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**Title: New approaches to neuroprotection in chronic cerebral hypoperfusion in the rat**

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Nowadays, the **Faculty of Medicine**, University of Szeged is one of the most outstanding medical schools in Hungary teaching health sciences in three languages. The Faculty has excellent scientific laboratories performing high standard researches supported by national and international grants. Students have a wide range of opportunities to join scientific research activities during the time of their studies. Experience gained during university years help many students to become successful researchers all around the world. The Faculty has four **Ph.D. Doctoral Schools** in which more than a hundred supervisors offer dissertation topic proposals. Notably, annually there are approximately 40 defended Ph.D. dissertations and graduations. It gained high reputation in research, education and practice of medical sciences. We are all proud of **Albert Szent-Györgyi**, former professor and dean of the Faculty who was awarded **Nobel Prize in 1937** for his research in Szeged. He is an idol both for lecturers and students, presenting the idea that world-wide results can be achieved in Hungary and Szeged.

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# New approaches to neuroprotection in chronic cerebral hypoperfusion in the rat

**Ph.D. Thesis**

Ádám Institoris, MD

Szeged
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# New approaches to neuroprotection in chronic cerebral hypoperfusion in the rat

By

Ádám Institoris, MD

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2. Farkas, E., Annaházi, A., Institóris, Á., Mihály, A., Luiten, P.G.M., Bari, F. (2005) Diazoxide and dimethyl sulphoxide alleviate experimental cerebral hypoperfusion-induced white matter injury in the rat brain. *Neurosci. Lett.*, 373(3), 195-199.

3. Farkas, E., Domoki, F., Institóris, Á., Annaházi, A., Busija, D.W., Bari, F. (2006) Neuroprotection by diazoxide in animal models for cerebrovascular disorders. *Vasc. Dis. Prev.* 3, 253-263.

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| | |
|---|---|
| 2VO | permanent, bilateral common carotid artery occlusion |
| 5-HD | 5-hydroxydecanoate |
| AA | arachidonic acid |
| ABC | avidin-biotin complex |
| AD | Alzheimer's disease |
| AMP | adenosine monophosphate |
| AMPA | DL-alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid |
| ANOVA | analysis of variance |
| am | amygdala complex |
| ATP | adenosine triphosphate |
| BBB | blood brain barrier |
| Br | bregma |
| CA | cornu ammonis |
| CADASIL | cerebral autosomal dominant arteriopathy with subcortical infarcts and leukencephalopathy |
| CBF | cerebral blood flow |
| cc | corpus callosum |
| CGRP | calcitonin gene-related peptide |
| COX | cyclooxygenase |
| COX-2-/- | COX-2 knockout mice |
| CNS | central nervous system |
| ctx | cerebral cortex |
| DAB | diaminobenzidine |
| DFU | 5,5-dimethyl-3-(3-fluorophenyl)-4-(4-methylsulphonyl) phenyl-2(5H)-furanone) |
| DIAZ | diazoxide |
| DG | dentate gyrus |
| DNA | dezoxyribonucleic acid |
| DMSO | dimethyl sulphoxide |
| GFAP | glial fibrillary acidic protein |
| $H_2O_2$ | hydrogen peroxide |
| HLA-DR | human leukocyte antigen-DR |
| hpc | hippocampus |
| HRP | horse radish peroxidase |
| ic | internal capsule |
| IgG | immunoglobulin G |
| i.m. | intramuscular |
| iml | inner molecular layer |
| i.p. | intraperitoneal |
| iNOS | inducible nitric oxide synthase |
| LDP | long-term depression |
| LPS | lipopolysaccharide |
| LSD | least significant difference |
| LTP | long-term potentiation |

| | |
|---|---|
| luc | lucidum |
| $NaHCO_3$ | sodium hydrogencarbonate |
| NaOH | sodium hydroxide |
| NMDA | N-methyl-D-aspartate |
| NPS | normal pig serum |
| NRS | normal rabbit serum |
| NS-398 | *N*-[2-cyclohexyloxy-4-nitrophenyl] methanesulphonamide |
| NShS | normal sheep serum |
| MAP-2 | microtubule-associated protein-2 |
| MCAO | middle cerebral artery occlusion |
| $mitoK_{ATP}$ | mitochondrial ATP-sensitive $K^+$ channel |
| MM | Morris watermaze |
| MMP-2 | matrix metalloproteinase-2 |
| mRNA | messenger ribonucleic acid |
| NaOH | sodium hydroxide |
| NGS | normal goat serum |
| oml | outer molecular layer |
| or | stratum oriens |
| ot | optic tract |
| PBS | phosphate buffer saline |
| PG | prostaglandin |
| pre | pretreatment |
| post | post-treatment |
| pyr | stratum pyramidale |
| rad | stratum radiatum |
| ROS | reactive oxygen species |
| RT | room temperature |
| RT-PCR | reverse transcription- polymerase chain reaction |
| S.E.M. | standard error of mean |
| SHAM | sham-operated control |
| SUR | sulphonylurea receptor |
| str. | stratum |
| th | thalamus |
| TNF-α | tumor necrosis factor-α |
| TUNEL | terminal deoxynucleotide transferase biotin-dUTP nick end labeling |
| UTP | uridine triphosphat |
| WM | white matter |

# ÖSSZEFOGLALÁS

Patkányban a két artéria carotis communis elzárásával ("two vessel occlusion"= 2VO) krónikus agyi hipoperfúzió jön létre. Ez egy széleskörben elfogadott és alkalmazott állatkísérletes modell a vaszkuláris eredetű kognitív zavarok és neuropathológiai eltérések vizsgálatára. A 2VO modellben létrejövő iszkémiás neuronkárosodás és memória deficit mértékét csökkentő gyógyszeres kezelések egyrészt segítik a cerebrovaszkuláris és kardiovaszkuláris eredetű demenciák természetének felderítését, másrészt új terápiás megfontolások alapjául is szolgálhatnak. Kísérleteink célja az volt, hogy megvizsgáljuk a mitokondriális ATP-szenzitív $K^+$ csatornanyitó diazoxid (DIAZ), valamint a nem szelektív- (indometacin) és szelektív ciklooxigenáz gátlók (NS-398 COX-2 gátló) hatását 2VO-val létrehozott agyi hipoperfúzió különböző fázisaiban. A DIAZ kezelés hatásait az agyi hipoperfúzió korai és késői időszakában nátrium-hidroxid (NaOH) és dimetil-szulfoxid (DMSO) oldószerek alkalmazásával vizsgáltuk, valamint összehasonlítottuk a DIAZ elő- és utókezelések következményeit. Végül az indometacin és az NS-398 hatásait vizsgáltuk az agyi hipoperfúzió korai szakaszában. Hím Wistar patkányokon 2VO-t (n=137) vagy áloperációt (n=105) végeztünk, majd vizsgáltuk a térbeli tanulási képességet (Morris watermaze teszt), a makroszkópos agyi léziók gyakoriságát, illetve szövettani módszerekkel a hippokampuszban és egyes fehérállományi régiókban a neuronális károsodás mértékét (krezil- ibolya festés és COX-2-pozitív neuronjelölés), a denritpusztulást (mikrotubulus asszociált protein-2 jelölés), asztrocita reakciót (gliális fibrilláris savas protein jelölés) és a mikroglia aktivációt (OX-42 jelölés). Eredményeink alapján a hipoperfúzió a korai időszakban térbeli tanulási zavart, progresszív neuronális- és dendritkárosodást, valamint a késői időszakban régió specifikus asztrocita proliferációt és mikroglia aktivációt okozott. A DMSO-ban oldott DIAZ kezelés után, a hipoperfúzió késői fázisában a tanulási képesség alig változott, csökkent a neuron pusztulás mértéke és elmaradt a mikroglia aktiváció. A DIAZ jótékony hatását a DMSO feltehetően fokozta. 2VO után 2 héttel a NaOH-ban oldott DIAZ csak előkezelésként alkalmazva mérsékelte a memóriazavart és az idegsejt pusztulást, ami a DIAZ prekondícionáló hatására utal. A COX-gátló kezelések közül az NS-398 kivédte a tanulásromlást, ugyanakkor mindkét anyag fokozta a hippokampális neuron pusztulást. Feltehetően a COX gátlók protektív hatásukat nem közvetlenül a hippokampusz védelmén keresztül fejtik ki. Megfigyeléseink szerint a DIAZ előkezelés hatékony lehet bizonyos vaszkuláris eredetű memóriazavarok megelőzésében. A szelektív COX-2 gátlók szintén hatásosak lehetnek a kognitív tünetek kezelésében, alkalmazásuk azonban további gondos vizsgálatokat igényel.

## SUMMARY

Permanent bilateral common carotid artery occlusion (2VO) is a suitable model with which to investigate chronic cerebral hypoperfusion-related cognitive disturbances and brain pathology in the rat. A number of drugs have been demonstrated to reduce ischemic neuronal damage and memory deficit in the 2VO model, but there are still relevant pharmacological approaches which might help to reveal the nature of dementia of cerebro- or cardiovascular origin and might additionally be of therapeutic value. The aim of our investigations was to characterize the potential neuroprotective effects of the mitochondrial ATP-sensitive $K^+$ channel opener diazoxide (DIAZ) and nonselective (indomethacin) and selective (COX-2) cyclooxygeanse-2 inhibitors (NS-398) in the 2VO model at different periods of cerebral hypoperfusion. The effect of DIAZ was examined in the early and late phases of cerebral hypoperfusion using sodium hydroxide (NaOH) or dimethyl sulphoxide (DMSO) as solvent, respectively. We also compared the effects of DIAZ pretreatment with those of post-treatment. Finally, we compared the effects of indomethacin and NS-398 in the early period of cerebral hypoperfusion. Male Wistar rats were exposed to 2VO (n=137) or sham operation (n=105). The observed parameters were visuospatial learning (Morris watermaze test), the incidence of macroscopic brain lesions, the degree of neuronal damage (cresyl violet staining and neuronal COX-2 labeling), dendritic degeneration (microtubule-associated protein-2 labeling), the astrocytic reaction (glial fibrillary acidic protein labeling) and microglia activation (OX-42 labeling) at the level of the hippocampus and some white matter areas, detected with histological methods. We found that an impaired learning capacity was accompanied by progressive neuronal and dendritic damage, region-specific astrocyte proliferation and microglia activation. DIAZ dissolved in DMSO preserved the learning capacity, reduced the neuronal damage and alleviated the microglia activation in the observed regions at 13 weeks of survival. We assume that the effect of DIAZ was potentiated by DMSO. Only pretreatment with DIAZ (dissolved in NaOH) alleviated the memory impairment and neuron loss at 2 weeks after 2VO, which suggests its preconditioning effect in protection. NS-398, but not indomethacin, prevented the learning impairment, but both drugs increased the damage to the hippocampal neuron layers, suggesting that the protective action was not directly related to the hippocampus. In conclusion, DIAZ pretreatment could be effective in preventing predictable memory failures of cardiovascular origin. Selective COX-2 inhibition might also be useful to treat cognitive disturbances, though it requires careful patient selection.

# INTRODUCTION

## Epidemiology of cerebral hypoperfusion-related cognitive disorders (incidence, Alzheimer's disease, vascular dementia, cardiac surgery and chronic heart failure)

Dementia is one of the major challenges in public health worldwide: currently around 24 million people suffer from various types of cognitive disorders and this number is projected to double every 20 years [1]. The regional prevalence of dementia in the European Union and in the USA is 4% and 6.4%, respectively [2]. Dementia is defined as a clinical syndrome characterized by progressive deteriorations in multiple cognitive domains that are severe enough to interfere with the daily functioning and finally result in a shortening of the lifespan in the elderly. Demented patients are compelled to endure a lower quality of life and they also impose a heavy burden on the family, the society and industrialized health care.

Besides unmodifiable risk factors, such as a genetic predilection [3] or the advance of age [4], vascular risk factors and disorders are also involved in the pathogenesis and clinical expression of dementia [5]. The net result of these vascular events is the development of a chronically reduced level of cerebral perfusion. Permanent disturbance of the cerebral circulation has been demonstrated in various clinical syndromes accompanied by dementia, such as Alzheimer's disease (AD), vascular dementia, Binswanger disease or cerebral autosomal dominant arteriopathy with subcortical infarcts and leukencephalopathy (CADASIL).

Patients with a mild cognitive impairment, which is the prodromic phase of AD, exhibit a decreased blood flow in the parietotemporal region, including the hippocampus [6,7]. The degree and pattern of reduction in the cerebral perfusion of various brain regions has been demonstrated to correlate well with the incidence of a later AD, and thus, it can be a predictive marker for the development of AD [8,9]. The causal relationship between the reduced cerebral perfusion and the development of neurodegenerative processes is unclear. However, an ever-increasing amount of evidence indicates that nongenetic AD is initiated by vascular factors that precede the neurodegenerative process [10]. A considerable number of experiments have revealed that chronic brain hypoperfusion can trigger energy metabolic deficits and memory loss before any neuronal structural pathology materializes [11-13]. The application of experimental models which approach neurodegenerative diseases from the aspect of the impaired cerebral circulation cab therefore be expected to be of great benefit in the development and testing of potential neuroprotective strategies.

A cerebral hypoperfusion-related cognitive impairment also frequently emerges following acute cardiovascular crisis states, as a consequence of the low cardiac output. Atrial fibrillation, myocardial infarction and coronary bypass surgery are predictors of cognitive disturbance and dementia [14-17]. An animal model which resembles a rapidly developed, but permanently existing global cerebral hypoperfusion can provide useful findings with a view to preventing or treating these conditions.

**Experimental model for chronic cerebral hypoperfusion in the rat (permanent, bilateral common carotid artery occlusion)**

To design an experimental setup that mimics the rheological, histological and cognitive disturbances observed in clinical practice, researchers have had to take into account considerations such as the question of species, the appropriate surgical method with which to induce cerebral hypoperfusion, an acceptable survival rate, good surgical recovery, and simple and reproducible behavioral testing [18]. The most appropriate model that best fits these requirements is the permanent ligation of both common carotid arteries in the rat ("two vessel occlusion" = 2VO)[18]. The rat is a suitable laboratory animal for this purpose as the well-developed circle of Willis ensures a chronically reduced, but constant blood flow toward the forebrain through the vertebral circulatory system following 2VO.

*Hemodynamic changes*

The temporal trace of cerebral blood flow (CBF) changes following 2VO is well described by experiments involving a use of an autoradiographic method [19,20], laser Doppler flowmetry [21-23] or an arterial spin labeling technique [24]. Immediately after the occlusion, there is a sudden drop in blood flow in several areas of the forebrain. The greatest reduction has been recorded in the parietotemporal cortex and the white matter (WM) areas, with 30-40% of the control CBF, while in the hippocampus it was 60% of the control level [19,21-23]. A 10-20% recovery of the CBF develops by approximately 1 week after 2VO [20], but the successive elevation of the CBF subsequently decelerates until it reaches the control level by 8 weeks to 3 months following 2VO [19,25]. Six months after 2VO, CBF measurements reflect a complete recovery of perfusion in all cerebral regions [24]. Overall, not every period of cerebral hypoperfusion after 2VO in rats reflects the CBF pattern observed in demented patients [26]. However, if we divide the time elapsed from 2VO until complete recovery of the CBF, then the first 2-3 days after 2VO is the phase of acute ischemia, followed by the chronic

hypoperfusion phase lasting for 3 months, and finally, during the third phase, the CBF returns to the original baseline [18]. Thereby, each period of the model permits the follow-up and the potential treatment of different clinicopathological entities. Although only the second phase resembles the hemodynamic, metabolic, histological and cognitive conditions of dementia, the changes in the acute period initiate various pathological events that are similar to rapid, but mild cerebral hemodynamic disturbances accompanied by memory disorders. Hence, the acute phase is useful for the investigation of intermittent acyclia-related memory disorders.

*Metabolic disturbances and oxidative stress*

The brain tissue has a high metabolic rate and is susceptible to reductions in oxygen and blood supply. The reduced availability of energy, and in particular of ATP and phosphocreatine, impairs the physiological functioning of the neuronal cells and subsequently the cellular integrity. In the 2VO model, the suddenly restricted blood flow in the forebrain is accompanied by a decreased tissue level of energy-rich phosphates (phosphocreatine and ATP), whereas the concentrations of adenosine, lactate and AMP increase immediately after the induction of 2VO and remains elevated for at least 2 weeks [27]. The glucose utilization recovers by the end of the third week, while an enhanced lactate concentration or reduced ATPase activity remains detectable from 1 to 8 months following 2VO [27,28]. The chronically impaired neuronal energy production initiates and sustains a cascade of neuropathologic events that underlie an impaired cognitive ability [29].

Other sequelae of tissue hypoperfusion include an imbalance between the production of oxidative agents and the persisting antioxidant capacity. The temporal alterations in the levels of reactive oxidative agents and antioxidant systems in the 2VO model correlate with cellular ravage and an inflammatory reaction, emphasizing the role of oxidative stress in the pathogenesis of hypoperfusion-induced neurodegeneration. One of the probable reasons for the development of oxidative stress even in an oxygen-poor environment is the diminished expression of antioxidant enzyme proteins and the failure of the mitochondrial function due to the hypometabolic conditions. Twenty-eight days after 2VO, a decreased activity of mitochondrial respiratory complex I, a low cytochrome C expression, an elevated hydrogen peroxide ($H_2O_2$) production, a decreased membrane potential and mitochondrial swelling have been demonstrated in isolated mitochondria from the rat hippocampus [30]. Another possible source of deleterious reactive oxygen species (ROS) production is the activated microglia as a major contributor to the ongoing inflammatory reaction during hypoperfusion [31]. These observations suggest that the restriction of oxidative stress by pharmaceutical

compounds is one of the mainstream treatment strategies via which to achieve neuroprotection in chronic cerebral hypoperfusion [30,32-34].

