muscimol ligand binding were examined autoradiographically in additional two groups without 2VO ($n = 5$/group). Animals were injected either with NaCl (vehicle) or CTX 200 mg/kg for 5 days in order to evaluate whether NMDA, AMPA and $GABA_A$ ligand binding densities were affected.**

***Results:* CTX pretreatment reduced infarct volume compared to vehicle pretreatment ($p < 0.05$). The effect of CTX pretreatment was attenuated by administration of the GLT-1 inhibitor, dihydrokainate (DHK) 30 min before 2VO. CTX had no effect on the number of spontaneous spreading depressions after 2VO. Analysis of quantitative receptor autoradiography showed no statistically significant difference between rats after administration with CTX compared to control rats.**

***Conclusions:* Pretreatment with CTX has neuroprotective potential without effect on NMDA, AMPA and $GABA_A$ receptor density and spontaneous spreading depression. This effect can be abolished by GLT-1 inhibition, indicating that upregulation of GLT-1 is an important mechanism for neuroprotective action in penumbra-like conditions, e.g. if neurosurgeons plan to occlude cerebral veins during tumor surgery.**

Key words: ceftriaxone, cortical spreading depression, glutamate transporter-1, penumbra, venous ischemia.

INTRODUCTION

Glutamate is the major excitatory synaptic neurotransmitter in the mammalian central nervous system. On the other hand, rises in the extracellular concentration of glutamate lead to neurotoxicity (e.g. Attwell et al., 1993), therefore, it is important to maintain glutamate at a low level. Normal concentrations of extracellular glutamate are maintained by glutamate transporters (Choi, 1988; Rothstein et al., 1996). At least five subtypes of excitatory amino acid transporters (EAATs) have been confirmed of which glutamate transporter-1 (GLT-1)–EAAT2 mainly exists in astrocytes (Pines et al., 1992; Rothstein et al., 1996). The predominant EAAT subtype in humans is EAAT2 and in rodents it is GLT-1 (Rothstein et al., 1994; Lehre et al., 1995). The transporter is important for human diseases such as addiction (Sari et al., 2009), Huntington (Miller et al., 2008), neonatal hypoxia–ischemia (Mimura et al., 2012) or focal brain ischemia (Chu et al., 2007; Zhang et al., 2007). Antisense knockdown of GLT-1 exacerbates transient focal ischemia-induced neuronal damage (Rao et al., 2001b). Some reports have shown that GLT-1 blockers can attenuate ischemia-induced glutamate release (Seki et al., 1999). Thus, the function of GLT-1 for the brain in ischemic conditions is discussed controversially especially in the ischemic penumbra where lesion development progresses. In contrast, ceftriaxone (CTX) exposure did not result in neuroprotection against oxygen–glucose deprivation (Lipski et al., 2007), contradicting efficacy of elevated GLT-1 under ischemia-like condition *in vitro*.

Pretreatment of stroke victims, however, is not possible. On the other hand, cerebral veins have to be

*Corresponding author. Tel: +49-6131-17-2373; fax: +49-6131-17-6640.

E-mail address: oliver.kempski@unimedizin-mainz.de (O. Kempski).

Abbreviations: 2VO, two-vein occlusion; CBF, cerebral blood flow; CSD, cortical spreading depression; CTX, ceftriaxone; DG, dentate gyrus; DHK, dihydrokainate; EAAT, excitatory amino acid transporters; GLT-1, glutamate transporter-1; HL, hindlimb area; MABP, mean arterial blood pressure; SD, standard deviation.

0306-4522/13 $36.00
http://dx.doi.org/10.1016/j.neuroscience.2013.03.018

occluded frequently in brain tumor surgery, a condition which can be planned and would allow pretreatment of respective patients. In contrast to arterial stroke models (e.g. middle cerebral artery occlusion) where the entire blood supply is cut off producing a large non-perfused core with a small penumbral region, occlusion of two bridging veins in our two-vein occlusion (2VO) model causes a small ischemic core but a widespread area of reduced cerebral blood flow (CBF) and a slower growing infarction (Nakase et al., 1995, 1996, 1997a, 1998) Therefore, this venous ischemia model is suited to study penumbra pathophysiology (Kempski et al., 1999; Otsuka et al., 2000).

Thus, in this study, we examined whether CTX-pretreatment reduces infarct growth in a rat venous ischemia model using rose bengal and focused fiberoptic illumination. Furthermore, we evaluated by quantitative *in vitro* receptor autoradiography whether altered NMDA, AMPA and $GABA_A$ ligand binding densities by CTX are involved in neuroprotection after 2VO.

EXPERIMENTAL PROCEDURES

Animal preparation

All studies were performed according to the German animal protection legislation. We used 67 male Wistar rats (weighing 260–340 g; Charles River Laboratories, Sulzfeld, Germany) for the experiments. Rats were kept in individual animal cages and allowed free access to food and water.

At first, rats were injected with CTX or vehicle intraperitoneally as a pretreatment for successive 5 days before venous ischemia. At the 6th day, rats were anesthetized with an intraperitoneal injection of chloral hydrate (36 mg/100 g body weight) before premedication with 1.0-mg atropine. Anesthesia was maintained with chloral hydrate (12 mg/100 g body weight/h) through a peritoneal silicon catheter. All animals were intubated with silicon tubing (outer diameter, 2.5 mm) and mechanically ventilated with 30% O_2 using a rodent ventilator (Model 683; Harvard Apparatus, South Natick, MA, USA) while monitoring end tidal CO_2 (Artema MM206C; Heyer, Sundbyberg, Sweden). Rectal temperature was kept at about 37.0 °C using a feedback-controlled heating pad (Harvard Apparatus). Polyethylene tubing (outer diameter, 0.80 mm; Portex; Smiths Industries Medical Systems Co., London, UK) was inserted into the tail artery to measure mean arterial blood pressure (MABP) and arterial blood gases with a blood gas analyzer (ABL System 615; Radiometer, Copenhagen, Denmark). Another polyethylene tubing was inserted into the left femoral vein. After rats were mounted in a stereotaxic frame (Stoelting, Wood Dale, IL, USA), a 1.5-cm midline skin incision was set up, and a small hole was made by an 18-G needle 2 mm right lateral from midline, and 2 mm below the bregma. The lateral ventricle cannula was made from an L-shaped 26-G needle connected to polyethylene tubing (volume, 10 µl; length, 7 cm ± 1 mm). The cannula was inserted with a micromanipulator. A left parietal cranial window was opened by using a high-speed drill (GD 604; AESCULAP, Tuttlingen, Germany) under an operating microscope (OP-Microscope; Zeiss, Wetzlar, Germany). During the craniotomy, the drill tip was cooled continuously with physiological saline to avoid thermal injury to the cortex. The dura mater was left intact.

Cortical vein occlusion by photochemical thrombosis

The occlusion of two adjacent cortical veins was induced by means of rose bengal dye (50 mg/kg body weight, Sigma Chemical Co., St. Louis, MO, USA) and fiberoptic illumination using a 50-W mercury lamp (6500–7500 1×, 540 nm) and a 100-µm fiber directly positioned over the veins, thus avoiding illumination of surrounding brain tissue (Nakase et al., 1995, 1996). Only rats with similar venous anatomy (i.e., with two prominent adjacent veins connecting into the superior sagittal sinus) were used (Fig. 1). The diameter of the occluded veins was approximately 100 µm. After the first vein was occluded for 10–20 min from starting of illumination, half of the initial rose bengal dose was injected intravenously and the second vein was illuminated until occlusion.

