Title Page

1. Manuscript title

Neuroprotective effect of Ceftriaxone on penumbra in the rat venous ischemia model

2. Authors name 3. Department

Toshitaka Inui, M.D. Institute for Neurosurgical Pathophysiology,

University Medical Center of the Johannes Gutenberg-University Mainz

Institute for Neurosurgical Pathophysiology,

Department of Neurosurgery, Nara Medical University, Nara, Japan

Beat Alessandri, Ph.D.

University Medical Center of the Johannes Gutenberg-University Mainz

Institute for Neurosurgical Pathophysiology,

Axel Heimann, D.V.M.

University Medical Center of the Johannes Gutenberg-University Mainz

Institute for Neurosurgical Pathophysiology,

Katrin Frauenknecht,M.D, Ph.D.

University Medical Center of the Johannes Gutenberg-University Mainz

Institute for Neuropathology,

Oliver Kempski, M.D., Ph.D. Institute for Neurosurgical Pathophysiology,

University Medical Center of the Johannes Gutenberg-University Mainz

Institute for Neurosurgical Pathophysiology,

4. Corresponding author's name

Oliver Kempski, M.D.

University Medical Center of the Johannes Gutenberg-University Mainz

Institute for Neurosurgical Pathophysiology,

55131 Mainz, Germany

Telephone: +49-6131-17-2373

Fax: +49-6131-17-6640

Email: oliver.kempski@unimedizin-mainz.de

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7. Running head

Effect of ceftriaxone on venous ischemia

Title

Neuroprotective effect of Ceftriaxone on the penumbra in a rat venous ischemia model

Laboratory investigation

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 in the ischemic penumbra. Recently, a neuroprotective effect of β-lactam antibiotics such as ceftriaxone (CTX) that increases expression of GLT-1 has been reported. 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) that is known to have a large penumbra.

Methods. CTX (100mg/kg, 200mg/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 2 adjacent cortical veins (2VO) by photothrombosis with rose bengal that was followed by KCl-induced cortical spreading

depression. Infarct volume was evaluated with 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 2 groups without 2-VO (n=5/group). Animals were injected either with NaCl (vehicle) or CTX 200mg/kg for 5 days in order to evaluate whether NMDA, AMPA and GABA receptor expression was 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) 30mins before 2VO. CTX had no effect on the number of spontaneous spreading depressions. 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 receptors. 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.

Key Words: Ceftriaxone, cortical spreading depression, glutamate transporter-1, penumbra, venous ischemia

Introduction

Glutamate is the major excitatory synaptic transmitter in the mammalian central nervous system. On the other hand, rises in the extracellular concentration of glutamate lead to neurotoxicity [1], therefore, it is important to maintain glutamate at a low level. Normal concentrations of extracellular glutamate are maintained by glutamate transporters [2,25]. At least five subtypes of excitatory amino acid transporters (EAATs) have been confirmed of which GLT-1－EAAT2 mainly exists in astrocytes [20,25]. The predominant EAAT subtype in humans is EAAT2 and in rodents it is GLT-1 [7,26]. Antisense knockdown of GLT-1 exacerbates transient focal ischemia-induced neuronal damage [22]. Some reports have shown that GLT-1 blockers can attenuate ischemia-induced glutamate release [28]. Thus, the function of GLT-1 for the brain in ischemic conditions is discussed controversially especially in the ischemic penumbra where lesion development progresses. Recently a neuroprotective effect in the ischemic penumbra via upregulation of GLT-1 has been shown for the beta-lactam antibiotic ceftriaxone [3,27] which easily crosses the blood brain barrier. In contrast, ceftriaxone exposure did not result in neuroprotection against oxygen-glucose deprivation [8],

contradicting efficacy of elevated GLT-1 under ischemia-like condition in vitro. Thus, in this study, we examined whether CTX-pretreatment reduces infarct growth in a rat venous ischemia model using rose bengal and focussed fiberoptic illumination [12-15]. This 2-vein occlusion (2VO) model produces widespread reduction of cortical cerebral blood flow (CBF) with a rather large penumbra area. Therefore, this venous ischemia model is suited to study penumbra pathophysiology.

Furthermore, we evaluated by quantitative in-vitro receptor autoradiography whether altered NMDA, AMPA and GABA receptor expression by CTX is involved in neuroprotection after 2VO.