*Learning and memory disturbance (experimental tests, types of learning and memory failures)*

Experimental tests designed for animal models of dementia mainly focus on the visuospatial learning skills relevant to a certain widely-used clinical tests [35-37]. These learning paradigms are suggested to reflect hippocampus-related learning processes [38,39]. Hence, the application of these tests is suitable in experimental models, *e.g.* the 2VO model, which affect the normal integrity of the hippocampus. One of the most commonly used setups for this purpose is the Morris watermaze. Several studies have documented that rats with 2VO display a longer swim pathway, and an increased escape latency in the watermaze from 7 days post-surgery up to 12 months of cerebral hypoperfusion. The memory disturbance is still relapsed when the CBF has already been restored [30,40,41]. The progressively deteriorated learning capacity is therefore presumed to correlate with the ongoing neuropathologic aberrations, rather than with the cerebral perfusion, at least in the 2VO model [18].

In summary, the histopathological disorders with the learning and memory impairments observed in experimental chronic cerebral hypoperfusion are relevant to the histological and cognitive alterations observed in clinical dementia, and make this model applicable for investigations of the development, progression and potential treatment of these diseases.

*Histopathologic changes (neuronal damage, white matter injury and inflammatory reaction)*

Various experiments have had the aim of determining which spatial and temporal histopathologic aberrations correlate well with impaired learning and memory. The neuropathologic changes in the 2VO model are most obvious in those parts of the brain which are particularly sensitive to ischemia or which are supplied primarily by the carotid arteries, *e.g.* the hippocampal formation or some WM areas. There are several possibilities to visualize the death or microscopic degeneration of neurons, the proliferation and activation of glia cells and inflammation. The earliest appearing neurodegeneration in the hippocampus 4 days after 2VO, was demonstrated with a quantitative RT-PCR method. In those studies, elevated amyloid precursor protein mRNA and γ-secretase mRNA levels were measured, markers that are also indicative of the pathogenesis of AD [42,43]. The earliest detection of neuronal

destruction with the conventional hematoxylin-eosin staining method was successful at 2 weeks, revealing necrotic pyramidal cell loss in the cortex [44]. Apoptotic cell death after week 2 was also detected with terminal deoxynucleotide transferase biotin-dUTP nick end labeling (TUNEL) and is localized to the CA1 and CA3 subfields of the hippocampus and to some WM areas, such as the optic tract and the fimbria fornix [45,46]. This damage gradually progresses with time, even up to 190 days following 2VO [40]. Immunocytochemical labeling of neuronal arborization with microtubule-associated protein-2 (MAP-2) and synaptophysin antibody reflected that cerebral hypoperfusion additionally triggers the progressive ruination of dendritic processes and synaptic integrity [47]. The presence of WM injury is also described in this model.

Chronic cerebral hypoperfusion is accompanied by a slowly-developing and persistent inflammatory reaction in the ischemic brain regions. The most noteworthy histological events observed after 2VO which hallmark the ongoing inflammation are reactive gliosis and activation of the microglia [48,49]. Astrocytic proliferation detected by means of glial fibrillary acidic protein (GFAP) immunocytochemistry revealed an augmented GFAP signal in the cortex and in WM areas starting from week 1, whereas it was observed only 6 months after 2VO in the hippocampus [32,50]. Immunohistochemical detection of activated ameboid-like microglia with lectin- and complement receptor-3 (OX-42) antibody demonstrated an early activation of the cells 20 minutes following permanent carotid ligation in the hippocampus [51]. Microglia activation was also visible in the WM 14 days and 13 weeks following 2VO [52,53]. In the 2VO model, the microglia is suggested to be responsible for the tissue damage through the release of tumor necrosis factor (TNF)-α by inducing apoptosis or via the production of the degrading enzyme, matrix metalloproteinase-2 (MMP-2) [12,52,54]. Therefore, pharmacological inhibition of microglia activation might exert neuroprotection by moderating inflammation-induced neuronal damage.

**Diazoxide – a mitochondrial ATP-sensitive $K^+$ channel opener**

In cerebral ischemia, the imbalance of cellular homeostasis initiates cascade mechanisms which finally result in cell death. The mitochondria are known to play an essential role in this process. Since they are the main energy suppliers of the cell and regulate the cellular $Ca^{2+}$ homeostasis, the dysfunction of the mitochondria is considered to be a pivotal event in brain tissue injury [55,56]. The depletion of oxygen and glucose impairs the proper functioning of the respiratory chain, leading to restricted ATP production, increased

$Ca^{2+}$ concentration of the mitochondrial matrix, cytochrome C release and the generation of free radicals [55]. These changes are disastrous for the cell and eventually lead to necrosis or apoptosis [57]. For this reason, pharmacological intervention regarding the mitochondrial function is a promising strategy through which prevent ischemic neuronal injury.

The membrane of the mitochondrial matrix is enriched in ATP-sensitive $K^+$ channels (mito$K_{ATP}$) [58]. These channels are composed of discrete pore-forming (Kir6.1/Kir6.2) and regulatory subunits (presumably the sulphonylurea receptor (SUR)-2). Under physiological conditions, at optimal energy and oxygen supply of the cell, the respiratory chain in the inner mitochondrial membrane builds up electric and osmotic gradients for the proton between the intermembrane space and the matrix [55]. Opening the mito$K_{ATP}$ induces early and delayed preconditioning effects against ischemic damage that can be abolished by the specific channel blocker 5-hydroxydecanoate (5-HD). The early events include $K^+$ influx into the matrix and a positive shift of the transmembrane potential. This action stabilizes the volume of both the matrix and the intermembrane space, which increases the activity of the electron transport chain and hence the chemical proton gradient leads to alkalinization of the matrix. Second, mito$K_{ATP}$-induced mitochondrial swelling is known to improve fatty acid oxidation and ATP production [59]. In the event of the elevation of the intracellular $Ca^{2+}$ level to above the mitochondrial buffer concentration, the entrance of $Ca^{2+}$ through the inner membrane initiates pernicious events in the matrix. However, the electrogenic transport of $Ca^{2+}$ is highly dependent on the membrane potential, and thus the reduced negativity of the inner membrane following the $K^+$ inflow via the mito$K_{ATP}$ prevents $Ca^{2+}$ accumulation in the matrix [60]. In the presence of sufficient oxygen, further electron transport generates a mild ROS production [61,61,62]. The concrete role of the free radicals arising from the reaction is not understood, but they initiate compensatory mechanisms involving protein kinase C activation, which provide protection for the cell against subsequent, otherwise lethal ischemia [63]. Short, repetitive sublethal ischemic episodes also lead to the opening of the mito$K_{ATP}$ and the emergent ROS from the accelerated electron transfer fortify the cell against ischemic damage. This phenomenon is called ischemic preconditioning. Pharmacological opening of the mito$K_{ATP}$ has also been found to provide ischemic tolerance [64]. The preconditioning effect of mito$K_{ATP}$ activation has emerged as a nothworthy feature in the development of cardioprotective strategies [65]. Since the brain contains nearly 7 times as much mito$K_{ATP}$ as does the heart, great importance has been attached to the neuroprotective role of mito$K_{ATP}$ for the prevention and treatment of ischemic neuronal damage [66].

The drug most widely accepted and used to mimic ischemic preconditioning by the opening of the mito$K_{ATP}$ is benzothiadizine, or as it is generally called diazoxide (DIAZ). This substance has a 2000-fold affinity to mito$K_{ATP}$ relative to the cell surface $K_{ATP}$ channels [64]. At first, DIAZ was used for the treatment of hypertensive emergencies [67] as a high concentration of the drug hyperpolarizes the cell membrane by opening cell surface $K_{ATP}$ channels and thereby relaxes the smooth muscles of the resistance vessels. Furthermore, DIAZ has proved to inhibit insulin release from pancreatic beta-cells, which has led to another application of the drug, in insulinoma therapy [68].

For the past twenty years, intensive research has been carried out to describe and explain the preconditioning feature of DIAZ in many organs, mostly in the heart and the brain. The neuroprotective effects of DIAZ have been demonstrated under *in vitro* hypoxic conditions, in experimental animal models for perinatal hypoxia/ischemia and for transient ischemic stroke in adults, respectively.

*In vitro* neuronal and astrocytic cell cultures preincubated with DIAZ exhibit better viability after oxygen-glucose deprivation or glutamate exposure [64]. The beneficial effects of DIAZ were confirmed in hippocampal and cortical slice preparations subjected to hypoxic/ischemic conditions [69,70]. The mechanism of protection by DIAZ has been associated with depolarization of the mitochondria, the prevention of $Ca^{2+}$ accumulation, anti-apoptotic properties and enhanced ROS production [64].

DIAZ has been tested in several experimental animal models of cerebral ischemia, in both newborn and adult specimens. In newborn piglets, the decrease in the vascular reactivity of the pial arterioles by ischemia/reperfusion was prevented by the topical administration of DIAZ prior to the ischemia. Pretreatment of 7-day-old rat pups with DIAZ significantly reduced the volume of the infarct induced by the reversible ligation of one carotid artery with hypoxia [64]. Under the same circumstances, DIAZ prevented the activation of the immediate-early genes c-Fos and c-Jun, down-regulated calpain, an enzymatic mediator of neuronal death, and decreased DNA fragmentation in the hippocampus and in the cortex [71]. Furthermore, the mitochondria in the CA1 area of the hippocampus of newborn piglets which underwent ischemia/hypoxia displayed extensive swelling and $Ca^{2+}$ accumulation which was abolished by DIAZ treatment [72]. Besides the testing of DIAZ in newborn models of cerebral ischemia, it has been approved for stroke models of adult mice and rats. After reversible occlusion of the middle cerebral artery, the volume of the infarcted area as indicated by 2,3,5-triphenyltetrazolium chloride staining, showed a 50% reduction when the rats were treated with an intracerebroventricular injection of DIAZ [73]. Mitochondria extracted from the neurons

of the ischemic penumbra revealed a decreased membrane potential and $Ca^{2+}$ influx, mitochondrial swelling, reduced complex-I damage and a decreased formation of transition pores through which $Ca^{2+}$ enters the mitochondria [73]. Furthermore, DIAZ also decreased the number of TUNEL-positive apoptotic cells in the infarcted territory [74]. DIAZ additionally improved the astrocytic survival compared to the respective controls [74]. Lenzsér et al. (2005) imposed reversible occlusion of both common carotid arteries of rats exposed to hypotension. Under these severe ischemic conditions, the integrity of the blood brain barrier (BBB) was disrupted. However, DIAZ limited the permeability of the BBB, as demonstrated by the injection of Evans blue and sodium fluoresceinate into the brain parenchyma [75]. Finally, all the beneficial effects of DIAZ discussed above could be reversed by the administration of 5-HD, confirming the contribution of the mito$K_{ATP}$ in the protective mechanisms [64].

In the studies of ischemic stroke has invariably been administered DIAZ before the onset of ischemia, emphasizing the prevention approach of treatment. These experiments have made use of the widely proven concept that DIAZ mediates ischemic preconditioning. However, post-treatment strategies may be more desirable, as the occurrence of a cerebral ischemic event is most often unpredictable.

**The role of the cyclooxygenase system in ischemic brain damage**

*The localization and physiological role of cyclooxygenase-2 in the central nervous system (CNS)*

Arachidonic acid (AA) is continuously released from membrane phospholipids by phospholipase A2. The AA released is further metabolized to bioactive eicosanoids via cyclooxygenases (COXs), lipoxygenases and epoxygenases. Under normal and pathological conditions COX-derived prostanoids – thromboxanes, prostacyclins and prostaglandins (PGs) are important mediators of cerebrovascular control in 3 ways: by causing vasodilation, by causing vasoconstriction, or by permitting the physiological effects of other vasoactive mediator systems.

In the central nervous system (CNS), three isoforms of the enzyme have been described: COX-1, COX-2 and COX-3, the latter being a splice variant of COX-1 [76]. COX-1 is expressed constitutively in most cells of the brain and participates in various homeostatic processes. [76,77]. The expression of COX-2 in the brain is due to both constitutive and induced production. Constitutive production is observed in discrete populations of excitatory neurons in the adult brain. There is a high basal level of COX-2 activity in the pyramidal layer of the

hippocampal CA1 region, and in the granule cells of the dentate gyrus (DG), while a mild level of COX-2 production has been described in the spinal cord and in the cerebral cortex [78,79]. At a subcellular level, the perinuclear region and dendritic spines are enriched in COX-2, indicating its contribution to synaptic signaling [80,81]. In the hippocampus, the presence of COX-2 in the glutamatergic neurons demonstrates its role in the formation of long-term potentiation (LTP) initiated by the activation of N-methyl-D-aspartate (NMDA)-sensitive receptors. COX-2 might therefore be important in the evolution of memory. The inhibition of COX-2 prevents LTP in the hippocampal dentate neurons, but the addition of $PGE_2$ reverses the COX-2-mediated suppression [82,83]. The COXs are normally present in the cerebral blood vessels. The endothelial cells process COX-1 and COX-2 and there is continuous prostanoid production, which may play a significant role in maintaining the resting cerebrovascular tone. COX-1 and COX-2 have also been detected in the smooth muscle cells [84]. There have been several reports that the glia cells produce prostanoids via the COX-2 pathway. Strong evidence was recently provided that perivascular axo-dentric synapses were immunopositive for COX-2, consequently, the simultaneous release of prostanoids and/or excitatory transmitters from a single terminal may affect the neuronal activity and CBF [85].

There have been numerous studies of whether the levels of COX-derived metabolites are in the vasoactive range in the brain under physiological conditions. Observations on piglets [86,87] and in rats [88] indicated that the selective inhibition of COX-2 results in only a minor reduction of the resting CBF. Similarly, no reduction in cortical blood perfusion was found in mice when COX-2 was inhibited selectively [89].

In the cerebral cortex, COX-2 is involved in neuronal activity-dependent functional hyperemia. This effect was first demonstrated in the somatosensory cortex of mice where selective COX-2 inhibition diminished the vibrissal stimulation-induced blood flow increase, whereas the vascular reactivity to other stimuli remained intact [89]. Furthermore, the flow response to vibrissal stimulation is impaired in the whisker-barrel cortex of COX-2 null-mice [89]. The highly selective COX-2 inhibitor rofecoxib significantly reduce the increases in regional CBF induced by somatosensory stimulation in rats [88].

*COX-2 expression in acute ischemic brain damage*

Various brain pathologies elicit a marked overexpression of COX-2, contributing to the development of neuronal injury. Cerebral COX-2 levels are induced globally after focal infarction in humans [90,91] and in various animal models alike [92-95]. In the course of focal cerebral ischemia, the upregulation of COX-2 mRNA can be detected at 3 hours of

reperfusion, while the increased protein levels become apparent after 6 hours, peaking at 12-24 hours. COX-2 overexpression in the ischemic penumbra is limited to the neurons, glia and vascular endothelial cells [92,96-98]. The release of glutamate and inflammatory cytokines from damaged neurons may participate in the induction of COX-2 expression under these conditions. In the early phase of ischemia, neuronal/glial COX-2 induction can be triggered by the glutamate-induced activation of NMDA receptors with a subsequent $Ca^{2+}$ inflow that activates the transcription of COX-2 mRNA [96,99]. The deleterious effect of COX-2 includes several downstream mechanisms. The major detrimental impact during an excitotoxic injury is putatively mediated by the production of $PGE_2$ through the PG EP1 receptor. The activation of the receptor impairs the exchange of $Ca^{2+}$ for $Na^{+}$, and the dying neuron is therefore unable to eliminate NMDA-induced $Ca^{2+}$ accumulation [100]. Besides the production of $PGH_2$, the conversion of AA by COX results in the simultaneous production of a superoxide anion. This can play a particularly important role in the brain pathology during the recovery from anoxic stresses such as ischemia or asphyxia, when large amounts of radicals are formed. These free radicals impair the cerebrovascular responsiveness to various stimuli such as NMDA, calcitonin gene-related peptide (CGRP) and the dilator prostanoid, iloprost, and damage the BBB transport [101-103], albeit the relative contributions of COX-1 and COX-2-derived mediators have not yet been determined. COX-2-dependent ROS production and oxidative damage have also been suggested to occur in the brain after excitotoxic brain injury and global cerebral ischemia [104,105] and in the brains of animals subjected to restraint stress [106,107].The relative parts played by COX-2-derived ROS and prostanoids in neurotoxicity have not been clarified either. Besides the direct contribution of COX-2-derived $O_2^{-\bullet}$ to excitotoxic damage, other sources of production of ROS are noteworthy [108,109]. The reaction between $O_2^{-\bullet}$ and nitric oxide produced by inducible nitric oxide synthase (iNOS) creates peroxynitrite, a highly reactive chemical species, which is capable of damaging lipids, proteins and DNA [110-112]. In the later stage of cerebral ischemia, an inflammatory reaction develops at the site of the ischemia. The damaged neurons release pro-inflammatory cytokines, such as interleukin-1 or TNF-α, which are activators of COX-2 expression in the microglia, and the consequential release of prostanoids ($PGE_2$ and thromboxane) and oxygen free radicals exacerbates the tissue injury [113-115].

The participation of the COX-2 pathway in experimental ischemic brain damage has been best revealed by studies in which selective COX-2 inhibitors and COX-2 knockout mice (COX-2-/-) or COX-2 overexpressing mice were used. Gene deletion studies of COX-2 pointed to several extracerebral abnormalities, while the CNS was shown to be unaffected in

COX-2-/- mice [116]. Additionally, these animals displayed reduced susceptibility to brain ischemia and to NMDA toxicity [99,117]. Mice overexpressing COX-2 in the brain are more susceptible to the brain damage produced by focal cerebral ischemia [118].

*COX-2 inhibitors in acute cerebral ischemia*

Selective COX-2 inhibitors successfully moderate cerebral ischemic lesions or excitotoxic injuries in a good number of animal or *in vitro* models. For example, NS-398, a commonly used selective COX-2 inhibitor, ameliorated lipopolysaccharide-induced neuronal damage and attenuated TNF-α release in neuronal cell cultures [119]. The intraperitoneal administration of NS-398 to mice and rats significantly reduced infarct size after transient middle cerebral artery occlusion (MCAO) [92]. Additionally, NS-398 decreased the lesion size, improved the functional outcome and induced dendritic hypertrophy in the pyramidal cells of the parietal cortex following the devascularizing lesion of the motor cortex in rats [120]. Nimesulide, another COX-2 inhibitor, reduced the frequency of neuronal damage in the hippocampus, are prevented glutathione depletion and lipid peroxidation in both mice and Mongolian gerbils exposed to transient 2VO [117,121]. Similar observations have been made for other selective COX-2 inhibitors, in particular rofecoxib, DFU and SC-58236 [104].