Measurement of CBF and tissue impedance

CBF was measured by laser Doppler flowmetry (Model BPM 403a; Vasomedics, St. Paul, MN, USA) with a 0.8-mm needle probe (Nakase et al., 1995, 1996). CBF which is expressed in Laser Doppler units (LDU) was measured at 25 points with the occluded veins lateral to the scanning field by using a stepping-motor-driven and computer-controlled micromanipulator (Nakase et al., 1996, 1997a). Thus, one scanning procedure yielded information from 25 different locations (square of 5 × 5 points) 300 µm apart from each other. Scanning was performed before and every 5 min for 75 min after venous ischemia. The median of observation frequency histograms correlates with absolute regional blood flow as determined by hydrogen clearance (Uranishi et al., 1999). Two impedance electrodes were inserted into the cortex (depth, 0.4–0.5 mm; distance, 3 mm) (Fig. 1) to measure cell swelling that occurs during induced cortical spreading depression (CSD) and venous ischemia (Otsuka et al., 2000). The impedance electrodes were made from two stainless steel wires (outer diameter, 0.5 mm) covered with polyvinyl chloride for electrical insulation except for the 0.3-mm sharp-pointed tips. Impedance was measured at 1 kHz (10 mV, bias-free) throughout the experiment by using a precision LCR monitor (4284A; Hewlett–Packard, Avondale, PA, USA).

Induction of cortical spreading depression

After insertion of impedance electrodes, a glass micropipette for KCl injection was placed into the lateral parietal cortex (Fig. 1). The micropipette which was linked to a microinjection pump (CMA/100; Carnegie Medicine, Stockholm, Sweden) was filled with 150 mmol/L KCl solution, and 10 5.0-µl KCl injections were administered every 7 min to induce cortical spreading depression (Otsuka et al., 2000; Nakagawa et al., 2005). Injection of KCl started after completion of 2VO and ended 70 min thereafter.

Experimental studies and treatment groups

Effects of CTX on CBF and infarct volume. Forty five rats were randomly assigned to the following five groups. Group A: vehicle-pretreatment (0.9% NaCl, $n = 9$); Group B: CTX 100 mg/kg-pretreatment ($n = 10$); Group C: CTX 200 mg/kg-pretreatment ($n = 10$); Group D: vehicle-pretreatment together with the GLT-1 inhibitor dihydrokainate (DHK, 0.14 mg/kg) ($n = 8$); Group E: CTX 200 mg/kg-pretreatment together with DHK 0.14 mg/kg (in 20 µl saline) ($n = 8$) (Fig. 2). All rats

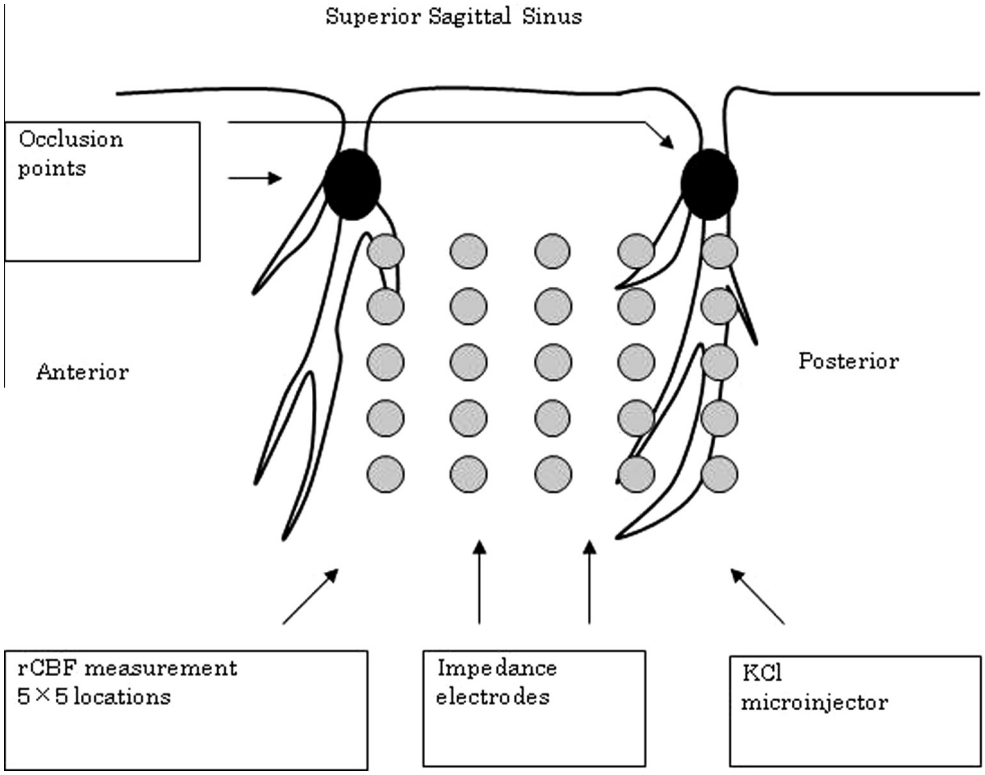

Fig. 1. Schematic drawing of the experimental setup after craniotomy. The location of scanning area for cerebral blood flow measurement, impedance electrodes, KCL microinjector, and occlusion points of two adjacent veins are shown. Every instrument was placed in parietal cortex to ensure that each distance was comparable.

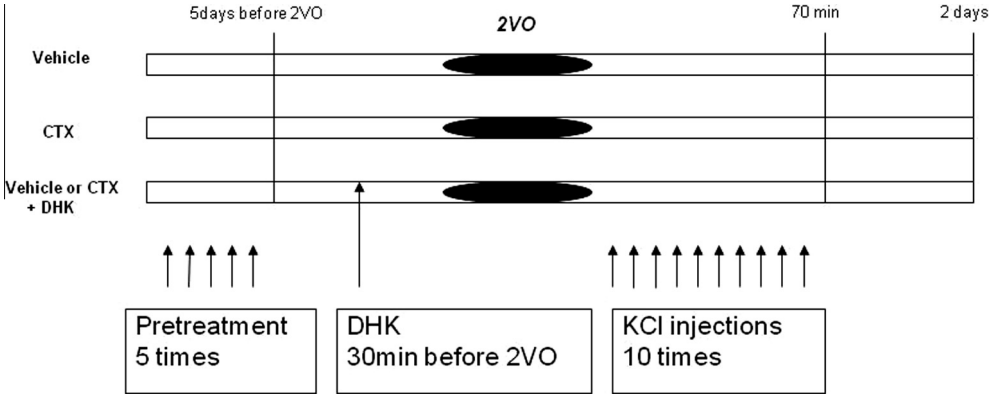

Fig. 2. Experimental time course showing administration of drugs, vein occlusion, and KCl injections.

received CTX or vehicle intraperitoneally for five successive days before venous ischemia. In groups D and E, DHK was intraventricularly injected once 30 min before venous occlusion (Zhang et al., 2007; Gong et al., 2012). CTX pre-treatment regimen was adapted from Chu et al. (2007). All drugs were purchased from Sigma Chemical Co.