Material and Methods

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/100g body weight) before premedication with 1.0mg atropine. Anesthesia was maintained with chloral hydrate (12 mg/100g body weight/h) through a peritoneal silicon catheter. All animals were intubated with silicon tubing (outer diameter, 2.5mm) and mechanically ventilated with 30% O_2 using a rodent ventilator (Model 683; Harvard Apparatus, South Natick, MA) 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), a 1.5 cm midline skin incision was set up, and a small hole was made by a 18G needle 2mm right lateral from midline, and 2mm below the bregma. The lateral ventricle cannula was made from an L shaped 26G needle connected to polyethylene tubing (volume:10μl, length 7cm±1mm). 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) and fiberoptic illumination using a 50W mercury lamp (6500-7500 1x, 540 nm) and a 100 μm fiber directly positioned over the veins, thus avoiding illumination of surrounding brain tissue [12,13]. 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 100μm. 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) with a 0.8-mm needle probe [12,13]. CBF which is expressed in LDU ((Laser Doppler units) was measured at 25 points with the occluded veins lateral to the scanning field by using a stepping-motor-driven and computer-controlled micromanipulator [12,14]. Thus, one scanning procedure yielded information from 25 different locations (square of 5 x 5 points) 300 μm apart from each other. Scanning was performed before and every 5min for 75 min after venous ischemia. The median of observation frequency histograms correlates with absolute regional blood flow as determined by hydrogen clearance [31]. Two impedance electrodes were inserted into the cortex (depth, 0.4-0.5 mm; distance, 3mm) (Fig. 1) to measure cell swelling that occurs during induced cortical spreading depression and venous ischemia [17]. 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 with using a precision LCR monitor (4284A; Hewlett-Packard, Avondale, PA).

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 ten 5.0-μl KCl injections were administered every 7 min to induce cortical spreading depression [11,17]. Injection of KCl started after completion of 2 vein occlusion and ended 70 minutes thereafter.

Experimental Design and Treatment Groups

Effect of CTX on CBF and infarct volume

45 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, 0.14mg/kg) (n=8); Group E: CTX 200 mg/kg-pretreatment together with DHK 0.14mg/kg (in 20μl saline) (n=8) (Fig. 2). All rats received CTX or vehicle intraperitoneally for 5 successive days before venous ischemia. In group D and E, DHK was intraventricularly injected once 30 minutes before venous occlusion. All drugs were purchased from Sigma Chemical Co.

Effect of CTX on cortical spreading depression

12 rats were randomized into 2 groups. Group 1: vehicle-pretreatment (0.9% NaCl;

n=6); Group 2: CTX 200mg/kg-pretreatment (n=6).

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 coronal sections were sliced in parietal region including the infarct area. Sections were stained with hematoxylin-eosin. The histological evaluation of the infarct volume was accomplished using a light microscope connected to a CCD camera (Sony, Tokyo, Japan) and Optimas 6.51 software (Optimas Corp., Seattle, WA). 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 (mm2) multiplied with the distance between sections (0.2 mm).

Effect of CTX on receptor density

Quantitative in-vitro receptor autoradiography was performed on 10 Wistar rats which were assigned to two groups, one injected (i.p.) with 200mg/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 analysed. 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 labelling of NMDA, AMPA and $GABA_A$ receptors, respectively [10,18,29]. Ligands were purchased from NEN™ Life Sciences Products Inc (Boston, MA, USA). Labelling and incubation procedures for the different binding sites were performed according to the protocols of Zilles et al. [33] as previously described [4].

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) n the presence of 30μM glycine and 50 μM spermidine at 22°C for 60 min. Incubation was terminated by washing in cold buffer (2 x 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 x 4 s) with cold buffer and fixation rinsing (2 x 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 x 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 five ([^{3}H]AMPA) or six weeks ([^{3}H]MK-801 and [^{3}H]Muscimol)- Autoradiographs were scanned in equal light conditions with the digital CoolSNAP camera (Roper Scientific, Photometrics CoolSNAP^{TM}cf, 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 ± SD) [4,5]

Mean ligand binding density was analyzed within layers I to 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 ± 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 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.