However, it must be noted that, even within various stroke models, the distal cellular effects of the stroke may be completely different, depending on the exact etiology of the injury. Thus, it is likely that the effects of COX-2 inhibitors will also vary, depending on the injury model used. Additionally, the time course of COX-2 inhibitor drug administration can result in differential cellular and behavioral effects [122] and should therefore be systematically investigated for each injury model.

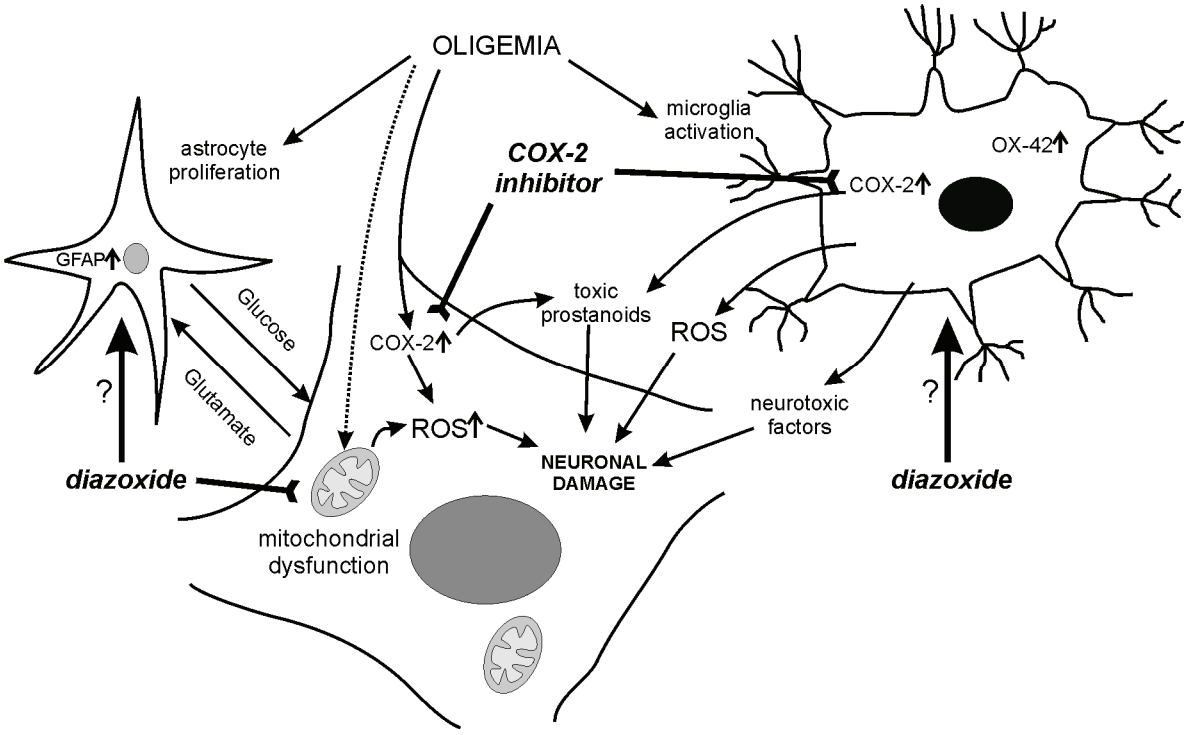



**Figure 1**. The hypothetic protective action of diazoxide and selective COX-2 inhibition in chronic cerebral hypoperfusion-induced neuronal damage. Abbreviations: COX-2: cyclooxygenase-2; GFAP: glial fibrillary acidic protein; ROS: reactive oxygen species.

## The purpose of our experiments

The aim of our experiments was to test the potential neuroprotective features of two types of widely investigated pharmacological agents (a mito$K_{ATP}$ opener and specific/nonspecific COX inhibitors) at various time points (3 days, 2 weeks and 13 weeks) during experimental chronic cerebral hypoperfusion. Previously, both treatments had proved to be beneficial in acute ischemic circumstances, but had not been investigated in chronic ischemia, such as created in the 2VO model.

First, we set out to determine the degree of learning dysfunction and neurodegeneration at various time points after 2VO induction. We determined the 2VO-induced visuospatial learning dysfunction, the presence of neuronal damage, dendritic ruination, astrocytic proliferation and microglia activation at the level of the hippocampus and in some WM areas. The early and late periods of cerebral hypoperfusion (resembling acute cardiovascular failures and dementia with vascular components, respectively) were compared.

Second, we aimed to describe the putative protective effect of the mito$K_{ATP}$ opener DIAZ. We compared the preconditioning effect of DIAZ (administered before 2VO onset) with the effect of post-treatment. Within this set of experiments, we proposed to characterize

the additive effect of DIAZ and the antithrombotic and ROS scavenger dimethyl sulphoxide (DMSO), widely applied as a solvent in the later period (13 weeks) of the 2VO model.

Third, we tested a nonselective and a selective COX-2 inhibitor (indomethacin and NS-398, respectively) on the visuospatial learning and histological changes during 2VO. COX inhibitor treatments were evaluated at 3 days and 2 weeks following 2VO.

Since both DIAZ and COX-2 inhibitors alleviate oxidative damage, we finally distinguished the neuroprotective properties of mito$K_{ATP}$ opening and COX inhibition during the early period (2 weeks) of cerebral hypoperfusion.

## MATERIALS AND METHODS

### Surgical procedure; the induction of chronic cerebral hypoperfusion

A total of 242 male Wistar rats (260 g± 26 g) were used. All animal experiments were approved by the Ethical Committee of the University of Szeged. The animals were anesthetized via the i.p. injection of chloral hydrate in a 400 mg/kg dose, followed by 0.05 ml 0.1% atropine intramuscularly (i.m.) to prevent an autonomic reaction during the operation (*i.e.* mucous secretion blocking the airways).

Chronic cerebral hypoperfusion was induced with the same surgical procedure in each experiment. Half of the animals underwent permanent bilateral common carotid artery occlusion (2VO), while the other half served as sham-operated controls (SHAM). The common carotid arteries were exposed via a ventral cervical incision, carefully separated from their sheaths and vagal nerves, and permanently ligated with silk sutures (5.0). Lidocaine (1%) was applied as local anesthetic. The same procedure was performed on the SHAM group, but without the actual ligation of the carotid arteries.

## Pharmacological treatments

**Table 1.** Summary of the treatments at different survival times and their clinical relevance during the three experiments.

| Experi-ment | Pharmacon | Time of treatment (pre-, post-) | Type of treatment (repeated, bolus) | Survival time | Effect | Relevance |
|---|---|---|---|---|---|---|
| 1 | diazoxide in DMSO | post | repeated (5x) | 13 weeks | mitoK$_{ATP}$ opening + antithrombotic effect and free radical scavenging | AD |
| | DMSO | | repeated (5x) | | antithrombotic effect and free radical scavenging | |
| 2 | diazoxide in NaOH | pre | repeated (5x) | 2 weeks | mitoK$_{ATP}$ opening | acute disruption of brain perfusion with gradual reperfusion (atrial fibrillation, coronary bypass surgery) |
| | diazoxide in NaOH | | bolus | | mitoK$_{ATP}$ opening | |
| | diazoxide in NaOH | post | repeated (5x) | | mitoK$_{ATP}$ opening | |
| 3 | NS-398 | post | repeated (3x) | 3 days | COX-2 inhibition | |
| | indomethacin | | repeated (3x) | | general COX inhibition | |
| | NS-398 | | repeated (3x) | 2 weeks | COX-2 inhibition | |
| | indomethacin | | repeated (3x) | | general COX inhibition | |

**Table 1**. In Experiment 1, nontreated animals served as controls for pharmacological treatments.In Experiments 2 and 3, the vehicle was administered as control. Abbreviations: AD: Alzheimer's disease; COX: cyclooxygenase; DMSO: dimethyl-sulphoxide; NaOH: sodium hydroxide; mitoK$_{ATP}$: mitochondrial ATP-sensitive $K^+$ channel.

Both the SHAM and the 2VO animals were subdivided into further experimental groups based on the surgery, the treatment and the survival time. For the experimental protocols, see Figure 2. Experiment 1 (Fig. 2A) involved rats with a survival time of 13 weeks. Group 1 received no treatment following the SHAM operation (SHAM-C-13w, n=6), group 2 animals were injected with 0.25 ml DMSO, i.p. (SHAM-DMSO-13w, n=6), while group 3 received 5 mg/kg DIAZ in DMSO, i.p. (SHAM-DIAZ+DMSO-13w, n=8). In groups 4-6, the 2VO rats were treated in the same way as the SHAM animals: the group 4 rats were not treated (2VO-C-13w, n=6), group 5 received 0.25 ml DMSO (2VO-DMSO-13w, n=6) and the group 6 animals were treated with 5 mg/kg DIAZ in the same volume of DMSO (2VO-DIAZ+DMSO, n=7). The treatment was applied on 5 consecutive days, starting immediately following surgery.

Experiment 2 (Fig. 2B) involved rats with a survival time of 2 weeks. Nine experimental groups were established. Treatment was performed with DIAZ or its vehicle (0.1 N NaOH) given as pretreatment (5 groups) or post-treatment (4 groups) to both SHAM and 2VO rats. During pretreatment, the first set of SHAM and 2VO animals received 0.5 mg/kg DIAZ (SHAM-DIAZ pre-2w, n=6; 2VO-DIAZ pre-2w, n=6) or vehicle (SHAM-C pre-2w, n=5; 2VO-C pre-2w, n=8) in 0.25 ml i.p. on 5 consecutive days prior to surgery. Another group was pretreated with one bolus i.p. injection of 5 mg/kg DIAZ 1 day before the 2VO operation (SHAM-DIAZ pre bolus-2w, n=5). The post-treated rats were administered daily

injections of 0.5 mg DIAZ (SHAM-DIAZ post-2w, n=7; 2VO-DIAZ post-2w, n=6) or the solvent (SHAM-C post-2w, n=6; 2VO-C post-2w, n=6) in 0.25 ml for 5 days after surgery, the first injection being applied directly after surgery.

In Experiment 3 (Fig. 2C), the rats were divided into 12 groups immediately after surgery. Brain specimens were collected 3 days after surgery in half of the animals (6 groups); the other half survived for 2 weeks (6 groups). The first set of these animals received 0.5 ml 5% sodium hydrogencarbonate ($NaHCO_3$) i.p. as the solvent control on 3 consecutive days (SHAM-C-3d, n=7 and 2VO-C-3d, n=8; SHAM-C-2w, n=9 and 2VO-C-2w, n=5). The second set of rats were treated with 3 mg/kg indomethacin (n=8) dissolved in 0.5 ml 0.9% saline i.p. (SHAM-indo-3d, n=8 and 2VO-indo-3d, n=10; SHAM-indo-2w, n=8 and 2VO-indo-2w, n=5). The third set of rats were injected i.p. with 15 mg/kg NS-398 (n=9) in 0.5 ml 5% $NaHCO_3$ (SHAM-NS-398-3d, n=9 and 2VO-NS-398-3d, n=8; SHAM-NS-398-2w, n=7 and 2VO-NS-398-2w, n=8). The first injection was given immediately following 2VO or SHAM surgery.

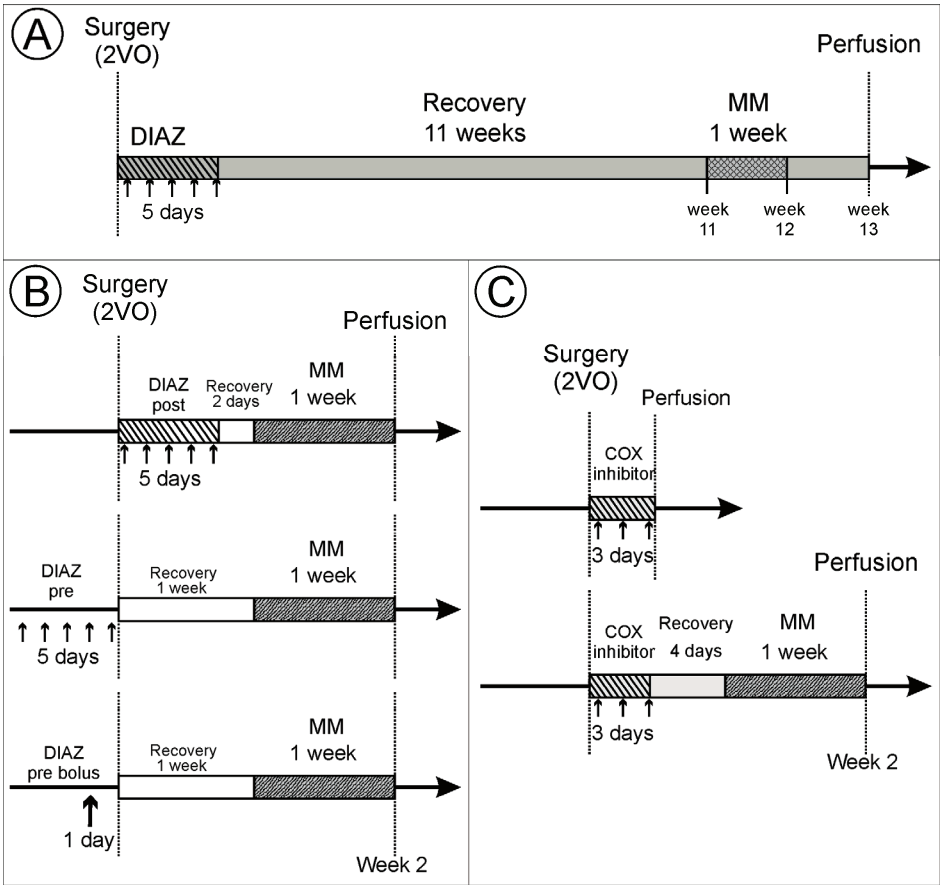



**Figure 2**. Time scales of the experimental protocols. Abbreviations: 2VO: bilateral common carotid artery occlusion; COX: cyclooxygenase; DIAZ: diazoxide; MM: Morris watermaze test; pre: pretreatment; post: post-treatment; SHAM: sham-operated control.

## Spatial learning test

Depending on the various experimental protocols, the rats were trained in the Morris watermaze spatial learning paradigm 1 week or 11 weeks after surgery (Fig. 2). The training protocol was the same throughout the whole study: for 5 consecutive days, 2 trials per day with a constant interval of 4 hours. In the watermaze, the rats were introduced into a swimming pool 160 cm in diameter, 35 cm in height, filled with water (22 $^{o}$C), facing the wall of the tank. The water was made opaque by the addition of milk, so that the rats were unable to see underwater. Several extra-maze visual cues and an auditory source were placed around the pool to help the orientation. The four quadrant points at which the rats were placed into the water were randomly varied at each trial. The animals were then given 2 minutes to find the location of a hidden platform submerged 2 cm below the water surface. Those rats which failed to find the platform were gently guided to it. After each trial, the animals were allowed to sit on the platform for 15 s. The length of the pathway swum by the rats and the escape latency were recorded and analyzed at each trial by a computerized video imaging analysis system (EthoVision, Noldus Information, The Netherlands).

## Immunocytochemistry

**Table 2**. Summary of the groups and observed parameters in the three experiments.

| | | | | | Histology | | | | |
|---|---|---|---|---|---|---|---|---|---|
| **Experiment** | **Surgery (SHAM, 2VO)** | **Treatment** | **Survival time** | **Morris water-maze** | **Cresyl violet** | **COX-2 immuno** | **MAP-2 immuno** | **GFAP immuno** | **OX-42 immuno** |
| 1 | SHAM 2VO | C (nontreated) | 13 weeks | + | | + | | + | + |
| | | DMSO | | + | | + | | + | + |
| | | DMSO+DIAZ | | + | | + | | + | + |
| 2 | SHAM 2VO | C (NaOH) | 2 weeks | + | + | | | + | + |
| | | DIAZ post | | + | + | | | + | + |
| | | DIAZ pre | | + | + | | | + | + |
| | | DIAZ pre bolus | | + | + | | | + | + |
| 3 | SHAM 2VO | C ($NaHCO_3$) | 3 days 2 weeks | + | + | | + | + | + |
| | | indomethacin | | + | + | | + | + | + |
| | | NS-398 | | + | + | | + | + | + |

Abbreviations: 2VO: two-vessel occlusion; C: control, COX: cyclooxygenase; DMSO: dimethyl sulphoxide; DIAZ: diazoxide; GFAP: glial fibrillary acidic protein; immuno: immunocytochemistry; MAP-2: microtubule associated protein-2; pre: pretreatment; post: post-treatment; SHAM: sham operation.

Depending on the survival times of the experimental protocols (Fig. 2), the animals were anesthetized with an overdose of pentobarbital and perfused transcardially with cold saline followed by 400 ml of 4% paraformaldehyde or of 3.5% paraformaldehyde and 0.5%

picric acid. Subsequently, the brains were carefully removed, and one hemisphere was postfixed in the same solution for 1 h, and then stored in 0.1 M phosphate buffer saline (PBS). All rat brains were processed for histological experiments. The incidence of macroscopic brain lesions was observed and evaluated immediately after the removal of the brain from the skull.

As the first step of the histological processing, 20-μm-thick free-floating coronal sections were cut on a cryostat at the level of the hippocampus (bregma -3.60)[123]. The first set of section from the 3-day and 2-week survival groups were mounted on gelatin-coated microscopic slides and stained with cresyl violet (Table 2).

Some of the sections from Experiment 1 were labeled with COX-2 antibody as follows. First, endogenous peroxidase activity was blocked with 0.3% $H_2O_2$. Nonspecific binding sites were covered with 5% normal goat serum (NGS), and the membrane permeability was enhanced with 0.3% Triton X-100. The sections were incubated for 2 days at room temperature (RT) in primary antibody solution containing rabbit anti-COX-2 antibody (Cayman), 1:2000, 1% NGS, 0.3% Triton X-100 and 0.1% sodium azide in 0.01 M PBS (pH 7.4). Next, the sections were rinsed and preincubated in 5% NGS for 1 hour. Incubation was performed in goat anti-rabbit biotinylated IgG (Santa Cruz) for 4 hours at RT. Finally, the signal was amplified with an ABC–Avidin Kit (1:400) (Vector) for 2 hours at RT. The color reaction was developed with diaminobenzidine (DAB) and $H_2O_2$.