Effects of CTX on cortical spreading depression. Twelve rats were randomized into two groups. Group 1: vehicle-pretreatment (0.9% NaCl; $n = 6$); Group 2: CTX 200 mg/kg-pretreatment ($n = 6$). All animals received 10 consecutive KCL injections in 7-min intervals starting after induction of 2VO.

Histological preparation for infarct volume

After surgery, the incised skin was closed with a skin stapler. The rats were returned to individual cages, and sacrificed after 2 days. All rats were perfusion-fixed with 4% paraformaldehyde under deep anesthesia with chloral hydrate, and the brain was carefully removed from the skull. Brains were embedded in paraffin and 3-μm-thick coronal sections were sliced in parietal region including the infarct area. All sections were blinded to the experimenter before analysis and stained with hematoxylin-eosin. The histological evaluation of the infarct volume was accomplished using a light microscope connected to a Charge-Coupled Device (CCD) camera (Sony, Tokyo, Japan) and

Optimas 6.51 software (Optimas Corp., Seattle, WA, USA). The infarct area was evaluated in serial sections of 200-μm steps. Finally, the infarction volume was calculated from the sum of all measured lesion areas (mm^2) multiplied with the distance between sections (0.2 mm).

Effects of CTX on in vitro neurotransmitter receptor autoradiography. Quantitative *in vitro* receptor autoradiography was performed on 10 Wistar rats which were assigned to two groups, one injected (i.p.) with 200 mg/kg CTX and one with vehicle for 5 days. No venous ischemia was induced.

After decapitation brains were frozen in isopentane at −30 °C for 10 min, and stored at −80 °C until analyzed. Coronal cryostat sections of 12-μm thickness were serially cut at −20 °C at the level of the dorsal hippocampus and mounted on TESPA-coated slides. Quantitative *in vitro* receptor autoradiography studies were performed using [^{3}H]MK-801, [^{3}H]AMPA and [^{3}H]Muscimol for labeling of NMDA, AMPA and $GABA_A$ receptors, respectively (Palacios et al., 1981; Monaghan and Cotman, 1985; Standley et al., 1995). Ligands were purchased from NEN Life Sciences Products Inc. (Boston, MA, USA). Labeling and incubation procedures for the different binding sites were performed according to the protocols of Zilles et al. (2000) as previously described (Diederich et al., 2012a,b).

Incubation with [^{3}H]MK-801, [^{3}H]AMPA and [^{3}H]Muscimol was always preceded by a preincubation period with the respective buffer to remove endogenous ligands. In order to demonstrate the maximum binding of [^{3}H]MK-801 to NMDA receptors, the binding assay was performed with 5 nM [^{3}H]MK-801 (specific activity 20.0 Ci/mmol) in a magnesium- and zinc-free solution (50 mM Tris–HCl buffer, pH 7.2) in the presence of 30 μM glycine and 50 μM spermidine at 22 °C for 60 min. Incubation was terminated by washing in cold buffer (2 × 5 min) and in H_2O (2 s). AMPA receptors were labeled with 10 nM [^{3}H]AMPA (specific activity 45.5 Ci/mmol) in 50 mM Tris–acetate buffer (pH 7.2, containing 100 mM KSCN) for 45 min at 4 °C. Incubation was terminated by rinsing (3 × 4 s) with cold buffer and fixation rinsing (2 × 2 s) with acetone/glutaraldehyde solution. $GABA_A$ receptors were incubated with 3 nM [^{3}H]Muscimol (specific activity 22.0 Ci/mmol) in 50 mM Tris–citrate buffer (pH 7.0) for 40 min at 4 °C. Incubation was terminated with 3 × 4-s rinses in cold buffer. Unspecific binding was determined by co-incubation of alternating sections with labeled ligands and excess of an appropriate unlabeled competitor. Subsequent to the final rinsing procedure, slides were carefully dried in either a stream of cool air ([^{3}H]MK-801 and [^{3}H]Muscimol) or hot air ([^{3}H]AMPA). Air-dried, tritium-labeled sections were co-exposed with [^{3}H]plastic standards (Microscales®; Amersham, Freiburg, Germany) to a [^{3}H]-sensitive film (Amersham Hyperfilm-^{3}H, GE Healthcare UK Ltd.) for 5 ([^{3}H]AMPA) or 6 weeks ([^{3}H]MK-801 and [^{3}H]Muscimol)-autoradiographs were scanned in equal light conditions with the digital CoolSNAP camera (Roper Scientific, Photometrics CoolSNAPcf, Ottobrunn/Munich, Germany) and digitized with the MCID image analysis system (Imaging Research Inc., St. Catharines, Ontario, Canada). Gray value images of the coexposed plastic standards were used to compute a nonlinear calibration curve, which defined a relationship between gray values in the autoradiographs and concentrations of radioactivity. Final values were normalized to vehicle treated control levels (mean ± standard deviation (SD)) (Diederich et al., 2012a,b).

Mean ligand binding density was analyzed within layers I–VI of the frontal cortex, area 1 (Fr1), parietal cortex hindlimb area (HL) and parietal cortex area 2 (Par2) as well as in the hippocampal subfields CA1, CA3 and dentate gyrus (DG) in both hemispheres.

STATISTICAL ANALYSIS

Data are expressed as means ± standard error of the mean (mean ± standard error of mean (SEM)). A one-way analysis of variance test was used to compare data. Statistical significance was assumed at an error probability of $P < 0.05$ (Sigmastat 3.1, Systat Software Inc.).

Ligand binding in vehicle and CTX-treated rats was analyzed by calculating mean concentration values for each ligand and region. Significant group effects were confirmed by an analysis of variance (ANOVA) and least significant difference (LSD) error protection. A P value < 0.05 was considered statistically significant. Analysis was performed using the general statistics module of Analyse-it for Microsoft Excel (Analyse-it Software, Ltd., Leeds, UK). Values are presented as % of NaCl-treated control rats.

Table 1. Physiological variables before and after venous ischemia

	MABP (mmHg)	pH	PaO_2 (mmHg)	$PaCO_2$ (mmHg)
Vehicle (group A)				
Before 2VO	95.2 ± 1.5	7.41 ± 0.01	117.9 ± 1.7	40.7 ± 0.5
After 2VO	94.6 ± 1.6	7.41 ± 0.01	117.7 ± 1.4	41.3 ± 0.4
CTX100 (group B)				
Before 2VO	98.0 ± 2.5	7.41 ± 0.02	120.1 ± 1.3	41.4 ± 0.4
After 2VO	94.5 ± 2.9	7.42 ± 0.01	120.3 ± 1.1	40.9 ± 0.3
CTX200 (group C)				
Before 2VO	94.8 ± 1.5	7.41 ± 0.01	121.5 ± 1.2	41.5 ± 0.3
After 2VO	93.5 ± 1.8	7.40 ± 0.02	122.0 ± 1.6	41.0 ± 0.4
Vehicle with DHK (group D)				
Before 2VO	98.0 ± 3.3	7.41 ± 0.02	119.9 ± 2.4	41.0 ± 0.2
After 2VO	96.8 ± 2.5	7.39 ± 0.02	120.1 ± 1.8	40.5 ± 0.4
CTX200 with DHK (group E)				
Before 2VO	99.0 ± 3.2	7.40 ± 0.02	120.1 ± 1.4	41.4 ± 0.3
After 2VO	104.7 ± 2.4*	7.41 ± 0.01	121.1 ± 1.8	40.7 ± 0.6

Vehicle = 0.9% NaCl; MABP = mean arterial blood pressure; PaO_2 = partial pressure of oxygen; $PaCO_2$ = partial pressure of carbon dioxide; 2VO = two-vein occlusion; CTX = ceftriaxone; DHK = dihydrokainate. The data are expressed as mean ± standard error of mean (SEM).