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 group 1 and 2 (*Effect of CTX on cortical spreading depression*) did not shop 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 minutes 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 was measured in group 1 and 2. Cortical impedance values showed no significant difference between both groups (vehicle, 4.4±0.27kΩ; CTX, 4.1±0.22kΩ) 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. 4A). There was no significant difference between the 2 groups (vehicle: 4.3±0.23kΩ; CTX: 3.9±0.22kΩ). 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. 4B).

Infarct volume

All rats had venous infarction. In group B (CTX100mg) and group C (CTX 200mg) infarct volume was significantly reduced to 7.48±0.64mm^{3}and 6.44±0.95mm^3, respectively, when compared with 8.84±1.94mm^3 in group A (vehicle) Lesion volume of the two CTX dosages did not differ statistically from each other (Fig. 5A). The reduction of infarct volume was significantly attenuated by intraventricular injection of DHK in group D (9.04±2.4mm^3) when compared to treatment with CTX 200mg/kg in

group C (6.44±0.95mm^3) (Fig. 5B).

Quantitative in vitro receptor autoradiography

Analysis of [^{3}H] MK-801, [^{3}H] AMPA and [^{3}H] Muscimol ligand binding values in the six cortical layers of the frontal cortex area 1 (Fr1), the parietal cortex hindlimb area (HL), the parietal cortex area 2 (Par2) as well as in the hippocampal subfields CA1, CA3 and in the dentate gyrus (DG) showed no significant differences between rats treated with ceftriaxone in comparison to saline-treated control rats (Fig. 6, Table 2).

Discussion

In this study we showed that a pretreatment with ceftriaxone reduced infarct volume in a rat venous ischemia model without influencing local cerebral blood flow 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 function furthers neuronal death in this model whereas induction of GLT-1 with CTX offers neuroprotection as seen in previous reports [3,27]. Quantitative receptor autoradiography showed no influence of 5 days

ceftriaxone preconditioning in absence of ischemia on expression of NMDA, AMPA and GABA receptors. This excludes the possibility that the induction of GLT-1 transporters via ceftriaxone, and, thereby reduced extracellular availability of glutamate might have caused upregulation of excitotoxic NMDA receptors.

Astrocytes play a crucial role in removal of extracellular glutamate to prevent glutamate excitotoxicity [2,25,26]. GLT-1 is the rat equivalent to the human homologue EAAT2 [16,22]. EAAT2 is responsible for more than 90% of the total glutamate uptake [9]. On the other hand, glutamate uptake may turn into release if the transporter reverses transport direction in ischemic conditions, and neuronal death is induced [24,30]. 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: transient focal ischemia downregulates GLT-1, and antisense knockdown of GLT-1 exacerbates ischemia-induced neuronal damage and causes infarct growth [21,22]. Ischemic preconditioning diminishes the increase in extracellular glutamate caused by oxygen-glucose deprivation and increases cellular glutamate uptake and expression of GLT-1 [23]. Conversely, reversal of GLT-1 may contribute to the ischemia-induced

increase of extracellular glutamate since glutamate uptake is electrogenic and reversed at elevated extracellular potassium and glial cell depolarization – conditions to be expected in the ischemic core [30]. Indeed, the GLT-1 blocker, DL-threo-β-benzyloxyaspartate reduces ischemia-induced glutamate release in a model of forebrain ischemia [19]. These considerations underline that glutamate transport is to be considered neuroprotective particularly in the penumbra zone, where glial cells are expected to still 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 [5]. In our venous ischemia model CBF mapping shows a widespread low flow region, whereas the ischemic core is hardly detectable in the acute phase [17]. Thus, this venous ischemia model has been proposed as a penumbra model [6,12,17]. The current data show that using this model upregulation of GLT-1 by pretreatment with CTX suppressed infarct growth which is very well in line with the idea that glutamate removal in the penumbra is acting protectively.

Spreading depression is known to be induced by increased extracellular glutamate [32]. 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 ceftriaxone improves functional outcome in patients with stroke is progressing [16]. We suggest additional trials in neurosurgery, where an intentional venous sacrifice is often necessary particularly in tumour 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 via upregulation of GLT-1. A future clinical use is suggested in conditions where an impaired venous drainage can be expected to occur.