Some sections from Experiment 3 (see Table 2) were immunocytochemically stained for MAP-2 to visualize the dendritic integrity. Pretreatment of the slices was performed with 0.3% $H_2O_2$, 20% normal pig serum (NPS), and 0.3% Triton X-100. The samples were then incubated overnight in a primary antibody solution containing mouse anti-MAP-2 antibody (Sigma), 1:10000, 20% NPS, and 0.03% merthiolate in 0.01 M PBS. The secondary antibody solution contained goat anti-mouse biotinylated IgG (Jackson) 1:400, 10% NPS, 0.03% merthiolate and 5% normal rabbit serum (NRS) in PBS. Finally, the sections were incubated in HRP-Streptavidin (Jackson) 1:1000, 1% NPS and 0.03% merthiolate in Tris buffer solution. The color reaction was developed with Ni-DAB and $H_2O_2$.

In some sections from all three experiments, the astrocytic reaction was immunocytochemically labeled with GFAP antibody. After pretreatment with 5% normal sheep serum (NShS), 0.3% $H_2O_2$ and 0.3% Triton X-100, the sections were incubated overnight in a solution containing the mouse anti-GFAP primary antibody (Sigma), 1:200, 1% NShS and 0.3% Triton X-100 in 0.01 M PBS. The secondary antibody solution consisted of goat anti-mouse biotinylated IgG (Jackson), 1:400, 10% NShS, 5% NRS and 0.03%

merthiolate in 0.01 M PBS. Finally, the sections were incubated in HRP-Streptavidin (Zymed), 1:200, and the color reaction was developed conventionally with DAB and $H_2O_2$.

To detect and analyze the microglial activation, OX-42 antibody was used on a further set of sections. The procedure started with rinsing and pretreatment of the sections with 0.5% Triton X-100 and 3% $H_2O_2$ in 0.01 M PBS, followed by reincubation in 20% NPS and 0.5% Triton X-100 in 0.01 M PBS for 1 hour. The sections were incubated overnight in a primary antibody solution containing biotinylated mouse anti-CD11b antibody (OX-42, Serotec), 1:500, 20% NPS and 0.03% merthiolate in 0.01 M PBS at RT. Next, the sections were rinsed and incubated in a solution of STA-PER (Jackson), 1% NPS and 0.03% merthiolate in 0.1 M Tris buffer for 1 hour at RT. Finally, the color reaction was developed with Ni-DAB and $H_2O_2$.

All the sections were mounted on gelatin-coated microscopic slides, air-dried, dehydrated, and coverslipped with Distrene 80 (dibutyl phthalate with xylene).

**Quantitative morphometry**

On the cresyl violet-stained sections, photographs of the hippocampus CA1 and CA3 stratum pyramidale and the DG granule cell layer were taken with a computerized image analysis system to identify neuronal damage (Olympus BX50 microscope, DP50 digital camera, ImagePro Plus software, Media Cybernetics, U.S.A.). On the sections from Experiment 2, the number of pyramidal neurons in the hippocampus CA1 region was counted with the help of an ocular grid at 40× magnification on a surface of 0.024 $mm^2$ (3 × 10 grid holes) that covered the entire width of the CA1 str. pyramidale. Cell counting was performed bilaterally on three consecutive brain sections. The six values were averaged, and the average was used for further statistical analysis. In Experiment 3, the proportion of animals displaying neuronal damage was determined with the help of cresyl violet-stained sections. Hippocampal neuronal damage was evaluated at 40x magnification with a light microscope. Damage to a hippocampal region was recorded when neurons showed typical signs of necrosis (dark staining, shrinkage and a dysmorphic shape). The number of animals exhibiting necrotic neuronal injury was counted and expressed as a percentage of the total number of animals in a given group. The ratio of affected animals was calculated for each hippocampal region and each group.

COX-2-labeled neuron counting was performed at 20x magnification with the help of an ocular grid with 1600 x $\mu m^2$ holes. Three consecutive coronal sections with a standard distance of 160 μm, were examined, starting at Br. -3.60 mm [123]. COX-2-positive neurons

were counted in the CA3 str. pyramidale and the DG inner and outer str. moleculare on an average surface of 0.018 $mm^2$ in each region. Regional cell counts of 3 sections per animal were averaged, and the average was used for further statistical analysis.

The percentage of the area covered by MAP-2-antibody-labeled dendritic processes to total area surface was quantified in the CA3 str. lucidum of the hippocampus. The percentage surface area of the GFAP-positive astrocytes was quantified in the dorsal hippocampus, the medial corpus callosum and the internal capsule. In the optic tract, the relative optical density was computed instead of the percentage area, since the homogenous labeling did not permit area measurements. The percentage surface area was measured for the individual hippocampal areas (the CA1 str. radiatum, the CA1 str. oriens, the CA3 str. radiatum, the CA3 str. oriens, the inner and outer molecular layers of the dentate gyrus, and the hilus) and for all three WM regions of interest by using a Quantimet Q-600HR computerized image analysis system (Leica, Cambridge, UK) [124]. As regards the OX-42 labeling, quantification of immunoreactive microglia was performed in a similar manner as for the GFAP-labeled sections on a computerized image analysis system (Olympus BX50, DP50; software: ImagePro Plus, Media Cybernetics).

Briefly, 3 consecutive coronal sections with a standard distance of 160 μm were selected for the analysis, starting at Br. -3.60 mm [123]. Hippocampal and WM regions of interest were manually delineated at 10x magnification, after background subtraction and gray scale threshold determination. The area covered by MAP-2-positive dendrites, GFAP-positive astrocytes or OX-42-positive microglia was computed as a percentage of the total area delineated. The measurements on the 3 sections per animal were averaged, and the average was used for further statistical analysis.

**Statistical analysis**

The Morris watermaze test results were analyzed statistically by means of a two-way repeated measurement model, followed by the least significant difference (LSD) *post hoc* test with the program SPSS. Individual day comparisons were performed with a univariate model and LSD *post hoc* analysis with SPSS. The proportions of failed trials to the total and the proportion of animals displaying neuronal damage in the hippocampus were analyzed statistically by a chi square test of the program Statistica. The MAP-2, GFAP and OX-42 immunocytochemical results were analyzed statistically with two-way or three-way ANOVA in correspondence with the number of variables (surgery, treatment and survival time), followed by the LSD *post hoc* test.

## RESULTS

### Spatial learning test

The swimming distance curves (Fig. 3) obtained from the Morris watermaze test showed a day-to-day improvement in the performance of the SHAM-control rats, while cerebral hypoperfusion resulted in a learning disability in all 2VO-control animals at a survival of either 2 weeks (**$P$<0.001) or 13 weeks (**$P$<0.001) (Fig. 3A). This difference was obvious on days 2, 3 and 5. No significant difference could be observed between the performance of the SHAM–C-2w vs the SHAM-C-13w rats or the 2VO–C-2w *vs* 2VO-C-13w rats. Neither DMSO nor DMSO+DIAZ treatment affected the spatial learning performance of the SHAM animals (Fig. 3B). However, DMSO alone and especially together with DIAZ significantly decreased the length of the swimming pathway of the 2VO animals throughout the whole period of learning in the Morris watermaze (*$P$<0.040 and **$P$<0.007, respectively) as compared with the control 2VO groups. DMSO+DIAZ treatment created an additional improvement in learning skill on days 2 and 3 as compared with DMSO in the 2VO animals (Fig. 3C). Both bolus and repeated pretreatment with DIAZ improved the spatial learning performance of the 2VO rats at 2 weeks throughout the whole period of the learning process, since these animals displayed the same performance as their respective SHAM controls (Fig. 3C). In contrast, DIAZ post-treatment was statistically ineffective in improving the memory capacity of the 2VO-DIAZ post-treated group. On days 2 and 5, these animals swam significantly longer than the SHAM-DIAZ post-treated rats (Fig. 3D).

The treatment with the COX inhibitors did not alter the learning ability of the SHAM animals. The indomethacin-treated 2VO rats (2VO-indo-2w) demonstrated a poor performance on days 3 and 4 as compared with the SHAM groups, similar to the vehicle-treated 2VO group (2VO-C-2w). This was also reflected by the ratio of failed trials (Fig. 3F). However, the 2VO-indo-2w animals (unlike the 2VO-C-2w group) attained the SHAM performance level by day 5 (Fig. 3E). The administration of NS-398 improved the learning capacity of the 2VO rats throughout the whole testing period in the Morris watermaze as compared with the 2VO-C-2w group (*$P$<0.024). The proportion of failed trials also indicated the beneficial effect of NS-398: the ratio of failed trials by the 2VO-NS-398-2w group was significantly lower than that by the 2VO-C-2w group, and not different from the SHAM groups (Fig. 3F).

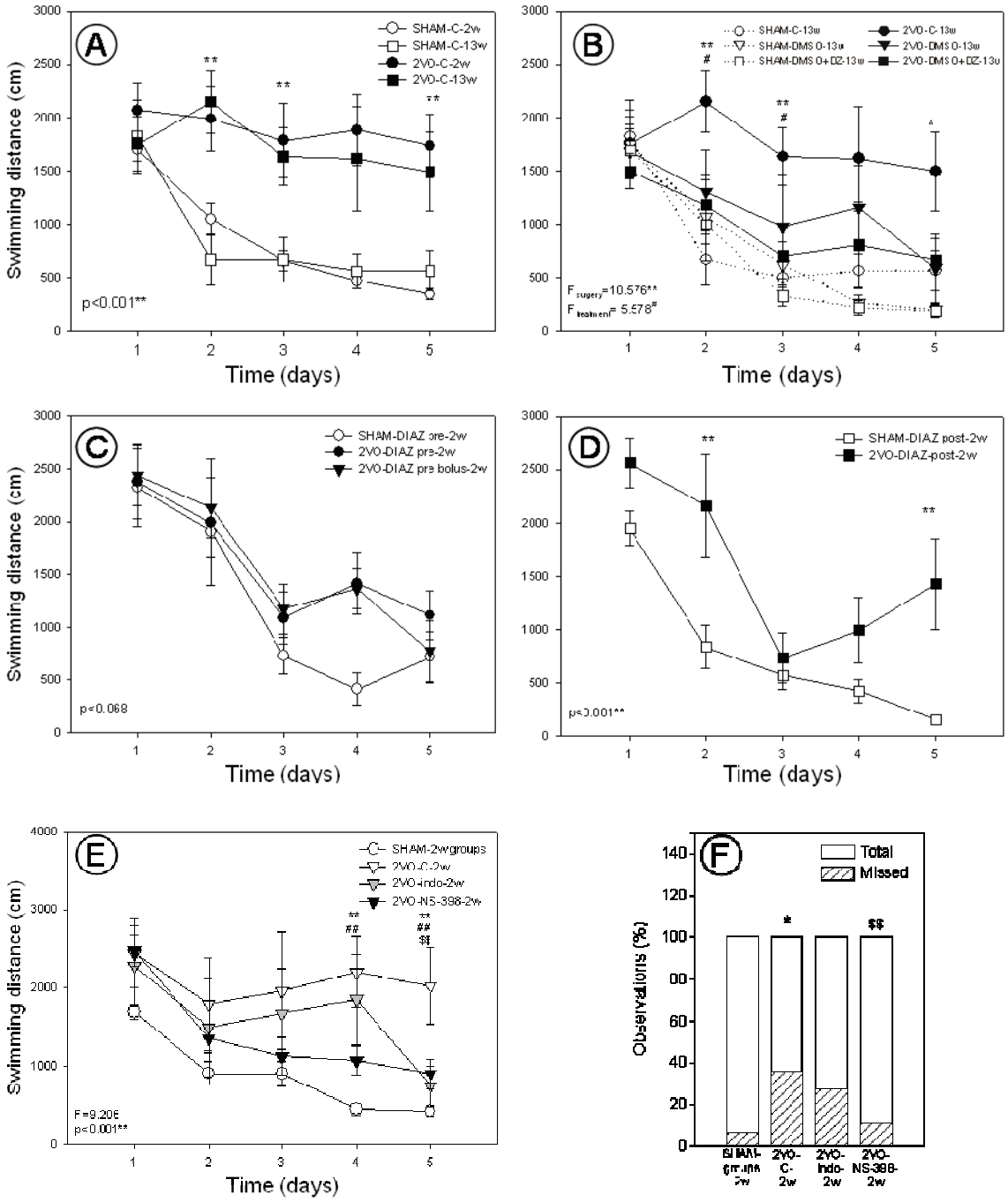



**Figure 3.** (A-E) Learning curves of the Morris watermaze spatial orientation test. Data are presented as means±S.E.M. (F): Proportion of missed trials to total between trials 2-10. Data are expressed as percentage averages (%). Statistical *F* values are based on a two-way repeated measurement ANOVA model (**P*<0.05). Individual days were analyzed with ANOVA and LSD *post hoc* test (**P*<0.05). *: indicates a difference between surgical intervention; # and $: stand for the difference between treatments. Abbreviations: 2VO: bilateral carotid artery occlusion; DMSO: dimethyl sulphoxide; DIAZ: diazoxide; indo: indomethacin; pre: pretreatment; post: post-treatment; SHAM: sham operation.

The administration of NS-398 resulted in a significantly lower distance swum by the 2VO rats throughout the whole testing period in the Morris watermaze as compared with the 2VO-C-

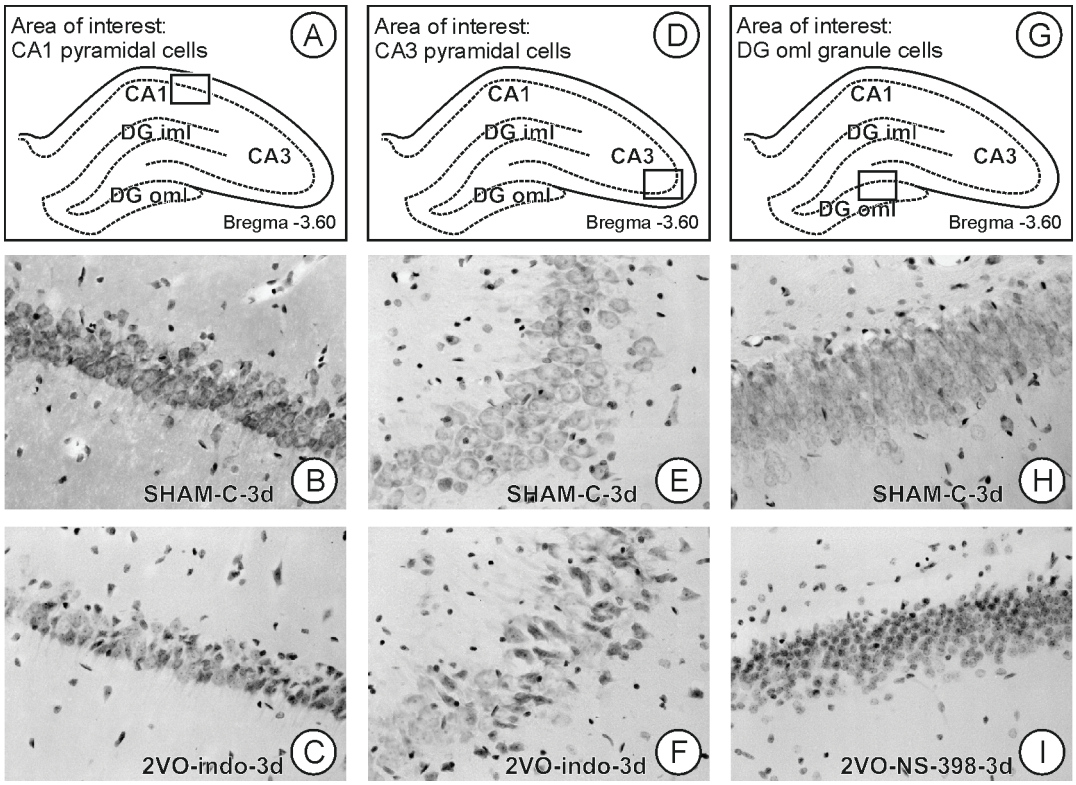
2w group (*$P$<0.024). A similar tendency could be observed as concerns the proportion of failed trials to the total number (Fig. 3F). The 2VO-solvent group failed to find the platform with a significantly higher incidence than the animals from the SHAM groups (*$P$<0.05), while indomethacin moderated, and NS-398 significantly reduced the proportion of failed trials relative to the control level (**$P$<0.0014).

**Neuronal damage, dendritic degeneration and macroscopic brain lesions**

The integrity of the hippocampal neuronal layers was evaluated by using different approaches. In Experiment 2, the number of cresyl violet-stained CA1 pyramidal cells was counted in a standard surface. In Experiment 1, the number of COX-2-positive neurons in the DG were counted and expressed as neuronal density with a similar method. In Experiment 3, the presence of a necrotic process was identified in cresyl violet-stained sections, and the proportion of animals displaying necrotic degeneration in the hippocampal CA1, CA3 and DG cell layers was calculated for each region and for each experimental group. The integrity of the dendritic arborizations was labeled with MAP-2 immunocytochemistry in Experiment 3. Finally, in Experiment 1, the presence of macroscopic lesions was noted.

The 2VO-related, progressive neuronal damage in the hippocampus can best be characterized in the CA1 region: while only 12.5% of the control 2VO animals displayed signs of necrosis, this ratio increased to 40% at the 2-week survival (Fig. 4). The quantitative evaluation of the degree of necrotic cell death at 2 weeks indicated an average of 7% loss of the CA1 pyramidal cells (Fig. 5). In the CA3 pyramidal cell and DG granule cell layers, the higher incidence of necrotic damage (37.5%) was present at 3 days, but then fell to 20% by week 2. This indicates a different temporal sensitivity of hippocampal areas to hypoperfusion. At 13 weeks, 66.7% of the 2VO rats had a complete hippocampal lesion (complete, unilateral loss of the hippocampus; Fig. 7F-I).