* $p < 0.05$.

RESULTS

Physiological variables

Blood gas analysis (PaO_2, $PaCO_2$, and pH) were within normal ranges in all groups throughout this study. MABP, rectal temperature, and brain temperature were not significantly changed before and after 2VO. Only MABP of group E (CTX + DHK) showed a significant increase by 5 mmHg after 2VO (Table 1). Physiological data from groups 1 and 2 (effect of CTX on cortical spreading depression) did not show any statistical difference between both groups (data not shown).

Changes of regional CBF

Regional CBF (rCBF) calculated as the median flow from the 25 locations in each animal did not show significant differences between groups during the control phase before venous ischemia. rCBF values were 39.5 ± 4.4, 50.1 ± 5.8, 41.6 ± 5.3, 43.5 ± 9.5, and 44.2 ± 5.8 LD units in groups A, B, C, D, and E, respectively (Fig. 3). rCBF values were reduced to 17.9 ± 4.4, 12.6 ± 1.5, 14.5 ± 2.4, 16.1 ± 2.4, and 15.2 ± 3.2 LD units in the respective groups 70 min after 2VO. Again there was no statistical difference between all groups after 2VO (Fig. 3).

Changes of tissue impedance and number of CSD

Impedance and number of CSD were measured in groups 1 and 2. Cortical impedance values showed no significant difference between both groups (vehicle, 4.4 ± 0.27 kΩ; CTX, 4.1 ± 0.22 kΩ) at baseline conditions. After venous occlusion a wave of CSD occurred spontaneously – before any injection of KCl. After each KCl injection into the cortex, a solitary wave of CSD was always observed as a sudden increase of tissue impedance (Fig. 4). There was no significant difference between the two groups (vehicle, 4.3 ± 0.23 kΩ; CTX, 3.9 ± 0.22 kΩ). The total number of KCl-elicited and spontaneously occurring CSDs was not significantly different between groups (saline, 20.1 ± 2.2; CTX, 18.3 ± 3.1) (Fig. 4).

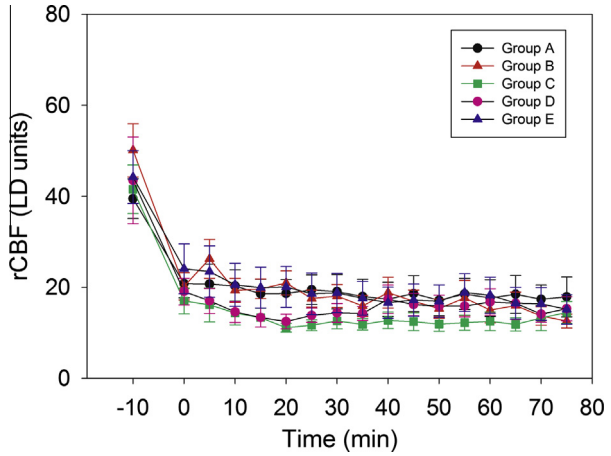

Fig. 3. Successive cerebral blood flow (CBF) change before and after cortical venous occlusion in group A (vehicle pretreatment), B (CTX 100 mg/kg pretreatment), C (CTX 200 mg/kg pretreatment), D (vehicle pretreatment + 0.14 mg/kg DHK) and E (CTX 200 mg/kg pretreatment + 0.14 mg/kg DHK). An approximately 50% reduction of rCBF after venous occlusion can be observed in all groups. There is no statistical significance between all groups. Values are given as mean ± standard error of mean (SEM). CTX = ceftriaxone, DHK = dihydrokainate.

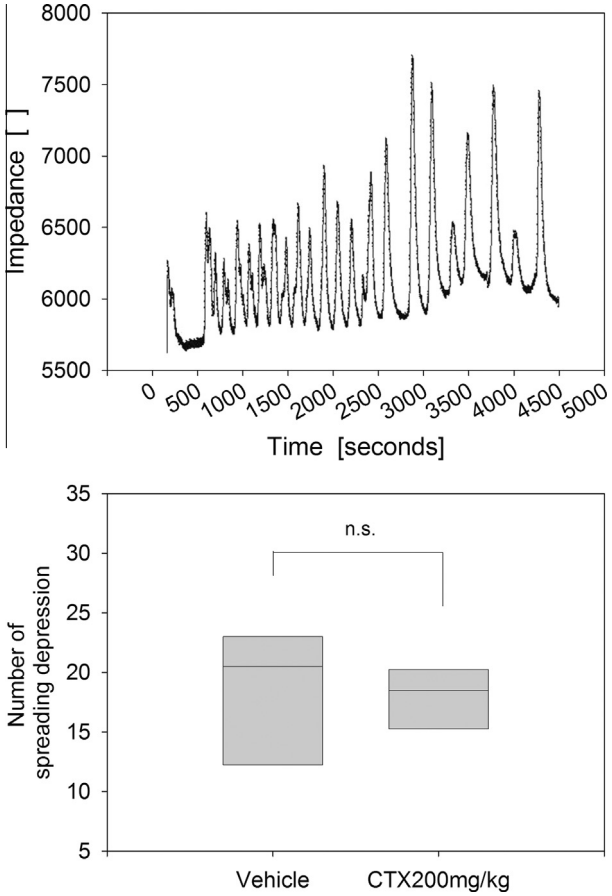

Fig. 4. Typical impedance change of KCl-induced CSDs (top) and number of CSDs in NaCl (vehicle) or CTX 200 mg/kg pretreatment group (bottom). Impedance during baseline and vessel occlusion is not shown. There is no statistical significance (n.s.) between groups. Values are given as mean ± standard error of mean (SEM).

Infarct volume

All rats had venous infarction. Fig. 5 shows examples of the clearly demarcated infarct area at 2 days after 2VO. In group B (CTX100 mg) and group C (CTX 200 mg) infarct volume was significantly reduced to 7.48 ± 0.64 and 6.44 ± 0.95 mm^3, respectively, when compared with 8.84 ± 1.94 mm^3 in group A (vehicle) Lesion volume of the two CTX dosages did not differ statistically from each other (Fig. 6A). The reduction of infarct volume was significantly attenuated by intraventricular injection of DHK in group D (9.04 ± 2.4 mm^3) when compared to treatment with CTX 200 mg/kg in group C (6.44 ± 0.95 mm^3) (Fig. 6B).