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References

1. Attwell D, Barbour B, Szatkowski M: Nonvesicular release of neurotransmitter. Neuron 11:401-407, 1993

2. Choi DW: Glutamate neurotoxicity and diseases of the nervous system. Neuron 1:623-634, 1988

3. Chu K, Lee ST, Sinn DI, Ko SY, Kim EH, Kim JM, et al: Pharmacological Induction of Ischemic Tolerance by Glutamate Transporter-1 (EAAT2) Upregulation. Stroke 38:177-182, 2007

4. Diederich K, Quennet V, Bauer H, Muller HD, Wersching H, Schabitz WR, et al: 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, 2012

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

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

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

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

9. Maragakis NJ, Dietrich J, Wong V, Xue H, Mayer-Proschel M, Rao MS, et al: Glutamate transporter expression and function in human glial progenitors. Glia 45:133-143, 2004

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

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

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

13. Nakase H, Kakizaki T, Miyamoto K, Hiramatsu K, Sakaki T: 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, 1995

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

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

16. Nederkoorn PJ, Westendorp WF, Hooijenga IJ, de Haan RJ, Dippel DW, Vermeij FH, et al: Preventive antibiotics in stroke study: rationale and protocol for a randomised trial. Int J Stroke 6:159-163, 2011

17. Otsuka H, Ueda K, Heimann A, Kempski O: 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, 2000

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

19. Phillis JW, Ren J, O'Regan MH: 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, 2000

20. Pines G, Danbolt NC, Bjoras M, Zhang Y, Bendahan A, Eide L, et al: Cloning and expression of a rat brain L-glutamate transporter. Nature 360:464-467, 1992

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

22. Rao VL, Dogan A, Todd KG, Bowen KK, Kim BT, Rothstein JD, et al: 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, 2001

23. Romera C, Hurtado O, Botella SH, Lizasoain I, Cardenas A, Fernandez-Tome P, et al: 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, 2004

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

25. Rothstein JD, Dykes-Hoberg M, Pardo CA, Bristol LA, Jin L, Kuncl RW, et al: Knockout of glutamate transporters reveals a major role for astroglial transport in excitotoxicity and clearance of glutamate. Neuron 16:675-686, 1996

26. Rothstein JD, Martin L, Levey AI, Dykes-Hoberg M, Jin L, Wu D, et al: Localization of neuronal and glial glutamate transporters. Neuron 13:713-725, 1994

27. Rothstein JD, Patel S, Regan MR, Haenggeli C, Huang YH, Bergles DE, et al: Beta-lactam antibiotics offer neuroprotection by increasing glutamate transporter expression. Nature 433:73-77, 2005

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

29. Standley S, Tocco G, Tourigny MF, Massicotte G, Thompson RF, Baudry M: 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, 1995

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

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

32. Van Harreveld A: Two mechanisms for spreading depression in the chicken retina. J

Neurobiol 9:419-431, 1978

33. Zilles K, Wu J, Crusio WE, Schwegler H: 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, 2000

Figure legends

FIGURE 1: Schematic drawing of the experimental setup after craniotomy. The location of scanning area for CBF 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.

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

FIG 3. Successive CBF change before and after cortical venous occlusion in group A (vehicle pretreamten), 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 ± SEM. CTX = ceftriaxone, DHK = dihydrokainate

FIGURE 4: Typical impedance change of KCl-induced CSDs (top) and number of CSDs in NaCl (vehicle) or CTX200mg/kg pretreatment group (bottom). There is no statistical significance between groups. Values are given as mean ± SEM.

FIGURE 5. Infarct volume 2 days after venous ischemia without (top panel) and with inhibition of GLT-1 by 0.14 mg/kg dihydrokainate (DK; lower panel). Values are given as mean ± SEM. Infarct volume was reduced by CTX pretreatment compared with vehicle administration group. *$p < 0.05$ versus CTX200.

FIGURE 6: Representative colour-coded autoradiographs of [^{3}H]MK-801, [^{3}H]AMPA and [^{3}H]Muscimol binding in rats after treatment with ceftriaxone 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 ceftriaxone compared to controls.

TABLE 1: Physiological variables before and after venous ischemia

	MABP (mmHg)	pH	PO_2 (mmHg)	PCO_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; PO_2=partial pressure of oxygen; PCO_2=partial pressure of carbon dioxide; 2VO=two vein occlusion; CTX=ceftriaxone; DHK=dihydrokainate. The data are expressed as mean ± SEM. *$p < 0.05$ vs. vehicle group ???.