The progressive disintegration of the dendritic arborization was followed from day 3 to week 2 after 2VO onset in the CA3 str. lucidum of the hippocampus (Fig. 6). While the difference in MAP-2-positive fiber density between the SHAM and 2VO groups was negligible at day 3, the density of labeled dendrites was decreased significantly in the 2VO groups as compared with the SHAM group at 2 weeks.




J The number and proportion of animals with neuronal damage in the hippocampal areas:

| | | | CA1 | | CA3 | | DG | |
|---|---|---|---|---|---|---|---|---|
| Time to post-operative sacrifice | Surgery | Treatment | n (damaged/ total) | % | n (damaged/ total) | % | n (damaged/ total) | % |
| 3 days | SHAM | control | 0/7 | 0.00 | 1/7 | 14.29 | 1/7 | 14.29 |
| | | indo | 0/8 | 0.00 | 0/8 | 0.00 | 1/8 | 12.50 |
| | | NS-398 | 1/9 | 11.11 | 2/9 | 22.22 | 4/9 | 44.44 |
| | 2VO | control | 1/8 | 12.50 | 3/8 | 37.50 | 3/8 | 37.50 |
| | | indo | 5/10 | 50.00* | 6/10 | 60.00** | 4/10 | 40.00 |
| | | NS-398 | 3/8 | 37.50 | 3/8 | 37.50 | 8/8 | 100.00## |
| 2 weeks | SHAM | control | 0/9 | 0.00 | 1/9 | 11.11 | 2/9 | 22.22 |
| | | indo | 0/8 | 0.00 | 1/8 | 12.50 | 2/8 | 25.00 |
| | | NS-398 | 0/7 | 0.00 | 4/7 | 57.14 | 6/7 | 85.71 |
| | 2VO | control | 2/5 | 40.00 | 1/5 | 20.00 | 1/5 | 20.00 |
| | | indo | 0/5 | 0.00 | 0/5 | 0.00 | 2/5 | 40.00 |
| | | NS-398 | 1/8 | 12.50 | 0/8 | 0.00$ | 4/8 | 50.00 |

**Figure 4.** Cresyl violet staining and the proportions of animals displaying neuronal damage in the dorsal hippocampus (bregma −3.60). Area of interest: (A): CA1 stratum (str.) pyramidale, (D): CA3 str. pyramidale and (G): dentate gyrus outer molecular layer (DG oml). (B-C): Representative photomicrographs of the hippocampal CA1, (E-F):CA3 and (H-I): DG at 40× magnification. (J): Semiquantitative data on the proportions of damaged CA1, CA3 and DG in the hippocampus. Statistical analysis was performed with a non-parametric chi-square test. Significance levels of the *post hoc* test are given as: $^{*}P<0.05$, $^{**}P<0.01$ (2VO-indo-3d vs SHAM-indo-3d), $^{\$}P<0.05$ (2VO-NS-398-2w *vs* 2VO-C-2w), $^{\#\#}P<0.01$ (2VO-NS-398-3d vs 2VO-C-3d). Abbreviations: CA: cornu ammonis; DG: dentate gyrus; 2VO; permanent, bilateral common carotid artery occlusion (two-vessel occlusion); SHAM: sham-operated controls.

DIAZ treatments showed a number of beneficial effects on hippocampal integrity. DIAZ pretreatment prevented the loss of CA1 pyramidal neurons at 2 weeks of survival (Fig.

5) Post-treatment with DIAZ in DMSO prevented the total, unilateral degeneration of the hippocampus observed in the control 2VO rats at 13 weeks (DMSO alone also reduced the incidence of hippocampal lesions) (Fig. 7). However, post-treatment with DIAZ in NaOH solution did not exert any beneficial effect on the CA1 pyramidal cell integrity 2 weeks after 2VO onset (data not shown).

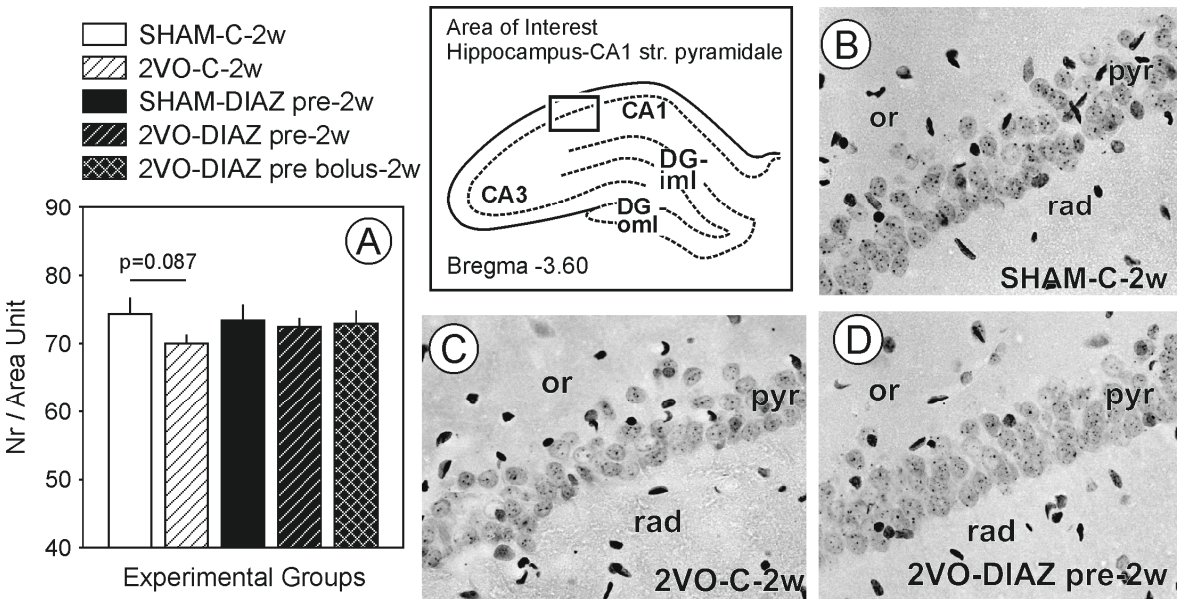



**Figure 5.** Cresyl violet staining and cell counting in the hippocampus CA1 str. pyramidale. (A): Quantitative data on pyramidal cell viability in the hippocampus CA1 str. pyramidale; area unit is 0.024 $mm^2$ (3×10 grid holes at 40× magnification). Data are presented as means ± SEM; **P*<0.05. The *P* value indicated in the graph was obtained with a Student *t* test. (B–D): Representative photomicrographs of the hippocampal CA1 area from pretreated animals. Abbreviations: 2VO: permanent, bilateral common carotid artery occlusion (two-vessel occlusion); C: control for treatment; DIAZ: diazoxide; pre: pretreatment; SHAM: sham-operated control.

Post-treatment paradigms with the COX inhibitors indomethacin and NS-398 yielded controversial data. Indomethacin increased the number of 2VO rats with CA1 and CA3 necrotic injury at 3 days (50-60%), but not at 2 weeks (0%) after 2VO onset (Fig. 1). On the other hand, NS-398 promoted DG injury, especially at 3 days (100%), but reduced the number of animals with CA3 damage at 2 weeks as compared with the 2VO-control animals (0% *vs* 20%) (Fig. 1). Neither of the COX inhibitors affected the 2VO-related injury of the MAP-2-positive dendrites (Fig. 6).

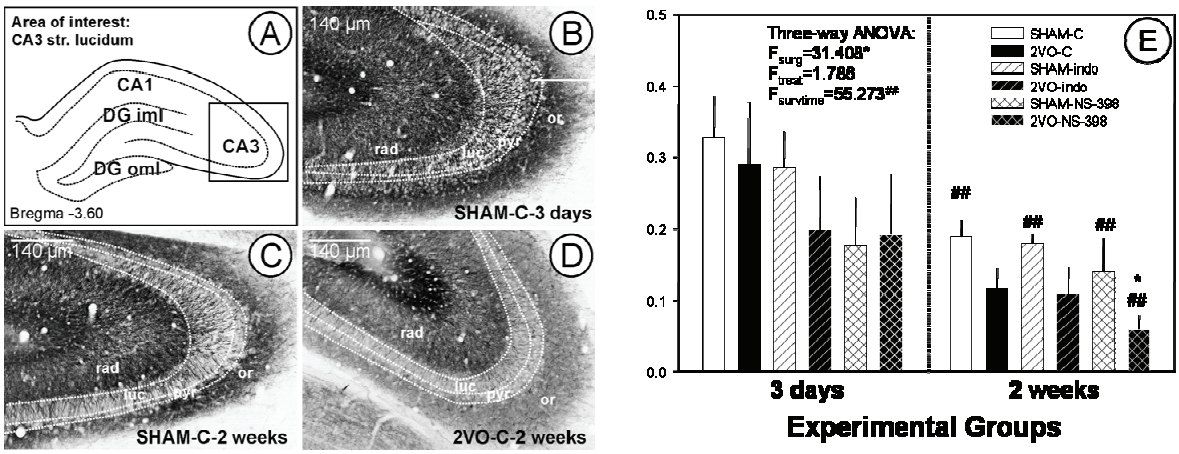



**Figure 6.** MAP-2 immunocytochemistry relating to synaptic density. Panel A: Area of interest: CA3 str. lucidum in the dorsal hippocampus (bregma −3.60). Panels B–D: Representative photomicrographs of the CA3 area of the hippocampus. Panel E: Quantitative data on MAP-2 immunocytochemistry in the hippocampal CA3 str. lucidum. Data are presented as means±S.E.M.; $^{*}P<0.05$; $^{\#\#}P<0.01$. The *F* value indicated in the graph was obtained with the LSD (least significant difference) *post hoc* test. Abbreviations: 2VO: permanent, bilateral common carotid artery occlusion (two-vessel occlusion); Sham: sham-operated controls; C: control for treatment; CA: cornu ammonis; or: stratum oriens; pyr: stratum pyramidale; rad: stratum radiatum, luc: str. lucidum.

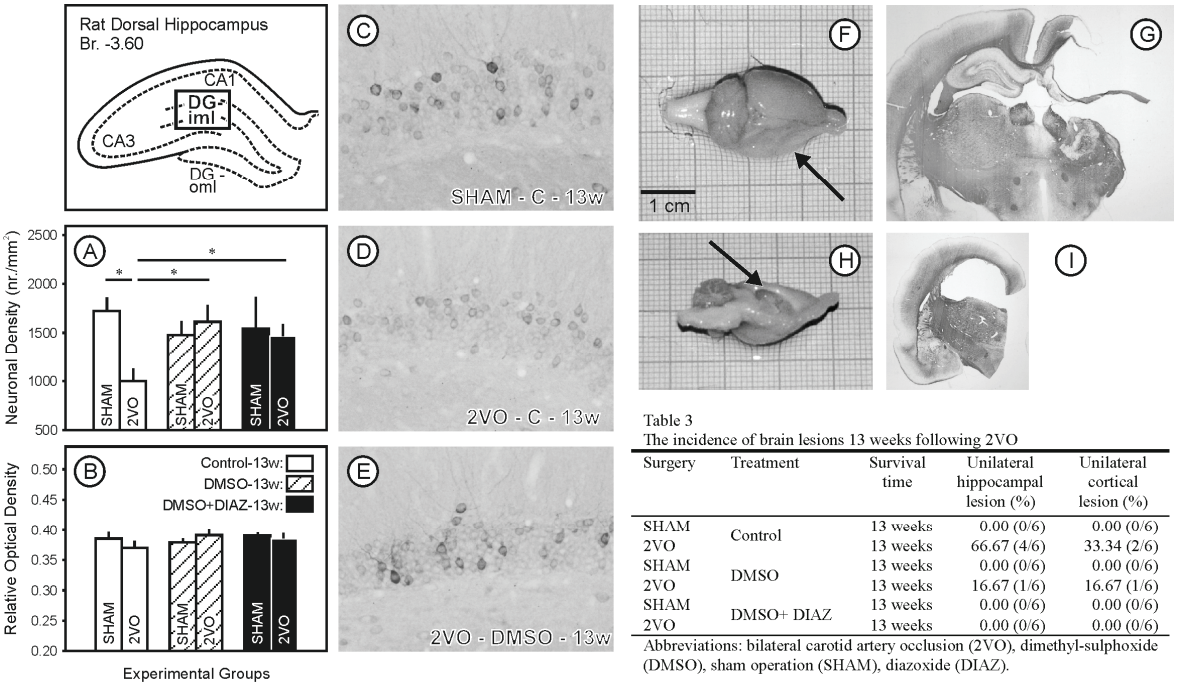



Table 3
The incidence of brain lesions 13 weeks following 2VO

| Surgery | Treatment | Survival time | Unilateral hippocampal lesion (%) | Unilateral cortical lesion (%) |
|---|---|---|---|---|
| SHAM | Control | 13 weeks | 0.00 (0/6) | 0.00 (0/6) |
| 2VO | | 13 weeks | 66.67 (4/6) | 33.34 (2/6) |
| SHAM | DMSO | 13 weeks | 0.00 (0/6) | 0.00 (0/6) |
| 2VO | | 13 weeks | 16.67 (1/6) | 16.67 (1/6) |
| SHAM | DMSO+ DIAZ | 13 weeks | 0.00 (0/6) | 0.00 (0/6) |
| 2VO | | 13 weeks | 0.00 (0/6) | 0.00 (0/6) |

Abbreviations: bilateral carotid artery occlusion (2VO), dimethyl-sulphoxide (DMSO), sham operation (SHAM), diazoxide (DIAZ).

**Figure 7.** Cyclooxygenase-2 (COX-2) immunocytochemistry in the rat hippocampus and macroscopic lesions of the brains. (A and B): show neuronal density and COX-2 expression of preserved neurons, respectively. Significance values were obtained with the Student *t*-test ($^{*}P<0.05$). (C, D and E): COX-2-positive neurons in the dentate gyrus inner granular cells. (F- I): representative macroscopic and microscopic images of cortical and hippocampal lesions. Abbreviations: 2VO: bilateral carotid artery occlusion (two-vessel occlusion); DMSO: dimethyl sulphoxide; DIAZ: diazoxide; SHAM: sham operation.

## Astrocytic reaction

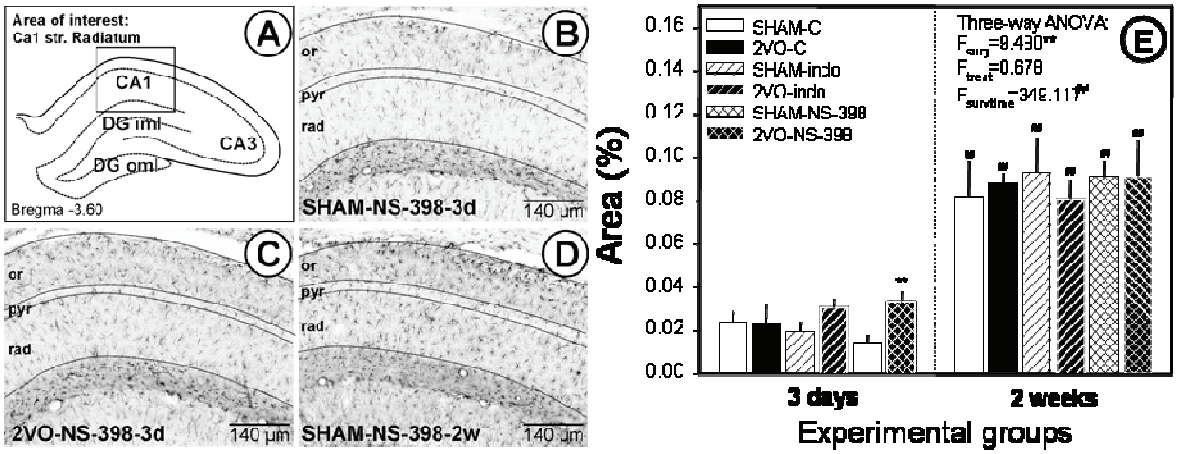



**Figure 8.** Astrocytic proliferation in the CA1 str. radiatum of the hippocampus labeled by GFAP immunocytochemistry. Images were taken at 10x magnification (B–E). Data are presented as means±S.E.M.; **P*<0.05; $^{\#\#}$*P*<0.01 (E). Abbreviations: 2VO: permanent, bilateral common carotid artery occlusion (two-vessel occlusion); SHAM: sham-operated controls; CA: cornu ammonis; or: stratum oriens; pyr: stratum pyramidale; rad: stratum radiatum.

The proliferation of GFAP-immunoreactive astrocytes was investigated in the hippocampus at all survival time, and in three WM regions (the corpus callosum, the internal capsule and the optic tract) 13 weeks after 2VO onset. GFAP immunoreactive astrocytes were present in all hippocampal and WM regions and in all experiments. At 3 days and 2 weeks following 2VO surgery, the GFAP activity was not altered by 2VO itself in any of the hippocampal areas (Fig. 8 and 9). Eleven weeks later (at 13 weeks of survival), the area covered by GFAP-positive astrocytes in the hippocampus was reduced in the 2VO animals as compared with the SHAM groups. The WM areas at 13 weeks displayed various patterns of astrocytic proliferation. The GFAP signal intensity in the corpus callosum and internal capsule of the 2VO animals was not different from that of their respective SHAM controls. Conversely, in the optic tract, cerebral hypoperfusion induced a consistent ~20% elevation of the GFAP signal in the 2VO groups as compared with the SHAM groups (Fig. 10A,B,E). This elevation appeared most prominent in the lateral portion of the optic tract, which contained more GFAP-positive immunoreactive material than the medial part (Fig. 10B).

None of the DIAZ pretreatments influenced the astrocytic proliferation at 2 weeks after 2VO onset. In turn, the post-treatment doubled the GFAP signal in both the str. oriens and the str. radiatum of the CA1 area in the hippocampus of the 2VO animals at this time point (**P*<0.05) (Fig. 9). Thirteen weeks after the 2VO surgery, the postsurgical treatment with DIAZ administered in DMSO significantly suppressed the GFAP immunoreactivity with about 33% in the corpus callosum of both the 2VO and the SHAM rats (Fig. 10F).