Effect of CTX on neurotransmitter receptor density

Analysis of [^{3}H] MK-801, [^{3}H] AMPA and [^{3}H] Muscimol ligand binding values in the six cortical layers of the

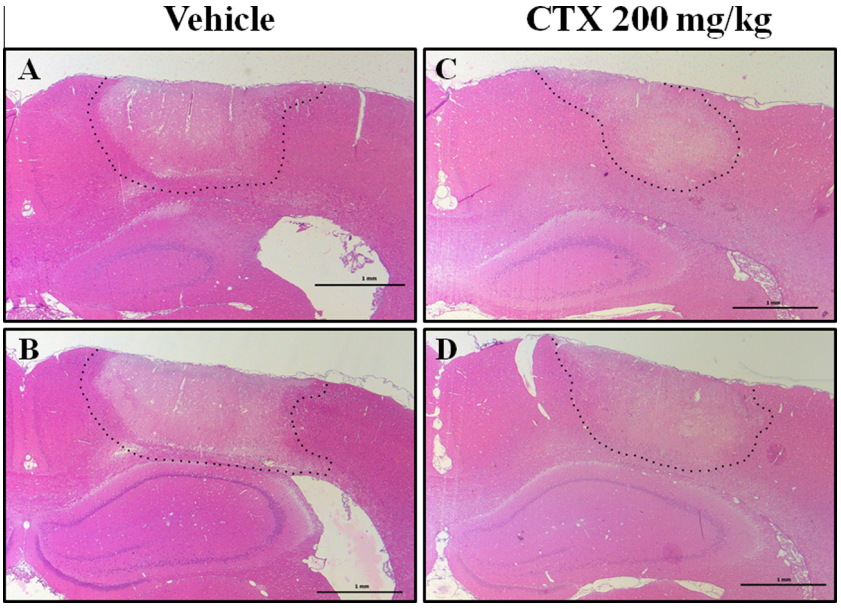

Fig. 5. Typical examples of hematoxylin-eosin stained sections from a vehicle- (A, B) and a ceftriaxone-treated (CTX 200 mg/kg) animal (C, D) at 2 days after venous ischemia. The infarct area is encircled by a dotted line. Coronal sections A and C are 0.7 mm anterior to sections B and D. Bar represents 1 mm.

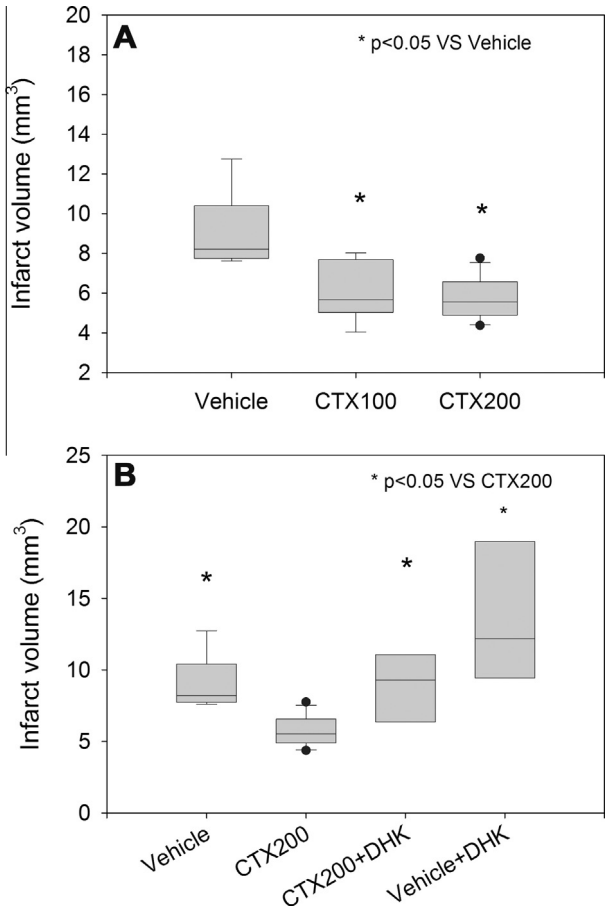

Fig. 6. Infarct volume (mm^3) at 2 days after venous ischemia without (A) and with (B) inhibition of glutamate transporter-1 by 0.14 mg/kg dihydrokainate (DHK). Values are given as mean ± standard error of mean (SEM). Infarct volume was reduced by CTX pretreatment compared with vehicle administration group. $^*p < 0.05$ versus CTX200.

frontal cortex area 1 (Fr1), the parietal cortex HL, the parietal cortex area 2 (Par2) as well as in the hippocampal subfields CA1, CA3 and in the DG showed no significant differences between rats treated with CTX in comparison to saline-treated control rats (Fig. 7, Table 2).

DISCUSSION

In this study we showed that a pretreatment with CTX reduced infarct volume in a rat venous ischemia model without influencing local CBF and the number of spreading depression waves induced by KCL injection and ischemia. This neuroprotective effect was attenuated by the GLT-1 inhibitor DHK. These results suggest that malfunction of GLT-1 transport worsens neuronal death in this model whereas induction of GLT-1 with CTX offers neuroprotection as seen in previous reports (Rothstein et al., 2005; Chu et al., 2007). Quantitative receptor autoradiography showed no influence of 5 days CTX treatment on ligand binding densities of NMDA, AMPA and $GABA_A$ receptors. The ligand binding autoradiography is a tool to detect the density of a particular functional receptor which might be affected by CTX pretreatment and, thereby, could influence ischemic brain damage. Data from protein and mRNA levels of these receptors do not correlate necessarily with ligand binding due to confounding factors such as turnover time, trafficking to synapses and internalization/externalization of receptors (e.g. Huh and Wenthold, 1999). Since CTX had no effect on ligand binding we can assume that CTX had also no effect on receptor density after 2VO. The lack of CTX effects on ligand binding excludes the possibility that the induction of GLT-1 transporters via CTX, and, thereby reduced extracellular availability of glutamate might have caused upregulation of excitotoxic NMDA

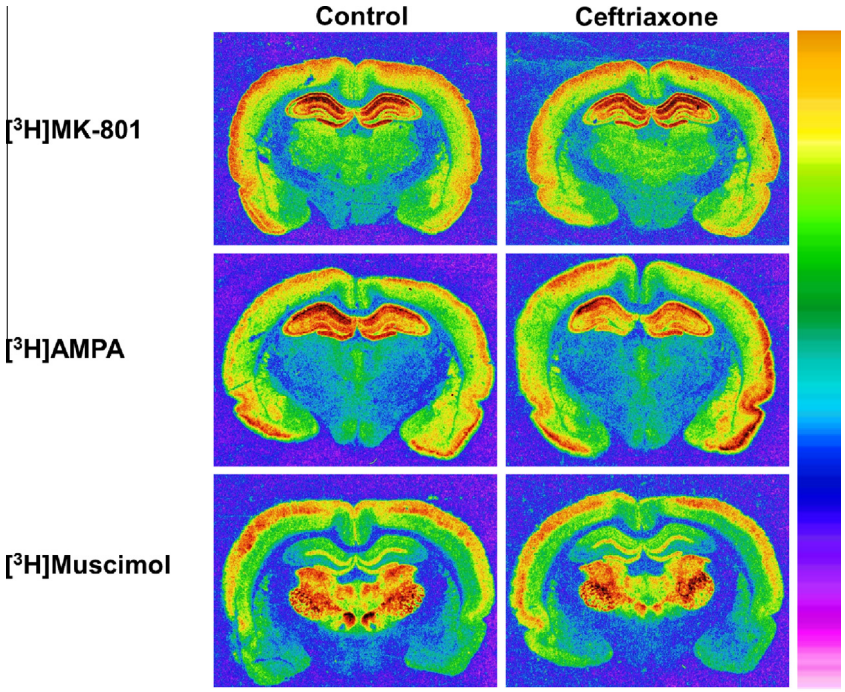

Fig. 7. Representative color-coded autoradiographs of [^{3}H]MK-801, [^{3}H]AMPA and [^{3}H]Muscimol binding in rats after treatment with ceftriaxone (CTX) compared to controls. The scale on the right indicates the relative densities from low (= purple) to high (red) density. Ligand binding values show no significant differences between rats after treatment with CTX compared to controls. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