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

[^{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

FIG 1

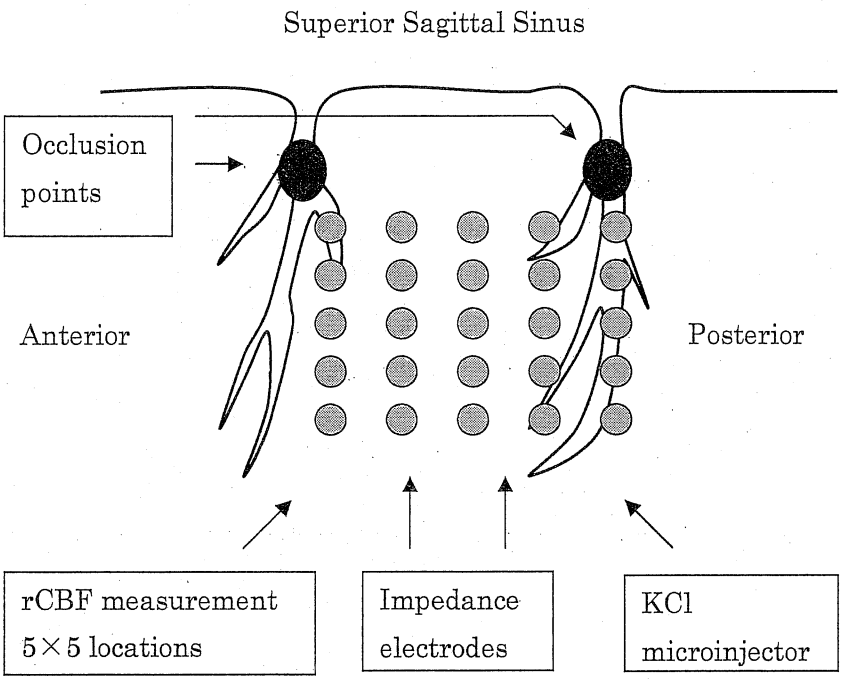

Superior Sagittal Sinus
Occlusion points
Anterior
Posterior
rCBF measurement 5×5 locations
Impedance electrodes
KCl microinjector

FIG. 2

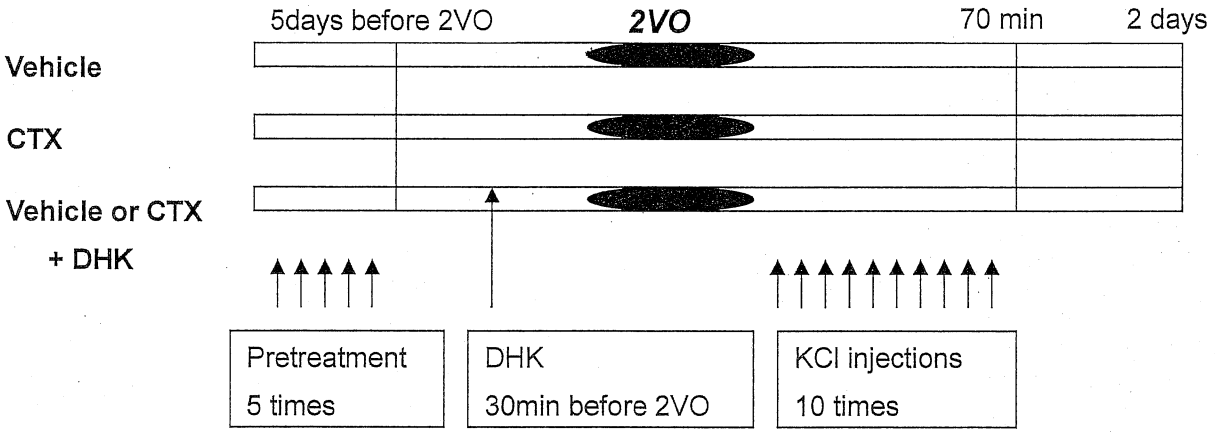

5days before 2VO
2VO
70 min
2 days
Vehicle
CTX
Vehicle or CTX
+ DHK
Pretreatment
5 times
DHK
30min before 2VO
KCl injections
10 times