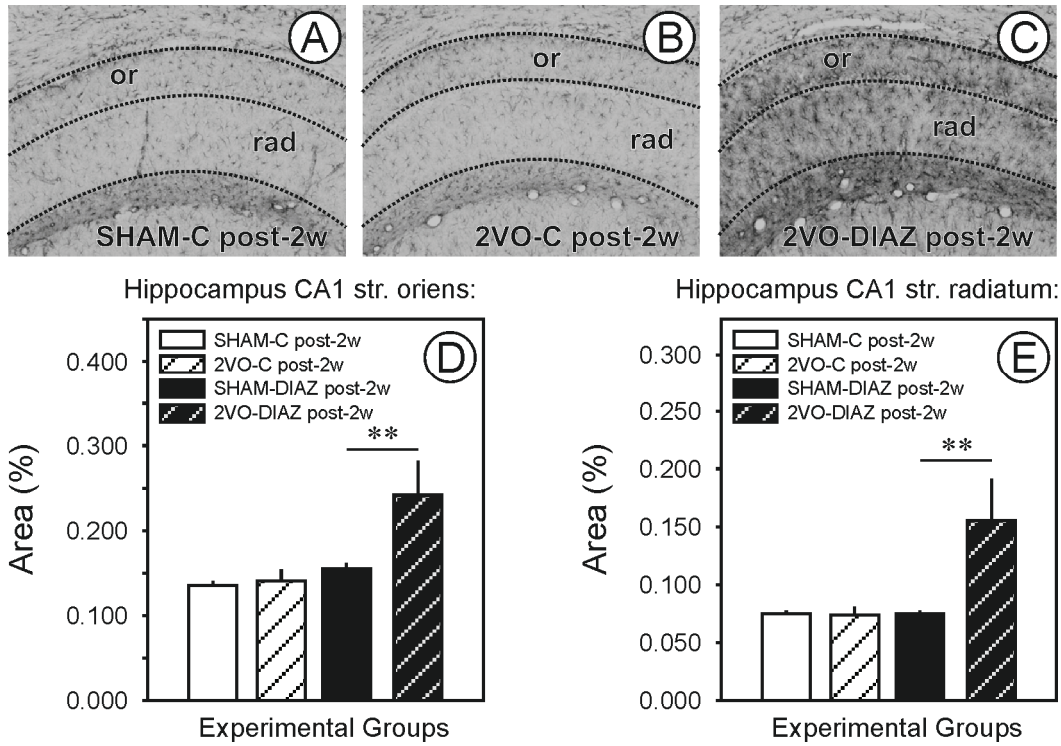



**Figure 9.** GFAP immunocytochemistry for astrocyte proliferation in the CA1 area of the hippocampus 2 weeks after surgery. (A–C) Representative photomicrographs of the hippocampal CA1 area from post-treated animals. (D) Quantitative data on GFAP immunocytochemistry in the hippocampal CA1 str. oriens of the diazoxide post-treated animals. (E) Quantitative data on GFAP immunocytochemistry in the hippocampal CA1 str. radiatum of the diazoxide post-treated animals. Data are presented as means ± SEM; **P*<0.05, ***P*<0.01. Abbreviations: 2VO: permanent, bilateral common carotid artery occlusion (two-vessel occlusion); C: control for treatment; DIAZ: diazoxide; post: post-treatment; SHAM: sham-operated control.

Indomethacin post-treatment did not alter the proliferation of astrocytes in the str. radiatum of area CA1 at 3 days of survival, while the treatment with NS-398 caused a significant elevation in the GFAP signal in the 2VO animals (Fig. 8). Conversely, neither of the COX inhibitor treatments influenced the hippocampal GFAP immunoglobulin at 2 weeks of survival.

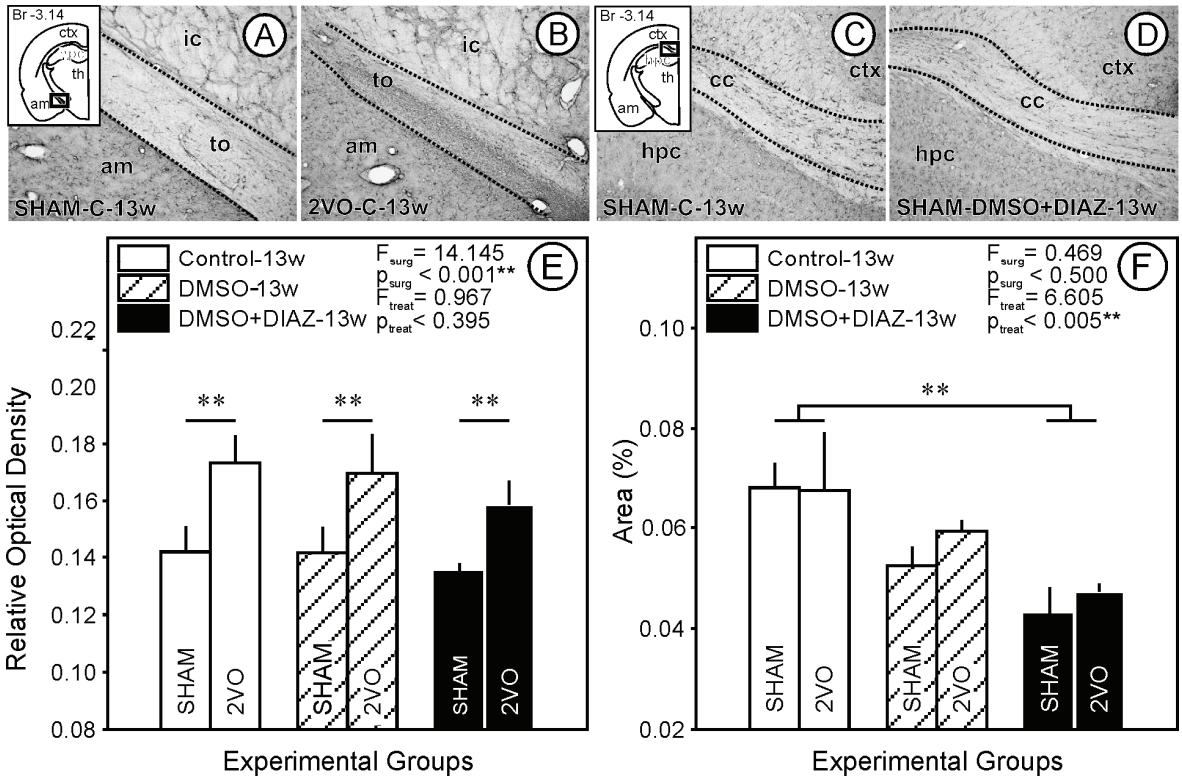



**Figure 10.** GFAP immunocytochemistry of the optic tract and corpus callosum 13 weeks after surgery. (A-B): Representative microscopic images of GFAP immunoglobulin in the optic tract at 10x magnification. (C-D): Representative microscopic images of GFAP immunoglobulin in the corpus callosum at 10x magnification. (E): Quantitative data on astrocytic proliferation in the optic tract (***P*<0.01). (F): Quantitative data on astrocytic proliferation in the corpus callosum (***P*<0.01). Abbreviations: 2VO: bilateral carotid artery occlusion; am: amygdala complex; cc: corpus callosum; ctx: cerebral cortex; DMSO: dimethyl sulphoxide; DIAZ: diazoxide; hpc: hippocampus; ic: internal capsule; SHAM: sham-operated control; th: thalamus.

## Microglia activation

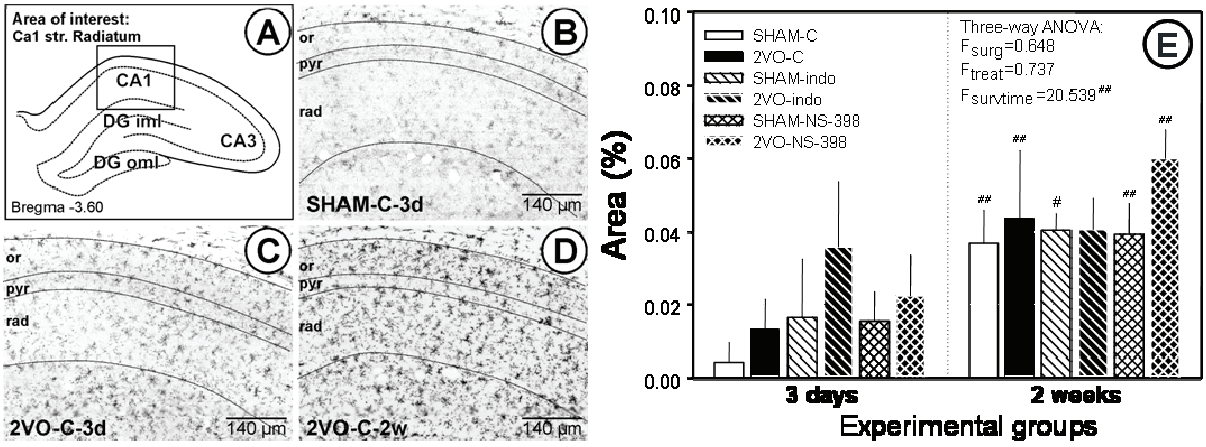



**Figure 11.** (A): Quantitative analysis of OX-42 immunocytochemistry indicating microglia activation in the hippocampus CA1 str. radiatum. Significance values were obtained with the LSD *post hoc* test (***P*<0.01; ##*P*<0.01). (B–D): Representative photomicrographs of the CA1 area of the hippocampus (10× magnification). Abbreviations: 2VO: permanent, bilateral common carotid artery occlusion (two-vessel occlusion); SHAM: sham-operated controls; CA: cornu ammonis; or: stratum oriens; pyr: stratum pyramidale; rad: stratum radiatum.

The activation of the microglia was labeled by means of OX-42 immunocytochemistry and was investigated in the hippocampus at all survival times and in 3 WM regions (the corpus callosum, the internal capsule and the optic tract) 13 weeks after 2VO onset.

The OX-42-labeled microglial activation was moderate or barely detectable in the hippocampal areas of the SHAM-control animals. Three days and 2 weeks after 2VO onset, the microglial activation in the 2VO nontreated groups remained similar to that in the SHAM controls (Figs. 11 and 12). Thirteen weeks after 2VO, the microglia activation was significantly enhanced in the CA1 area (*$F$=4.477) (Fig. 13E). At this time point, the microglia activation was augmented by 56% by cerebral hypoperfusion in the corpus callosum of the 2VO-C-13w rats, as compared with the SHAM-C-13w group (Fig. 13M). The most marked elevation in microglia activation was that in the optic tract of the 2VO-C-13w group (**$P$<0.001) (Fig. 13L). The internal capsule was unaffected by 2VO 13 weeks following surgery.

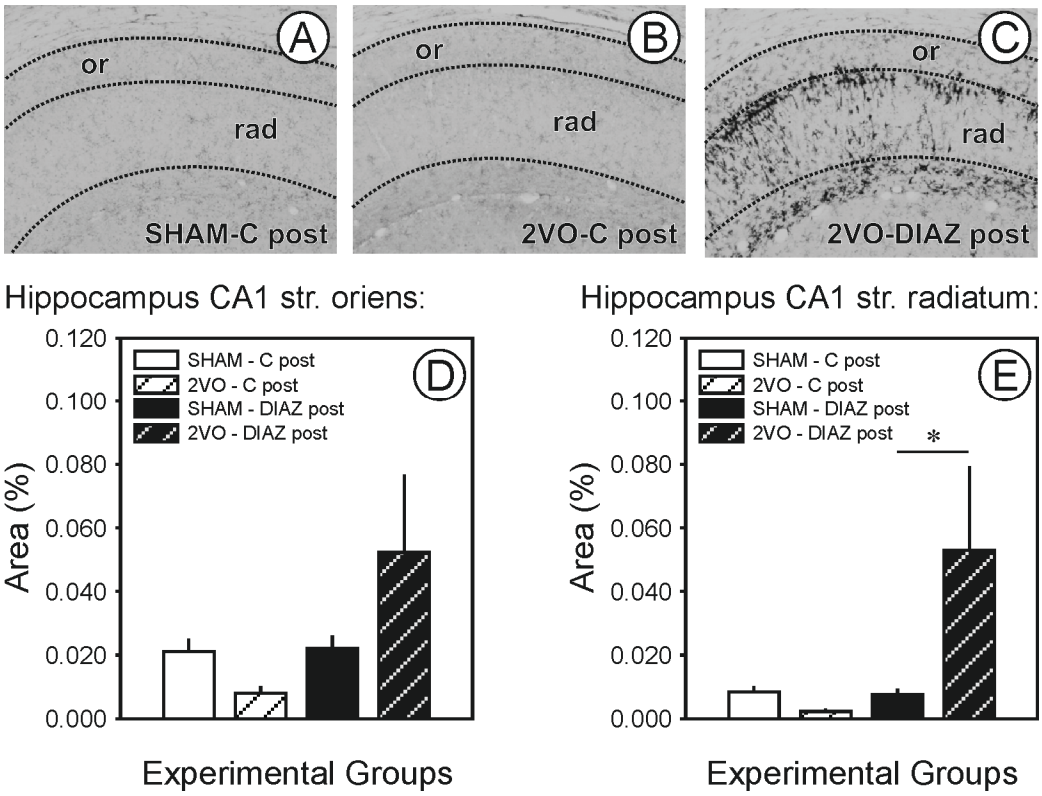



**Figure 12.** OX-42 immunocytochemistry related to microglia activation. (A–C): Representative photomicrographs of the hippocampal CA1 area from post-treated animals. (D): Quantitative data on OX-42 immunocytochemistry in the hippocampal CA1 str. oriens of the post-treated animals. (E): Quantitative data on OX-42 immunocytochemistry in the hippocampal CA1 str. radiatum of the post-treated animals. Data are presented as means ± SEM; *$P$<0.05, **$P$<0.01. Abbreviations: 2VO: permanent, bilateral common carotid artery occlusion (two-vessel occlusion); C: control for treatment; DIAZ: diazoxide; post: post-treatment; SHAM: sham-operated control.

The pretreatments with DIAZ did not modify the OX-42 labeling in the hippocampus of the 2VO rats at 2 weeks of survival (Fig. 12D,E).

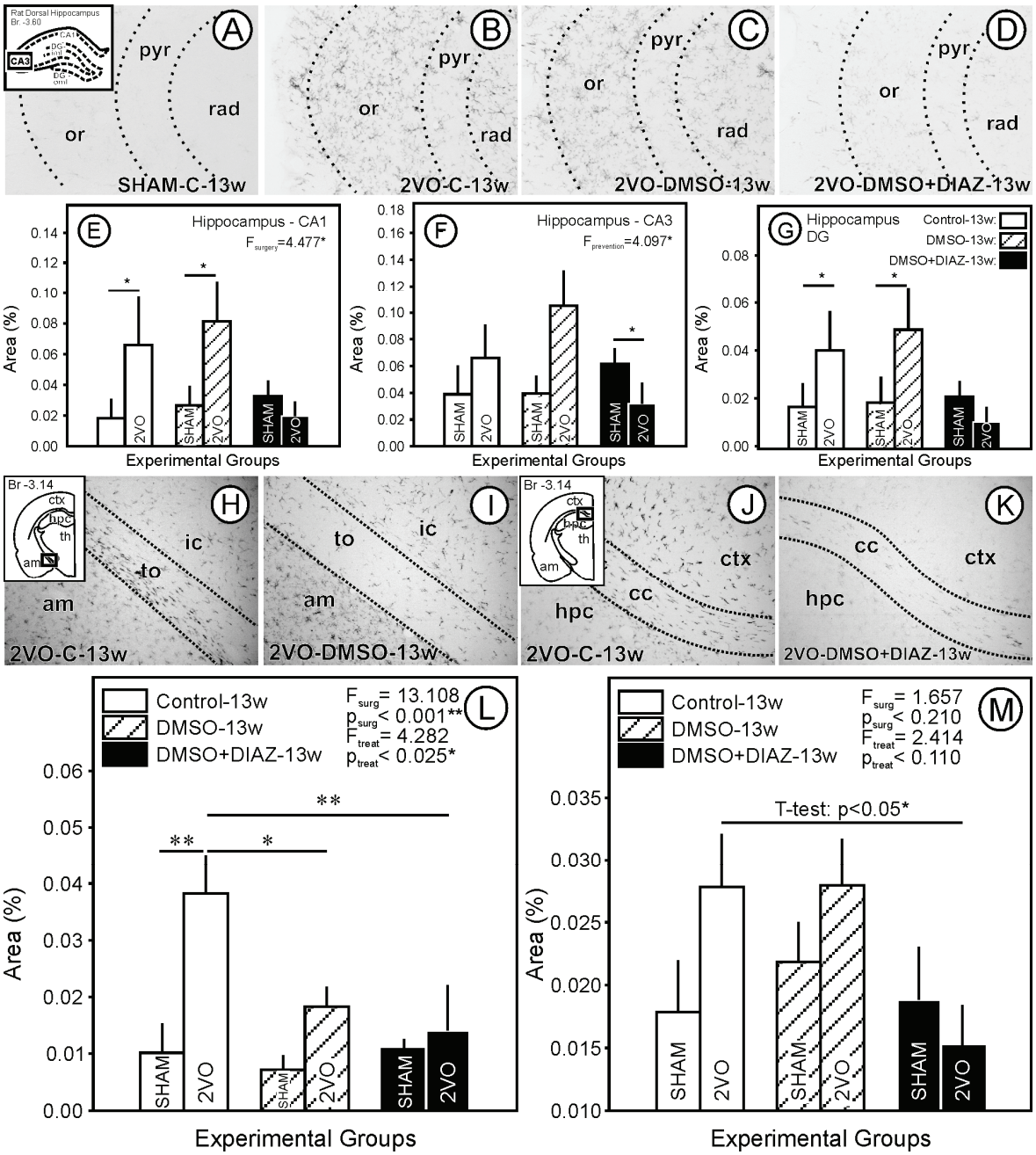



**Figure 13.** Microglial activation in the rat hippocampus CA3 region, optic tract and corpus callosum labeled by means of OX-42 immunocytochemistry. (A-D): Representative photomicrographs of the hippocampus CA3 area. (E-G): Quantitative data on microglia activation in the hippocampus CA1, CA3 and DG regions (**P*<0.05). (H-I): Representative photomicrographs of the optic tract. (L): Quantitative data on microglia activation in the optic tract (***P*<0.01). (J-K): Representative photomicrographs of the corpus callosum. (M): Quantitative data on microglia activation in the corpus callosum (**P*<0.05). All images were taken at 10x magnification. Abbreviations: 2VO: permanent, bilateral carotid artery occlusion; am: amygdala complex; cc: corpus callosum; ctx: cerebral cortex; DMSO: dimethyl sulphoxide; DIAZ: diazoxide; hpc: hippocampus; ic: internal capsule; or: str. oriens; pyr: str. pyramidale; rad: str. radiatum; SHAM: sham operation; th: thalamus.