receptors and consequently an increased excitability of neurons. The possibility that CTX interacted directly with infarct development at the time of 2VO is also excluded by its short-life time. Half-life time of CTX in healthy volunteers is around 6 h (Patel et al., 1981). Using microdialysis in rats Granero (Granero et al., 1995) found half-life time of 42.5 (plasma), 51.9 (striatum) and 64.7 min (lateral ventricle) for CTX. Only a small portion of injected CTX penetrates into the brain parenchyma. However, the used dosages seem to be high enough to induce GLT-1 transporter upregulation as demonstrated in various animal models of human diseases (Rothstein et al., 2005; Chu et al., 2007; Miller et al., 2008; Sari et al., 2009; Verma et al., 2010; Mimura et al., 2012).

Astrocytes play a crucial role in the removal of extracellular glutamate to prevent glutamate excitotoxicity (Choi, 1988; Rothstein et al., 1994, 1996). GLT-1 is the rat equivalent to the human homolog EAAT2 (Rao et al., 2001b; Nederkoorn et al., 2011). EAAT2 is responsible for more than 90% of the total glutamate uptake (Maragakis et al., 2004). Clearance of excessive glutamate is especially important during the early phase after brain ischemia. Using a glutamate electrode Guyot et al. (2001) showed an initial increase of extracellular glutamate in the penumbral region (peak 11-fold) that lasted for 2 h in most animals. One third displayed a second, prolonged sixfold elevation that lasted for at least 4 h. As also shown by studies using NMDA receptor antagonists the critical phase in experimental stroke concerns the first couple of hours. Therefore, pretreatment with CTX to increase numbers of GLT-1 is a crucial step. On the other hand, glutamate uptake may turn into release if the transporter reverses transport direction in ischemic conditions, and neuronal death is induced (Szatkowski et al., 1990; Rossi et al., 2000). In consequence, an increased availability of transporter molecules after upregulation of GLT-1 could also be dangerous.

There are indeed reports suggesting that GLT-1 is essential for uptake of extracellular glutamate to protect neurons in ischemic conditions. For instance, transient focal ischemia downregulates GLT-1, and antisense knockdown of GLT-1 exacerbates ischemia-induced neuronal damage and causes infarct growth (Rao et al., 2001a,b). Ischemic preconditioning diminishes the increase in extracellular glutamate caused by oxygen–glucose deprivation and increases cellular glutamate uptake and expression of GLT-1 (Romera et al., 2004). Similarly, preconditioning by intermittent hypobaric hypoxia elevated GLT-1 expression and reduced ischemia-induced brain injury that can be reversed by the GLT-1 blocker DHK (Gong et al., 2012). Conversely, reversal of GLT-1 may contribute to the ischemia-induced increase of extracellular glutamate since glutamate uptake is electrogenic and is reversed at elevated extracellular potassium and glial cell depolarization – conditions to be expected in the ischemic core (Szatkowski et al., 1990). Indeed, the GLT-1 blocker, DL-threo-β-benzyloxyaspartate reduces ischemia-induced glutamate release in a model of forebrain ischemia (Phillis et al., 2000). These considerations underline that glutamate transport is to be considered neuroprotective particularly in the penumbra zone, where glial cells are expected to still

Table 2. Values are means of ligand binding densities in ceftriaxone-treated rats presented as % of vehicle-treated rats ± SD

[^{3}H]MK801			[^{3}H]AMPA			[^{3}H]Muscimol		
Fr1	Mean (%)	SD	Fr1	Mean (%)	SD	Fr1	Mean (%)	SD
I	108.3	19.1	I	114.3	38.3	I	98.0	14.4
II	108.0	20.2	II	107.9	37.4	II	108.6	13.9
III	102.0	14.3	III	105.5	37.4	III	113.7	16.8
IV	95.4	9.5	IV	107.9	29.8	IV	107.8	26.0
V	98.7	10.5	V	106.3	42.7	V	98.9	17.6
VI	97.7	13.3	VI	99.6	52.6	VI	104.9	15.7
HL	Mean (%)	SD	HL	Mean (%)	SD	HL	Mean (%)	SD
I	97.8	15.6	I	130.3	47.9	I	99.6	18.9
II	100.8	11.7	II	137.9	44.7	II	102.3	14.9
III	101.5	19.3	III	144.1	40.7	III	96.4	14.9
IV	107.0	11.9	IV	134.6	55.2	IV	95.6	25.7
V	98.6	13.8	V	125.4	53.3	V	101.5	17.7
VI	104.8	20.2	VI	119.9	57.1	VI	105.5	20.3
Par2	Mean (%)	SD	Par2	Mean (%)	SD	Par2	Mean (%)	SD
I	105.7	21.2	I	99.65	65.6	I	118.5	21.5
II	108.3	14.1	II	104.2	61.7	II	114.7	17.6
III	106.9	15.0	III	115.4	56.3	III	109.1	15.2
IV	106.3	16.2	IV	107.7	57.5	IV	107.6	17.1
V	102.6	13.7	V	108.4	66.0	V	116.1	14.7
VI	107.4	19.9	VI	104.3	71.6	VI	119.8	23.4
Hippocampus	Mean (%)	SD	Hippocampus	Mean (%)	SD	Hippocampus	Mean (%)	SD
CA1 Or	106.3	16.8	CA1 Or	97.9	37.7	CA1 Or	111.8	16.9
CA1 Rad	106.9	18.5	CA1 Rad	96.8	38.9	CA1 Rad	114.3	16.9
CA1 Py	95.7	13.8	CA1 Py	99.9	38.3	CA1 Py	114.1	14.4
CA3 Or	110.0	14.7	CA3 Or	118.4	59.9	CA3 Or	109.9	31.2
CA3 Rad	105.8	17.6	CA3 Rad	96.6	49.2	CA3 Rad	122.4	35.8
CA3 Py	98.5	14.7	CA3 Py	112.0	45.9	CA3 Py	101.4	35.4
DG Mol	102.5	10.6	DG Mol	90.6	34.5	DG Mol	110.2	13.4
DG Gr	100.0	20.1	DG Gr	82.8	37.4	DG Gr	110.5	17.8

Mean control value = 100% (data not shown). *Abbreviations:* Fr1, frontal cortex area 1; HL, hindlimb area; Par1, primary somatosensory cortex/parietal Cortex; Or, stratum oriens; Rad, stratum radiatum; Py, stratum pyramidale; DG, dentate gyrus; Mol, molecular layer; Gr, granular layer; SD, standard deviation.