FIG. 3

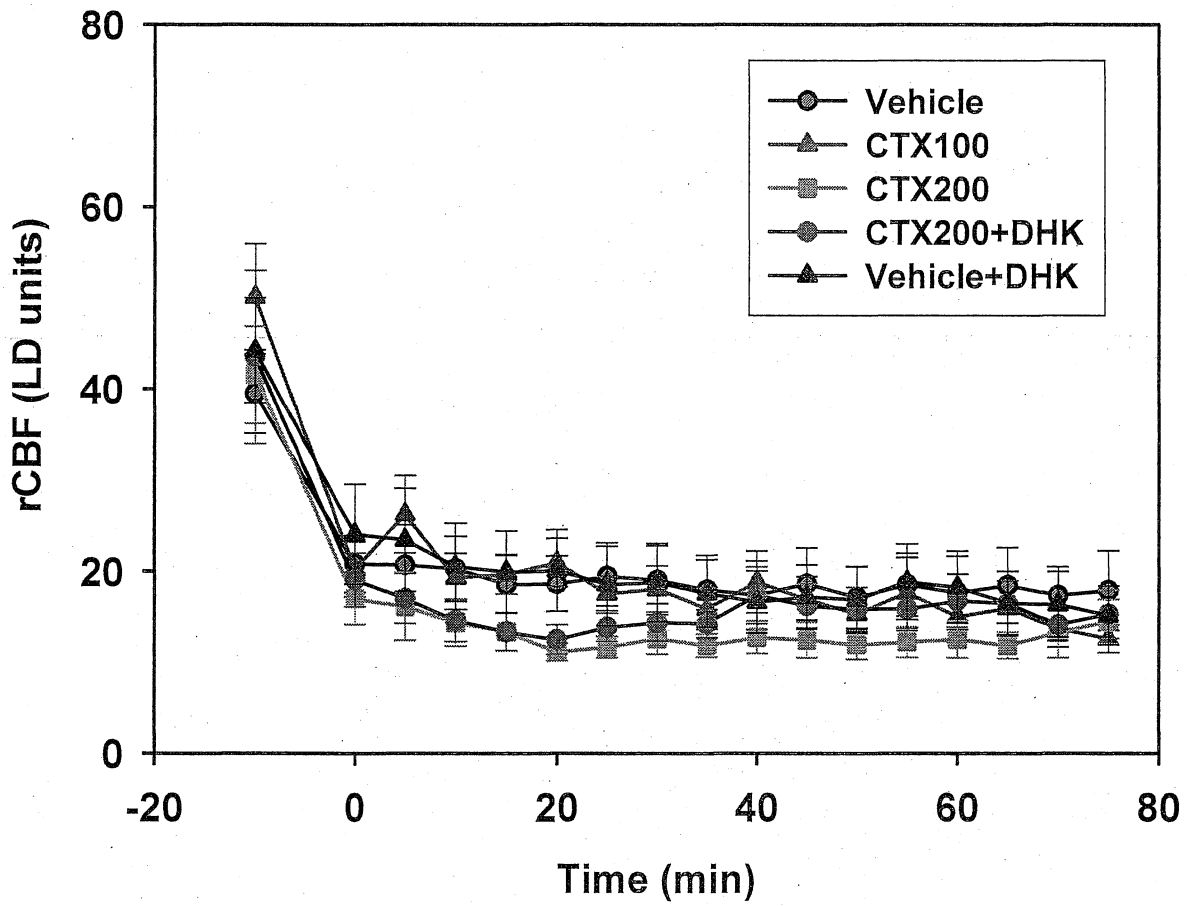
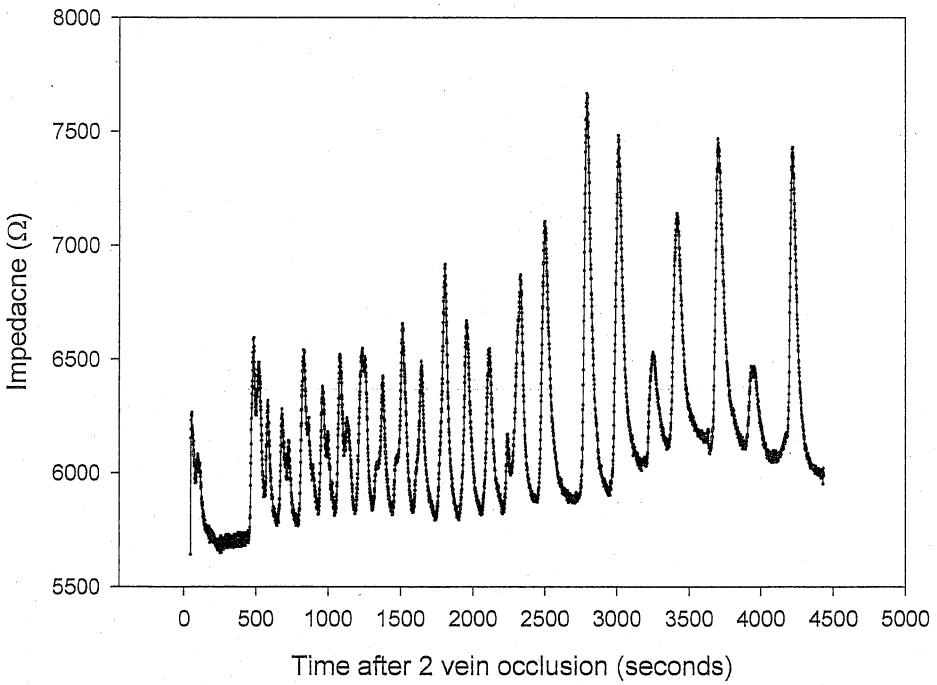