In contrast, DIAZ post-treatment doubled the level of microglia activation in the 2VO animals as compared with both the SHAM and the vehicle-treated 2VO groups (Fig. 12D,E).

At 13 weeks, DIAZ administered in DMSO completely abolished the increase in OX-42 signal (*$P$<0.05) (Fig. 13M), which was especially significant in the CA3 region (*$F$=4.097)(Fig. 13F). Similarly, DIAZ dissolved in DMSO reduced the microglia activation in the corpus callosum of the 2VO rats (Fig. 13F). In the optic tract, both DIAZ in DMSO and DMSO alone reduced the level of microglia activation in the 2VO rats to the SHAM control level (*$P$<0.05 and **$P$<0.01) (Fig. 13L).

COX inhibitor treatments were ineffective in combating microglial activation (Fig. 11E).

## DISCUSSION

### The effects of 2VO-induced chronic cerebral hypoperfusion on the observed parameters

*Changes in the later phase of cerebral hypoperfusion with relevance to AD*

The clinical symptoms of AD and other dementia consisted in the deficit of several domains of cognitive function. The early symptoms are built up from the following elements: difficulty in learning semantic and visuospatial information, an impaired retrieval memory, disturbance of executive function, and problems in attention and behavioral symptoms [125]. The neuropathological background of AD exhibits a progressive neurodegeneration involving the presence of senile plaques, neurofibrillary tangles, synaptic degeneration and neuronal loss in the cortex and the hippocampus [126]. Neuroinflammation is responsible for the progression of the neuropathological aberrations, but it has recently been suggested to account for the initiation of the disease [127]. Whether the inflammatory reaction is triggered by neurodegeneration or by cerebral hypoperfusion, which is also observed in the early stage of AD, has not been elucidated.

In Experiment 1, the observed cerebral hypoperfusion-related aberrations correspond to the cognitive and histopathological disturbances of AD in several domains, leading to the suspicion that the impaired cerebral perfusion might initiate progressive inflammation and neurodegeneration in coherence with memory failure. Eleven weeks of mild forebrain ischemia resulted in a significant learning impairment in the rats, as evidenced by the Morris watermaze paradigm. From 3 days to 2 weeks after 2VO, a mild reduction in neuron number, progressive dendritic degeneration and increasing incidence of damaged cell layers were present in the hippocampus, while 13 weeks following 2VO surgery, even macroscopic brain lesions could be detected with high frequency, which indicates the progressive nature of the

neurodegenerative process. Thirteen weeks of cerebral hypoperfusion also reduced the numbers of COX-2-positive CA1 pyramidal and DG granule cells. Since these cell types are known to produce COX-2 constitutively, an ischemia-related nonspecific loss of these neurons might contribute to the impaired synaptic plasticity and hence to the impaired memory of 2VO rats [82].

The GFAP immunocytochemistry in the investigated areas revealed region-specific differences as concerns the cerebral hypoperfusion. The astrocytic proliferation was significantly elevated in the optic tract and also enhanced in the corpus callosum, which is in concert with the observation of other investigators [50]. In contrast, the GFAP signal in the hippocampus was reduced by 13 weeks of hypoperfusion. Schmidt-Kastner et al. (2005) found no change in astrocyte proliferation at the level of the hippocampus until 6 months after 2VO [50]. This result might indicate that the severity of ischemia in the different brain regions determines the activity pattern of the astrocytes, since the greatest elevation in the GFAP signal was in the optic tract, which receives the lowest perfusion in the 2VO brain. Albeit, the persistent hypoperfusion in the hippocampus might caused progressive astrocytic degeneration and eventually cell death [53]. Interestingly, in the white matter and the frontal cortex of demented patients, the GFAP-labeled astrocytes also exhibited disintegration and regression, which was associated with ischemia [53].

The data obtained from OX-42 immunocytochemistry demonstrated a pronounced microglia activation in the hippocampal CA1, CA3 and DG regions, the corpus callosum and the optic tract, providing evidence for the existence of a continuous neuroinflammatory process. In concordance, a significantly elevated microglia activation was detected in the hippocampus and enthorinal cortex gray and WM in AD brains, as demonstrated by the human leukocyte antigen (HLA)-DR immunostaining of the microglia [128]. Our observations thus confirm that various neuropathological symptoms of AD and vascular dementia are closely connected to chronic cerebral hypoperfusion.

*Changes in the early phase of cerebral hypoperfusion with clinical relevance to acute disruption of cerebral perfusion*

In clinical cases, the appearance of memory disturbances is related to an acute disruption of the systemic circulation. These states can be predictable (planned cardiac surgery) or unpredictable (cardiac arrest, atrial fibrillation and myocardial infarction. Following coronary bypass surgery, half of the patients suffer a cognitive decline, which

persist even 6 months postoperatively in a quarter of the patients. The symptoms include primarily an impaired visuospatial orientation, but also the failures in attention and concentration [17]. In our model, 2 weeks of experimental cerebral hypoperfusion in rats likewise resulted in a reproducible visuospatial learning impairment, as demonstrated in Experiments 2 and 3. Under clinical conditions, the detection of consequential neuronal damage after surgery is restricted to neuroimaging techniques and to the measurement of neuronal (neuron-specific enolase) and glia-specific (protein S-100B) chemical markers from blood samples to estimate the progression of the cognitive decline [129,130].

Our histological observations between 3 days and 2 weeks following carotid-occlusion demonstrated mild and selective damage of the hippocampal neurons. Surprisingly, the DG outer molecular layer displayed the highest sensitivity to ischemia and to the treatments not the CA1 area, which was suggested to be the most ischemia-sensitive region in the hippocampus, since it is less safely supplied with an arterial inflow than the CA3 area or the DG [131].

2VO induced progressive dendritic degeneration in the hippocampus. The decrease in the MAP-2 signal already appeared on day 3 of 2VO, and 11 days later we found a significant loss of dendrites in the CA3 str. lucidum, similarly to our previous observation [34].

The GFAP and OX-42 labeling in the hippocampus remained unaltered following 2VO alone in the early period of cerebral hypoperfusion.

Thus, it can be concluded that the early period of the 2VO model resembles a similar cognitive disturbance to that experienced after cardiac surgery and is applicable to characterize the neurohistological changes in mild cerebral ischemia in a temporal manner. This model is therefore of considerable benefit in the testing of pharmacological preconditioning before cardiac surgery, with the aim of alleviating global cerebral ischemia-related cognitive failure and neuronal damage.

**The effects of DIAZ treatments**

*Effects of DIAZ post-treatment, and comparison of treatments at different periods of cerebral hypoperfusion*

The effects of the post-treatment with DIAZ were investigated after 2 and 13 weeks of cerebral hypoperfusion. At the 2-week follow-up, DIAZ given after 2VO onset did not prevent the spatial memory disturbance and the reduction in the hippocampal CA1

pyramidal cell number. In contrast, 13 weeks after 2VO, DIAZ administered in DMSO preserved the learning capacity and abolished the appearance of macroscopic brain lesions.

The immunocytochemical detection of astrocyte proliferation demonstrated no change in the hippocampus after 2 weeks of cerebral hypoperfusion; in turn, the GFAP signal was enhanced when DIAZ was administered after the induction of 2VO. In general, the astrocytes play an essential role in the excitability and homeostasis of the nervous tissue [132]. They mediate the synaptic plasticity and, as part of the neurovascular unit, provide optimal energy metabolism for the proper functioning of the neurons [133]. Data on the effects of DIAZ on the astrocytes are scarce. When DIAZ was applied to an astrocyte culture, it increased the glutamate uptake to 150% relative to the control, but 5-HD reversed this effect, indicating the contribution of mito$K_{ATP}$ in the process [134], These data imply a higher functional activity and proliferation of the astrocytes on DIAZ treatment. A different approach for the development of the astrocytic reaction is based upon a study which proved that, when exposed to ischemia, astrocytes express a nonspecific ATP-sensitive $Ca^{2+}$ channel, which contains the SUR-1 receptor subunit, the major target of DIAZ [135]. Opening of the channel postulates the swelling and migration of astrocytes into the hypoxic region [135]. In summary, we propose that DIAZ is able to augment the activity of astrocytes directly when they have previously been sensitized by ischemia.

At 13 weeks of cerebral hypoperfusion, DIAZ in DMSO reduced the GFAP signal in the corpus callosum of SHAM and 2VO rats, but not in the optic tract or in the hippocampus. This suggests a region-specific sensitivity of the astrocytes to DIAZ and ischemia.

Similarly to its action on astrocyte proliferation, DIAZ post-treatment increased the activation of the microglia in the 2VO rats 2 weeks after 2VO onset. During ischemia, the microglia act as a double-edged weapon. They provide protection by the phagocytosis of cellular debris, but may exert cytotoxicity through the excessive production of ROS and cytokines [136,137]. Microglia (cultured BV-2 murine microglia cells) express mito$K_{ATP}$ channels (Liu et al., 2006), which offers a direct target for DIAZ. DIAZ administered to toxin-activated microglia cultures decreased TNFα production and iNOS activity, which indicates that DIAZ reduced the activation of the microglia [138]. Since DIAZ enhanced the microglial activation in our study at the 2-week survival time, we propose that this was not a direct action of DIAZ on the microglia cells, but a reaction to a primary event, which cannot be identified with certainty.

In contrast, the microglia activation in the hippocampus and in the WM areas after 13 weeks of brain hypoperfusion was alleviated by DIAZ dissolved in DMSO. This suggests that

although DIAZ post-treatment in NaOH did not prevent neuron death, but enhanced microglia reaction at 2 weeks of survival, the DIAZ together with DMSO probably alleviated the microglia activation by preventing the progressive neuronal damage-related neuroinflammation.

The observations that DIAZ in NaOH solution was not neuroprotective when examined 2 weeks after 2VO onset, whereas in DMSO exerted beneficial effects at 13 weeks after 2VO surgery, strongly suggest that DMSO potentiated the action of DIAZ. This suggestion is especially relevant because DMSO alone achieved some memory improvement, as assessed in the Morris watermaze, prevented the loss of COX-2 positive neurons in the hippocampus and reduced the number of animals with macroscopic brain lesions. A number of beneficial effects of DMSO have been described which may be responsible for the additional neuroprotection. These activities include hydroxyl scavenging, anti-inflammatory and anti-edema effects, the impairment of platelet aggregation and adhesiveness, and the prevention of glutamate-induced neuronal cell death [139]. As the solvent DMSO also provided neuroprotective properties, we repeated this experiment exactly the same way as in Experiment 1, but with the use of the inorganic vehicle 0.1 N NaOH instead of DMSO to dissolve the DIAZ [140]. This experiment showed that DIAZ in this inorganic solvent (1) failed to prevent the learning impairment of 2VO rats in the Morris watermaze, and (2) did not modify the astrocytic proliferation in the hippocampus, but (3) reduced the microglia activation induced by 2VO in the CA1 and DG regions of the hippocampus [140]. The results of the two experiments together allow the conclusion that DMSO potentiated the effect of DIAZ in restoring the spatial memory, preventing the formation of brain lesions and reducing the astrocytic proliferation, while the mitigation of microglia activation seems to be a DIAZ-mediated effect.

*The effects of pretreatment with DIAZ, and the comparison of pretreatment with post-treatment*

Experiment 2 provided evidence, that repeated pretreatment with 0.5 mg/kg DIAZ affords neuroprotection in chronic cerebral hypoperfusion in rats by preventing the spatial learning disturbance and pyramidal cell loss in the CA1 area of the hippocampus, 2 weeks after 2VO. In contrast, post-treatment did not furnish neuroprotection, but enhanced the astrocyte proliferation and microglial activation. The most probable reason for the memory-preserving effect of DIAZ is its preconditioning effect, through which it prevented hippocampal neuron loss. In this case, DIAZ caused a transient increase in the level of ROS, which activated their scavenging enzymes in the brain.

A probable pathway of this action is the facilitating effect of DIAZ and its derivatives on DL-alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors. DIAZ has been shown to enhance LTP in the hippocampus both *in vitro* and *in vivo* by slowing the rate of AMPA-receptor deactivation of the neurons [141]. This possibility is questionable since the post-treatment was ineffective. However, if we consider that the DIAZ dissolved in NaOH did not necessarily reach the brain areas of learning in the 2VO animals in sufficient concentration to produce its effect, then DIAZ pretreatment at the optimal brain perfusion could act on the learning capacity via the AMPA receptors.

**The effects of COX inhibitors in chronic cerebral hypoperfusion**

*The comparison of nonselective and selective COX-2 inhibition in chronic cerebral hypoperfusion*

The major finding of Experiment 3 is that NS-398, but not indomethacin, abolished the learning disability induced by 2VO without corresponding, clear protection of the hippocampal neurons. Both indomethacin and NS-398 treatment increased the incidence of damage in the hippocampal pyramidal and granular cell layers, particularly in the 2VO animals, which was especially obvious 3 days after 2VO onset. The drugs did not affect the synaptic density, astrocytic proliferation and microglia activity.

The lack of a correlation between the learning scores and the neuronal injury in the hippocampus may stem from the variation in the regional cerebral representation of the spatial memory, and the ischemia-related electrophysiological properties of the morphologically intact neurons. The area most frequently associated with spatial learning is the CA1 region of the hippocampus. A number of studies have demonstrated a direct correlation between a cerebral hypoperfusion-induced memory deficit and CA1 damage [40,41,142]. On the other hand, others have found no or only a weak correlation between the diminishing CA1 neuron number and Morris watermaze performance [143-145]. In our Experiment 2, cresyl violet staining revealed only a mild reduction in CA1 neuronal cell number in the hippocampus 2 weeks after 2VO, which was accompanied by significantly impaired visuospatial learning. These findings suggest that areas other than the hippocampal CA1 must also be involved in spatial learning, *e.g.* the entorhinal cortex, the parahippocampal gyrus, and the rhinal and cingular gyri [146,147]. An interesting study by Mumby et al. (1996) demonstrated in rats that transient global cerebral ischemia-induced delayed non-matching to sample task deficits were due to extrahippocampal damage, and that prior hippocampal ablation protected against the effects

of ischemia by interrupting pathogenic processes that require the presence of the hippocampus [148]. Moreover, the memory deficit correlates with the white matter damage in 2VO rats [149,150]. In conclusion, a direct link cannot be established between ischemia-induced memory failure and a decreased neuron count in the hippocampus CA1.

Even though the hippocampal neurons are not lost in great numbers early after 2VO induction, ischemia can have an impact on the electrophysiological properties of these cells. In a model of global cerebral ischemia, Henrich-Noack et al. (2005) demonstrated that population spike generation in the DG granular cell layer was greatly decreased as early as 1 day postischemia, as compared with the pre-ischemic values and those in sham-operated animals. The functional impairment was detected despite an apparently intact morphology the of granular cells, as evidenced by Nissl staining [151]. Therefore, it is probable that at least a proportion of the surviving neurons in 2VO animals may be functionally impaired and may not exhibit proper LTP. This, in turn, may also limit spatial learning.

The protective potential of NS-398 on the memory capacity of 2VO rats suggests the involvement of COX-2 in the ischemia-induced damage in the brain regions responsible for spatial learning. However, the administration of NS-398 to *in vitro* CA1 pyramidal and DG granular cell cultures significantly reduced LTP and long-term depression (LTD) [152]. This finding suggests that the memory-preserving effect of the COX-2 inhibitor NS-398 is probably not related directly to the electrical disturbances of the surviving hippocampal cells. Accordingly, we can suggest that NS-398 limited the COX-2-related inflammatory process, but preserved the COX-1-related tissue perfusion in the learning areas of the brain.

The neuronal damage in the hippocampus showed different temporal and spatial distributions following the treatments, reflecting the different properties of the two drugs. Although the dose for indomethacin was chosen to be low enough to avoid general vasoactive side-effects (3 mg/kg), the detrimental effect of the drug appeared mainly in the ischemic 2VO rats close in time to the last injection of indomethacin on day 3, but was not present 9 days after the end of treatment. NS-398 damaged all of the investigated cell layers, especially the granule cells in the DG, in both the SHAM and 2VO rats at 3 days and 2 weeks after 2VO, respectively. These layers contain a large amount of constitutive COX-2-producing cells [78]. It is possible that COX-2 inhibition selectively damaged these COX-2-positive cells, which appeared as general neuronal damage with cell layer disruption on the cresyl violet-stained sections. If this is relevant, the basal COX-2 expression is therefore obligate for the viability of these neurons. The role of COX in ischemic neuronal damage has long been controversial. Some studies dispute the beneficial effects of selective COX-2 inhibitors due to their general

and cerebral side-effects. COX-2 activity is not uniformly deleterious, and treatments based on inhibition of the COX-2 pathway should focus on the mediators responsible for the deleterious effects of the enzyme, sparing those mediating the beneficial actions [153,154]. Although COX-2-derived $PGE_2$ has been found to mediate excitotoxic injury in ischemia via the prostanoid EP1 receptor [100], $PGE_2$ provides neuroprotection against excitotoxic injury, LPS-induced cytotoxicity or oxygen deprivation through the prostanoid EP2 receptor in hippocampal and cortical neuronal cultures [155-159]. Interestingly, the neuroprotective effects are generally observed at concentrations consistent with $PGE_2$ receptor activation (< l M), and lower than those required for toxic effects. $PGD_2$, another prostanoid, has been shown to prevent neuron loss in response to glutamate excitotoxicity *in vitro* [160]. Treatment with iloprost (a stable $PGI_2$ analog) after 2VO prevented hypoperfusion-induced lipid peroxidation [161]. COX-2 participates in memory formation. This results in another disadvantage of the drugs. Selective COX-2 inhibitors block the induction of LTP [162] and LDP [152]. Administration of the selective COX-2 inhibitor NS-398 to adult rats within 2 hours of behavioral training was also found to impair memory formation, though delaying the injections until 2 hours after the training had no effect on the memory [162,163].