maintain their potential and are still able to repolarize after spreading depression. Indeed glutamate release in less severely ischemic brain occurs mainly via volume activated channels and not via GLT-1 reversal (Feustel et al., 2004). In our venous ischemia model CBF mapping shows a widespread low flow region, whereas the ischemic core is hardly detectable in the acute phase (Otsuka et al., 2000). Thus, this venous ischemia model has been proposed as a penumbra model (Nakase et al., 1996; Kempski et al., 1999; Otsuka et al., 2000). Although a small infarction can be seen within 24 h in the 2VO model it grows continuously for at least 4 days (Nakase et al., 1997a,b; Kimura et al., 2005; Takeshima et al., 2011; Wajima et al., 2011). The current data show that using this model modification of GLT-1 by pretreatment with CTX suppressed infarct growth which is very well in line with findings after arterial ischemia (Chu et al., 2007) and the idea that glutamate removal in the penumbra is acting protectively. This notion is further supported by the reversal of neuroprotection by DHK in CTX-treated animals and by the tendency to exacerbate infarct in vehicle-treated animals. Neuroprotection, however, can be caused by increasing GLT-1 transporters or from improved inward transport by the pre-existing GLT-1 transporters by CTX treatment as reported by Lipski et al. (2007).

Spreading depression is known to be induced by increased extracellular glutamate (Van Harreveld, 1978). Therefore, we assumed that upregulation of GLT-1 could have an effect on spreading depression generation in the penumbra. However, our data did not show any significant changes in the number of spreading depressions after CTX pretreatment. As a possible explanation it is suggested that in our model potassium ions could be the major source of spontaneous spreading depressions occurring rather than glutamate which is mostly released in the small ischemic core. This remains to be verified in future experiments.

Currently, a clinical randomized trial whether the preventive use of CTX improves functional outcome in patients with stroke is progressing (Nederkoorn et al., 2011). We suggest additional trials in neurosurgery, where an intentional venous sacrifice is often necessary particularly in tumor surgery. Here a preoperative treatment with CTX may actually improve patient outcome when venous sacrifice can be expected

preoperatively. Likewise, CTX treatment might also be envisaged in conditions such as sinus-vein thrombosis.

CONCLUSIONS

This study provides evidence that pretreatment with CTX has a neuroprotective effect on the ischemic penumbra. A future clinical use is suggested in conditions where an impaired venous drainage can be expected to occur such as cases of brain tumor surgery.

Acknowledgments—This study was supported by staff of our laboratory. The authors thank Fatemeh Kafai for excellent secretarial assistance and Annett Ehlert, Michael Malzahn, Magdeleine Herkt and Laszlo Kopacz for their technical help and support.

REFERENCES

Attwell D, Barbour B, Szatkowski M (1993) Nonvesicular release of neurotransmitter. Neuron 11:401–407.

Choi DW (1988) Glutamate neurotoxicity and diseases of the nervous system. Neuron 1:623–634.

Chu K, Lee ST, Sinn DI, Ko SY, Kim EH, Kim JM, Kim SJ, Park DK, Jung KH, Song EC, Lee SK, Kim M, Roh JK (2007) Pharmacological induction of ischemic tolerance by glutamate transporter-1 (EAAT2) upregulation. Stroke 38:177–182.

Diederich K, Frauenknecht K, Minnerup J, Schneider BK, Schmidt A, Altach E, Eggert V, Sommer CJ, Schabitz WR (2012a) Citicoline enhances neuroregenerative processes after experimental stroke in rats. Stroke 43:1931–1940.

Diederich K, Quennet V, Bauer H, Muller HD, Wersching H, Schabitz WR, Minnerup J, Sommer C (2012b) Successful regeneration after experimental stroke by granulocyte-colony stimulating factor is not further enhanced by constraint-induced movement therapy either in concurrent or in sequential combination therapy. Stroke 43:185–192.

Feustel PJ, Jin Y, Kimelberg HK (2004) Volume-regulated anion channels are the predominant contributors to release of excitatory amino acids in the ischemic cortical penumbra. Stroke 35:1164–1168.

Gong SJ, Chen LY, Zhang M, Gong JX, Ma YX, Zhang JM, Wang YJ, Hu YY, Sun XC, Li WB, Zhang Y (2012) Intermittent hypobaric hypoxia preconditioning induced brain ischemic tolerance by up-regulating glial glutamate transporter-1 in rats. Neurochem Res 37:527–537.

Granero L, Santiago M, Cano J, Machado A, Peris JE (1995) Analysis of ceftriaxone and ceftazidime distribution in cerebrospinal fluid of and cerebral extracellular space in awake rats by in vivo microdialysis. Antimicrob Agents Chemother 39:2728–2731.

Guyot LL, Diaz FG, O'Regan MH, McLeod S, Park H, Phillis JW (2001) Real-time measurement of glutamate release from the ischemic penumbra of the rat cerebral cortex using a focal middle cerebral artery occlusion model. Neurosci Lett 299:37–40.

Huh KH, Wenthold RJ (1999) Turnover analysis of glutamate receptors identifies a rapidly degraded pool of the *N*-methyl-D-aspartate receptor subunit, NR1, in cultured cerebellar granule cells. J Biol Chem 274:151–157.

Kempski O, Seiwert T, Otsuka H, Heimann A, Nakase H (1999) Modelling of the ischemic penumbra. Acta Neurochir Suppl 73:41–44.

Kimura R, Nakase H, Tamaki R, Sakaki T (2005) Vascular endothelial growth factor antagonist reduces brain edema formation and venous infarction. Stroke 36:1259–1263.

Lehre KP, Levy LM, Ottersen OP, Storm-Mathisen J, Danbolt NC (1995) Differential expression of two glial glutamate transporters in the rat brain: quantitative and immunocytochemical observations. J Neurosci 15:1835–1853.

Lipski J, Wan CK, Bai JZ, Pi R, Li D, Donnelly D (2007) Neuroprotective potential of ceftriaxone in in vitro models of stroke. Neuroscience 146:617–629.

Maragakis NJ, Dietrich J, Wong V, Xue H, Mayer-Proschel M, Rao MS, Rothstein JD (2004) Glutamate transporter expression and function in human glial progenitors. Glia 45:133–143.

Miller BR, Dorner JL, Shou M, Sari Y, Barton SJ, Sengelaub DR, Kennedy RT, Rebec GV (2008) Up-regulation of GLT1 expression increases glutamate uptake and attenuates the Huntington's disease phenotype in the R6/2 mouse. Neuroscience 153:329–337.

Mimura K, Tomimatsu T, Minato K, Jugder O, Kinugasa-Taniguchi Y, Kanagawa T, Nozaki M, Yanagihara I, Kimura T (2012) Ceftriaxone preconditioning confers neuroprotection in neonatal rats through glutamate transporter 1 upregulation. Reprod Sci 18:1193–1201.

Monaghan DT, Cotman CW (1985) Distribution of *N*-methyl-D-aspartate-sensitive L-[^{3}H]glutamate-binding sites in rat brain. J Neurosci 5:2909–2919.