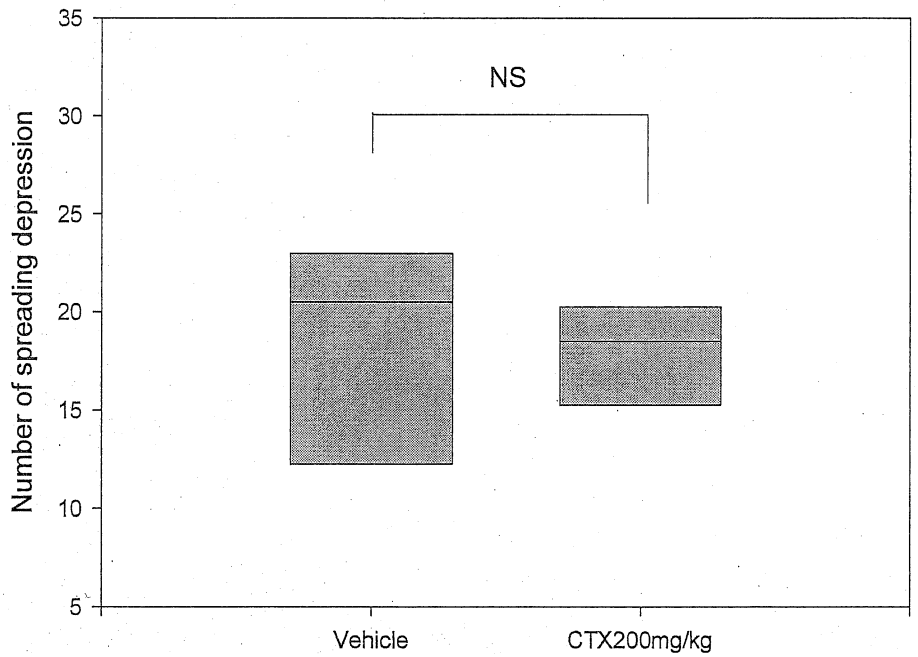
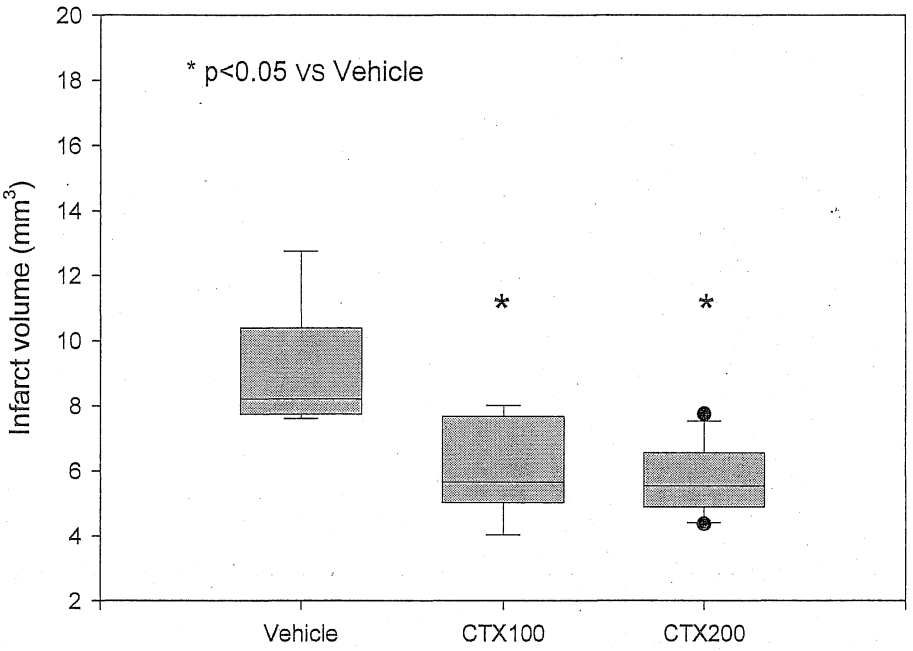

80
60
40
20
0
rCBF (LD units)
-20
0
20
40
60
80
Time (min)
Vehicle
CTX100
CTX200
CTX200+DHK
Vehicle+DHK

FIG 4


8000
7500
7000
6500
6000
5500
Impedacne (Ω)
0
500
1000
1500
2000
2500
3000
3500
4000
4500
5000
Time after 2 vein occlusion (seconds)


35
30
25
20
15
10
5
Number of spreading depression
NS
Vehicle
CTX200mg/kg


* p<0.05 vs Vehicle
Infarct volume (mm3)
20
18
16
14
12
10
8
6
4
2
*
*
Vehicle
CTX100
CTX200

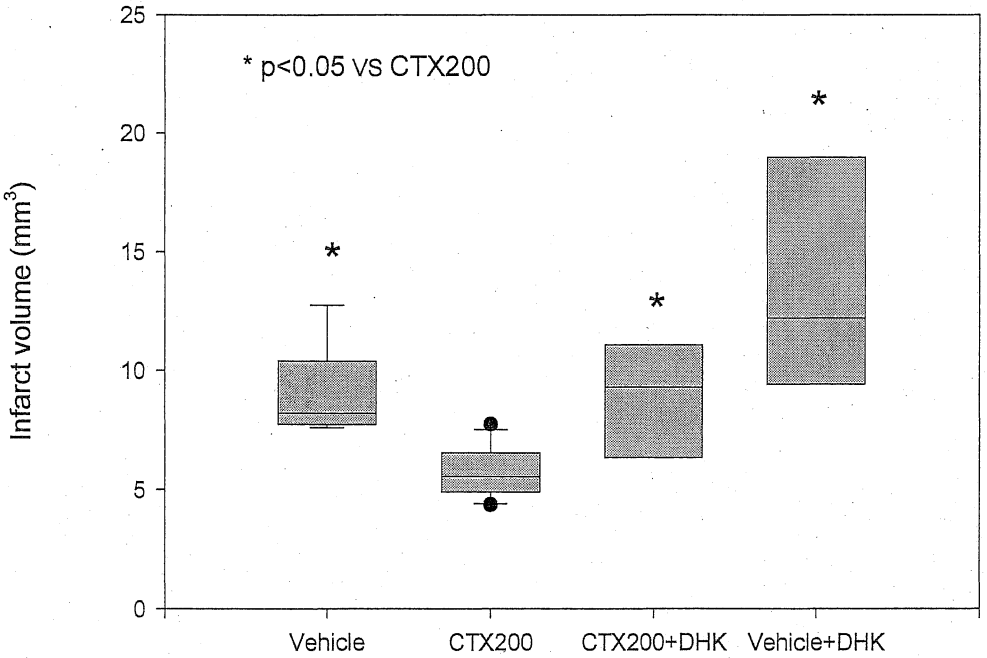

* p<0.05 vs CTX200
Infarct volume (mm3)
25
20
15
10
5
0
*
*
*
Vehicle
CTX200
CTX200+DHK
Vehicle+DHK

FIG 6