Nakagawa I, Alessandri B, Heimann A, Kempski O (2005) MitoKATP-channel opener protects against neuronal death in rat venous ischemia. Neurosurgery 57:334–340. discussion 334–340.

Nakase H, Kakizaki T, Miyamoto K, Hiramatsu K, Sakaki T (1995) Use of local cerebral blood flow monitoring to predict brain damage after disturbance to the venous circulation: cortical vein occlusion model by photochemical dye. Neurosurgery 37:280–285. discussion 285–286.

Nakase H, Heimann A, Kempski O (1996) Local cerebral blood flow in a rat cortical vein occlusion model. J Cereb Blood Flow Metab 16:720–728.

Nakase H, Kempski OS, Heimann A, Takeshima T, Tintera J (1997a) Microcirculation after cerebral venous occlusions as assessed by laser Doppler scanning. J Neurosurg 87:307–314.

Nakase H, Nagata K, Ohtsuka H, Sakaki T, Kempski O (1997b) An experimental model of intraoperative venous injury in the rat. Skull Base Surg 7:123–128.

Nakase H, Nagata K, Otsuka H, Sakaki T, Kempski O (1998) Local cerebral blood flow autoregulation following "asymptomatic" cerebral venous occlusion in the rat. J Neurosurg 89:118–124.

Nederkoorn PJ, Westendorp WF, Hooijenga IJ, de Haan RJ, Dippel DW, Vermeij FH, Dijkgraaf MG, Prins JM, Spanjaard L, van de Beek D (2011) Preventive antibiotics in stroke study: rationale and protocol for a randomised trial. Int J Stroke 6:159–163.

Otsuka H, Ueda K, Heimann A, Kempski O (2000) Effects of cortical spreading depression on cortical blood flow, impedance, DC potential, and infarct size in a rat venous infarct model. Exp Neurol 162:201–214.

Palacios JM, Wamsley JK, Kuhar MJ (1981) High affinity GABA receptors-autoradiographic localization. Brain Res 222:285–307.

Patel IH, Chen S, Parsonnet M, Hackman MR, Brooks MA, Konikoff J, Kaplan SA (1981) Pharmacokinetics of ceftriaxone in humans. Antimicrob Agents Chemother 20:634–641.

Phillis JW, Ren J, O'Regan MH (2000) Transporter reversal as a mechanism of glutamate release from the ischemic rat cerebral cortex: studies with DL-threo-beta-benzyloxyaspartate. Brain Res 868:105–112.

Pines G, Danbolt NC, Bjoras M, Zhang Y, Bendahan A, Eide L, Koepsell H, Storm-Mathisen J, Seeberg E, Kanner BI (1992) Cloning and expression of a rat brain L-glutamate transporter. Nature 360:464–467.

Rao VL, Bowen KK, Dempsey RJ (2001a) Transient focal cerebral ischemia down-regulates glutamate transporters GLT-1 and EAAC1 expression in rat brain. Neurochem Res 26:497–502.

Rao VL, Dogan A, Todd KG, Bowen KK, Kim BT, Rothstein JD, Dempsey RJ (2001b) Antisense knockdown of the glial glutamate transporter GLT-1, but not the neuronal glutamate transporter EAAC1, exacerbates transient focal cerebral ischemia-induced neuronal damage in rat brain. J Neurosci 21:1876–1883.

Romera C, Hurtado O, Botella SH, Lizasoain I, Cardenas A, Fernandez-Tome P, Leza JC, Lorenzo P, Moro MA (2004) In vitro ischemic tolerance involves upregulation of glutamate

transport partly mediated by the TACE/ADAM17-tumor necrosis factor-alpha pathway. J Neurosci 24:1350–1357.

Rossi DJ, Oshima T, Attwell D (2000) Glutamate release in severe brain ischaemia is mainly by reversed uptake. Nature 403:316–321.

Rothstein JD, Martin L, Levey AI, Dykes-Hoberg M, Jin L, Wu D, Nash N, Kuncl RW (1994) Localization of neuronal and glial glutamate transporters. Neuron 13:713–725.

Rothstein JD, Dykes-Hoberg M, Pardo CA, Bristol LA, Jin L, Kuncl RW, Kanai Y, Hediger MA, Wang Y, Schielke JP, Welty DF (1996) Knockout of glutamate transporters reveals a major role for astroglial transport in excitotoxicity and clearance of glutamate. Neuron 16:675–686.

Rothstein JD, Patel S, Regan MR, Haenggeli C, Huang YH, Bergles DE, Jin L, Dykes Hoberg M, Vidensky S, Chung DS, Toan SV, Bruijn LI, Su ZZ, Gupta P, Fisher PB (2005) Beta-lactam antibiotics offer neuroprotection by increasing glutamate transporter expression. Nature 433:73–77.

Sari Y, Smith KD, Ali PK, Rebec GV (2009) Upregulation of GLT1 attenuates cue-induced reinstatement of cocaine-seeking behavior in rats. J Neurosci 29:9239–9243.

Seki Y, Feustel PJ, Keller Jr RW, Tranmer BI, Kimelberg HK (1999) Inhibition of ischemia-induced glutamate release in rat striatum by dihydrokinate and an anion channel blocker. Stroke 30:433–440.

Standley S, Tocco G, Tourigny MF, Massicotte G, Thompson RF, Baudry M (1995) Developmental changes in alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionate receptor properties and expression in the rat hippocampal formation. Neuroscience 67:881–892.

Szatkowski M, Barbour B, Attwell D (1990) Non-vesicular release of glutamate from glial cells by reversed electrogenic glutamate uptake. Nature 348:443–446.

Takeshima Y, Nakamura M, Miyake H, Tamaki R, Inui T, Horiuchi K, Wajima D, Nakase H (2011) Neuroprotection with intraventricular brain-derived neurotrophic factor in rat venous occlusion model. Neurosurgery 68:1334–1341.

Uranishi R, Nakase H, Sakaki T, Kempski OS (1999) Evaluation of absolute cerebral blood flow by laser-Doppler scanning – comparison with hydrogen clearance. J Vasc Res 36:100–105.

Van Harreveld A (1978) Two mechanisms for spreading depression in the chicken retina. J Neurobiol 9:419–431.

Verma R, Mishra V, Sasmal D, Raghubir R (2010) Pharmacological evaluation of glutamate transporter 1 (GLT-1) mediated neuroprotection following cerebral ischemia/reperfusion injury. Eur J Pharmacol 638:65–71.

Wajima D, Nakamura M, Horiuchi K, Takeshima Y, Nishimura F, Nakase H (2011) Cilostazol minimizes venous ischemic injury in diabetic and normal rats. J Cereb Blood Flow Metab 31:2030–2040.

Zhang M, Li WB, Geng JX, Li QJ, Sun XC, Xian XH, Qi J, Li SQ (2007) The upregulation of glial glutamate transporter-1 participates in the induction of brain ischemic tolerance in rats. J Cereb Blood Flow Metab 27:1352–1368.

Zilles K, Wu J, Crusio WE, Schwegler H (2000) Water maze and radial maze learning and the density of binding sites of glutamate, GABA, and serotonin receptors in the hippocampus of inbred mouse strains. Hippocampus 10:213–225.

(Accepted 7 March 2013)
(Available online 21 March 2013)