Another aspect of neuronal damage in COX-2 inhibition is the elevated turnover of AA to eicosanoids by an enhanced lipoxygenase function. 5-Lipoxygenase-derived eicosanoids cause neuronal damage in the cortex during 14 days after 1 hour of transient MCAO [164]. The general disadvantage of the chronic use of COX-2 inhibitors, which led to the withdrawal of these drugs from the market in 2004, is that COX-2 inhibitors downregulate $PGI_2$ and upregulate thromboxane $A_2$, resulting in a cardiovascular hazard by producing a prothrombotic state in several organs [165]. Selective COX-2 inhibition can therefore induce microvascular thrombosis in the brain. One efficacious approach to restrict COX-mediated neuronal damage without considerable side-effects might be the selective targeting of prostanoid receptors which mediate cellular damage.

In conclusion, no relationship could be discerned between the advantageous effects of NS-398 on the memory capacity and the histological changes in the early period of cerebral hypoperfusion. To elucidate the mechanism of action of NS-398 on spatial learning, and to detect the differences between NS-398 and indomethacin treatment, further investigations are necessary. However, short-term treatment with a selective COX-2 inhibitor might provide memory protection in short and mild ischemic brain damage.

## CONCLUSION

First, our experiments have provided evidence that pretreatment, but not post-treatment with a safe concentration of DIAZ is able to alleviate the cerebral hypoperfusion-related memory deficit. However, further experiments are necessary to characterize the effects of DIAZ *in vivo* on the ischemic brain tissue. Moreover, our findings provide a possibility for physicians to apply DIAZ in conditions under which the occurrence of an ischemia-related memory disturbance is predictable, such as long-term cardiac surgery.

Second, targeting the COX-2-mediated pathway of neuroinflammatory reaction during cerebral hypoperfusion is efficacious in repelling a cognitive disturbance. Furthermore, our findings support the hypothesis that the hippocampal pyramidal and granular cells are not crucial for visuospatial memory. Finally, we suggest that preserving the constitutive COX-2 production in the brain should be considered when the COX-mediated pathway is targeted in the treatment of brain ischemia.

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Research report

# Diazoxide and dimethyl sulphoxide prevent cerebral hypoperfusion-related learning dysfunction and brain damage after carotid artery occlusion

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**Abstract**

Chronic cerebral hypoperfusion, a mild ischemic condition is associated with advancing age and severity of dementia; however, no unanimous therapy has been established to alleviate related neurological symptoms. We imposed a permanent, bilateral occlusion of the common carotid arteries of rats ($n = 18$) to create cerebral hypoperfusion. A mitochondrial ATP-sensitive $K^+$ channel opener diazoxide (DZ, 5 mg/kg) or its solvent dimethyl sulphoxide (DMSO) were administered i.p. (0.25 ml) on five consecutive days after surgery. Sham-operated animals ($n = 18$) served as control for the surgery, while nontreated rats were used as control for the treatments. Three months after the onset of cerebral hypoperfusion, the rats were tested in a hippocampus-related learning paradigm, the Morris water maze. Subsequently, the animals were sacrificed and neurons, astrocytes and microglia were labeled with immunocytochemistry in the dorsal hippocampus. DMSO and diazoxide dissolved in DMSO restored cerebral hypoperfusion-related learning dysfunction and prevented cyclooxygenase-2-positive neuron loss in the dentate gyrus. Cerebral hypoperfusion led to reduced astrocyte proliferation, which was not clearly affected by the treatment. Microglia activation was considerably enhanced by cerebral hypoperfusion, which was completely prevented by diazoxide dissolved in DMSO, but not by DMSO alone. We conclude that diazoxide can moderate ischemia-related neuroinflammation by suppressing microglial activation. Furthermore, we suggest that DMSO is a neuroprotective chemical in ischemic conditions, and it must be considerately used as a solvent for water-insoluble compounds in experimental animal models.


*Theme:* Disorders of the nervous system
*Topic:* Ischemia

*Keywords:* Cerebral hypoperfusion; Diazoxide; Dimethyl sulphoxide; Microglia; Spatial learning

## 1. Introduction

The incidence of chronic cerebral hypoperfusion increases with advancing age and dementia [15]; moreover, some researchers have found that decreasing cerebral blood flow (CBF) values correlate with an increasing degree of cognitive impairment in Alzheimer's disease patients [26,43]. In addition, reduced CBF has been recently suggested as an indicator for the progression of Alzheimer's disease [14,41]. Whether reduced CBF is one of the triggers or the consequence of neuronal dysfunction cannot be conclusively decided, but experimental evidence suggests that decreased CBF can lead to cognitive impairment and neuronal injury [15].

The use of bilateral, permanent occlusion of the common carotid arteries of rats (2VO) is a well-characterized model to investigate the cognitive and histopathologic consequences of chronic cerebral hypoperfusion [15]. The hippocampus has been shown to be particularly affected because hippocampus-related spatial memory, neuronal and microvascular integrity were predominantly compromised in the region [8,15,52]. Several studies based on this model were designed

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to test potentially beneficial strategies to delay the progression of dementia associated with reduced CBF. For example, pharmacological treatment with cholinesterase inhibitors improved 2VO-related memory dysfunction, CBF, and cerebral metabolism [36,49]. Administration of plant extracts gained from *Ginkgo biloba* or huperzine-A also attenuated 2VO-induced learning deficit [30,60]. Alternatively, dietary supplements such as polyunsaturated fatty acids and antioxidants could moderate 2VO-imposed learning impairment or increase G-protein-coupled receptor densities in the hippocampus [11,16]. In our present study, we also made use of the 2VO model of cerebral hypoperfusion to test the potentially neuroprotective effect of diazoxide (DZ), a putative, mitochondrial ATP-sensitive potassium channel (mitoK$_{ATP}$) opener.

Diazoxide has proved to be neuroprotective in several ischemia models. For instance, pretreatment with the agent could reduce infarct volume after middle cerebral artery occlusion in rats and mice [31,55]. Further in vivo experiments have provided evidence that diazoxide preserves NMDA-induced cortical arteriolar dilation after ischemia/reperfusion in piglets [12]. The mechanism behind these neuroprotective properties appears to be a selective opening of mitoK$_{ATP}$ because diazoxide effect can be abolished by the mitoK$_{ATP}$ blocker 5-hydroxydecanoate (5-HD) [24,32]. Hippocampal CA1 and CA3 interneurons are richly endowed by mitoK$_{ATP}$ that express region-specific distribution [18]. The activation of mitoK$_{ATP}$ in these cells under glucose deprivation proved to be neuroprotective as demonstrated in hippocampal slices [61]. Thus, the hippocampus appears to be a potential target and diazoxide a promising drug to achieve neuroprotection in chronic cerebral hypoperfusion.

Because diazoxide dissolves poorly in inorganic solvents, dimethyl sulphoxide (DMSO) was chosen in this study to prepare diazoxide solution. DMSO is widely used as a solvent for water-insoluble compounds in experimental animal research, yet DMSO itself possesses several vascular and neuroprotective properties [22,33,51]. For instance, DMSO can suppress platelet aggregation by antagonizing vasoactive substance release from platelets, or can scavenge neurotoxic free radicals [22,51]. Keeping these effects in mind, we also aimed to characterize and further explore the beneficial properties of DMSO in experimental cerebral hypoperfusion.

Our objective in the present study was to test the potentially neuroprotective effect of diazoxide in the 2VO model of cerebral hypoperfusion. Our prediction was that daily administration of the drug following carotid occlusion had beneficial effect on behavioral and neurological outcome. We were interested to investigate a late onset of complex changes caused by cerebral hypoperfusion.

In order to demonstrate drug effect in our experimental setup, we designed a Morris water maze paradigm frequently used as a standard test to asses hippocampus-related spatial memory function in 2VO [15]. Furthermore, we aimed to develop a comprehensive description of cerebral histopathologic changes by using neuronal, astroglial and microglial markers, of which the latest has not yet been employed in experimental, chronic cerebral hypoperfusion. Cyclooxygenase-2 (COX-2) labeling was chosen to identify hippocampal neurons involved in ischemic injury because this inducible form of the enzyme is markedly expressed in transient ischemia [25,37,42]. Glial fibrillary acidic protein (GFAP) immunocytochemistry was employed to label astrocytes that can react by degeneration to reduced CBF in the hippocampus [57]. Finally, OX-42 antibody known to recognize CR3 complement receptors (CD11b) on microglia was used to identify microglial activation, which is an early event in ischemia [1,57].

## 2. Materials and methods

### 2.1. Surgery and treatment

Fifty-one male Wistar rats (210 ± 10 g) were used for the study. All animal experiments were approved by the ethical committee of the University of Szeged. Experimental cerebral hypoperfusion was imposed [16] on half of the animals by permanent bilateral occlusion of the common carotid arteries (2VO), the other half served as sham-operated controls (SHAM). Prior to surgery, the animals were anesthetized by 400 mg/kg chloral-hydrate i.p., followed by 0.05 ml atropine i.m. The common carotid arteries were exposed via a ventral cervical incision, and separated from their sheaths and vagal nerves. Silk sutures were used for the ligation. The same procedure was performed on the SHAM group without the actual ligation. Survival rates for each group are presented in Table 1.

Both groups were divided into three subgroups ($n=6$) based on postsurgical treatment. The first set of animals of both SHAM and 2VO groups received 0.25 ml DMSO i.p., (SHAM–DMSO, 2VO–DMSO, respectively). The second set of animals was treated with 5 mg/kg diazoxide [7,31] given in 0.25 ml DMSO, i.p., (SHAM–DMSO + DZ, 2VO–DMSO + DZ). The animals were injected on five consecutive days. The first injection was applied directly after surgery. The last set of animals received no treatment after the operation and served as controls (SHAM–nontreated, 2VO–nontreated). The final composition of the experimental groups is presented in Table 1.

Table 1
Survival rate and the incidence of CNS lesions

| Experimental group | | Survival rate (%) | Unilateral hippocampal lesion (%) | Unilateral cortical lesion (%) |
|---|---|---|---|---|
| Nontreated | SHAM | 75 (6/8) | 0.00 (0/6) | 0.00 (0/6) |
| | 2VO | 66.6 (6/9) | 66.67 (4/6) | 33.34 (2/6) |
| DMSO | SHAM | 100 (6/6) | 0.00 (0/6) | 0.00 (0/6) |
| | 2VO | 50 (6/12) | 16.67 (1/6) | 16.67 (1/6) |
| Diazoxide | SHAM | 100 (8/8) | 0.00 (0/6) | 0.00 (0/6) |
| | 2VO | 87.5 (7/8) | 0.00 (0/6) | 0.00 (0/6) |

Abbreviations: bilateral carotid artery occlusion (2VO), dimethyl sulphoxide (DMSO), sham operation (SHAM).

### 2.2. Spatial learning test

Eleven weeks after surgery, the animals were trained in the Morris water maze [11]. The water maze consisted of a polyester circular pool (diameter: 160 cm, height: 35 cm) filled with water (22 °C), which was made opaque by milk so that the rats were unable to see an underwater platform. The hidden platform was submerged 2 cm below water surface. The water tank was located in an experimental room with various extra maze cues to enable the rats to learn the location of the platform. All rats performed two trials per day with a constant intertrial interval of 4 h, for five consecutive days. The animals were placed in the water at one of four starting quadrant points, which was varied randomly over the trials. The rats were given 2 min to find the platform and sit on it for 15 s. Rats that failed to find the location within the given time were guided to the platform and were allowed to stay on it for 15 s. Swimming paths were recorded by a computerized video imaging analysis system (EthoVision, Noldus Information Technology BV, Wageningen, The Netherlands). At each trial, escape latency, and swimming distance traveled before reaching the platform were analyzed.

### 2.3. Immunocytochemistry

Ten days after the Morris water maze experiments, the animals were anaesthetized with an overdose of pentobarbital, and perfused transcardially with 100 ml saline followed by 400 ml 3.5% paraformaldehyde and 0.5% picric acid in 0.1 M phosphate buffer (PB, pH 7.4). The brains were removed, one hemisphere was postfixed in the same solution for up to 1 h, and then stored in 0.1 M PB. Six brains per group were processed for immunocytochemical investigation.

Free floating coronal sections at the level of the hippocampus were cut at 20-μm thickness on a cryostat. COX-2 labeling was performed on the first set of sections as follows. First, endogenous peroxidase activity was blocked with 0.3% $H_2O_2$. Nonspecific binding sites were covered with 5% normal goat serum (NGS) and membrane permeability was enhanced by 0.3% Triton X-100. The sections were incubated for 2 days at room temperature (RT) in primary antibody solution containing rabbit anti-COX-2 antibody (Cayman), 1:2000, 1% NGS, 0.3% Triton X-100 and 0.1% sodium azide in 0.01 M PBS (pH 7.4). Next, the sections were rinsed and preincubated in 5% NGS for 1 h. Incubation was performed in goat anti-rabbit biotinylated IgG (Santa Cruz) for 4 h at RT. Finally, the signal was amplified by an ABC–Avidin Kit (1:400) (Vector) for 2 h at RT. Color reaction was developed by diaminobenzidine (DAB) and $H_2O_2$.

A second set of sections was immunocytochemically stained for glial fibrillary acidic protein (GFAP) to visualize astrocytic proliferation. Briefly, sections were treated with 0.3% $H_2O_2$ in PBS, and preincubated in 5% normal sheep serum (NShS). The samples were then incubated in a primary antibody solution containing mouse anti-GFAP antibody (Sigma), 1:200, 1% NShS, and 0.3% Triton X-100 in 0.01 M PBS, overnight at 37 °C. The secondary antibody solution consisted of sheep anti-mouse biotinylated IgG (Jackson), 1:200, and 0.3% Triton X-100 in 0.01 M PBS. Finally, the sections were incubated in HRP-Streptavidine (Zymed), 1:200, and color reaction was conventionally developed with DAB and $H_2O_2$.

To detect and analyze microglial activation over the hippocampal areas, OX-42 antibody was used on a third set of sections. The procedure started with washing and pretreating the sections with 0.5% Triton X-100 and 3% $H_2O_2$ in 0.01 M PBS, followed by preincubation in 20% normal swine serum (NSS) and 0.5% Triton X-100 in 0.01 M PBS for 1 h. The sections were incubated in a primary antibody solution containing biotinylated mouse anti-CD11b antibody (OX-42, Serotec), 1:500, 20% NSS, and 0.03% mertiolate in 0.01 M PBS, overnight at RT. Next, the sections were rinsed, and incubated in a solution of STA-PER (Jackson), 1% NSS, and 0.03% mertiolate in 0.1 M Tris-buffer for 1 h at RT. Finally, the color reaction was developed by Ni-DAB and $H_2O_2$. All sections were mounted on gelatin-coated microscopic slides, air-dried, dehydrated and coverslipped with DPX.

### 2.4. Analysis

Percentual surface area of GFAP-positive astrocytes was quantified in the dorsal hippocampus by using a Quantimet

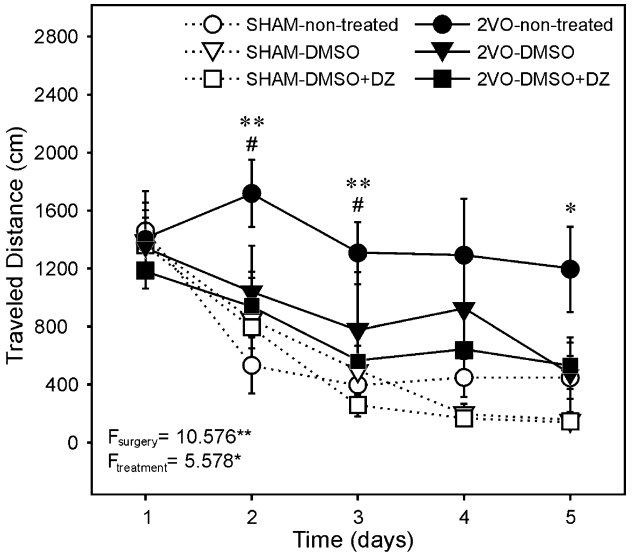


Fig. 1. Learning curves plotted of the Morris maze spatial orientation test. Statistic $F$ values are based on a two-way repeated measurement ANOVA model (*$p < 0.05$). Individual days were analyzed with ANOVA and LSD post hoc test (*$p < 0.05$). (*) indicates difference between SHAM–nontreated and 2VO–nontreated, (#) stands for the difference between 2VO–nontreated and 2VO–DMSO+DZ. Abbreviations: bilateral carotid artery occlusion (2VO), dimethyl sulphoxide (DMSO), diazoxide (DZ), sham operation (SHAM).

Q-600HR computerized image analysis system (Leica, Cambridge, UK) with a 469.4-nm emission filter [20]. Briefly, three consecutive coronal sections with a standard distance of 160 μm, starting at Br. − 3.60 mm [46] were selected for the analysis. Hippocampal regions of interest were manually delineated at 10 × magnification, after background subtraction and gray scale threshold determination. The area covered by GFAP-positive astrocytes was computed as percentage of the total area delineated. Measurements of the three sections per animal were averaged, and the average was used for further statistical analysis.

COX-2-labeled neuron counting was performed at 20 × magnification with the help of an ocular mesh with 1600-$\mu m^2$ holes. Three consecutive coronal sections with a standard distance of 160 μm, starting at Br. − 3.60 mm [46] were examined. COX-2-positive neurons were counted in the CA3 stratum (str.) pyramidale and the dentate gyrus (DG) inner and outer str. moleculare on an average surface of 0.018 $mm^2$ in each region. Regional cell counts of three

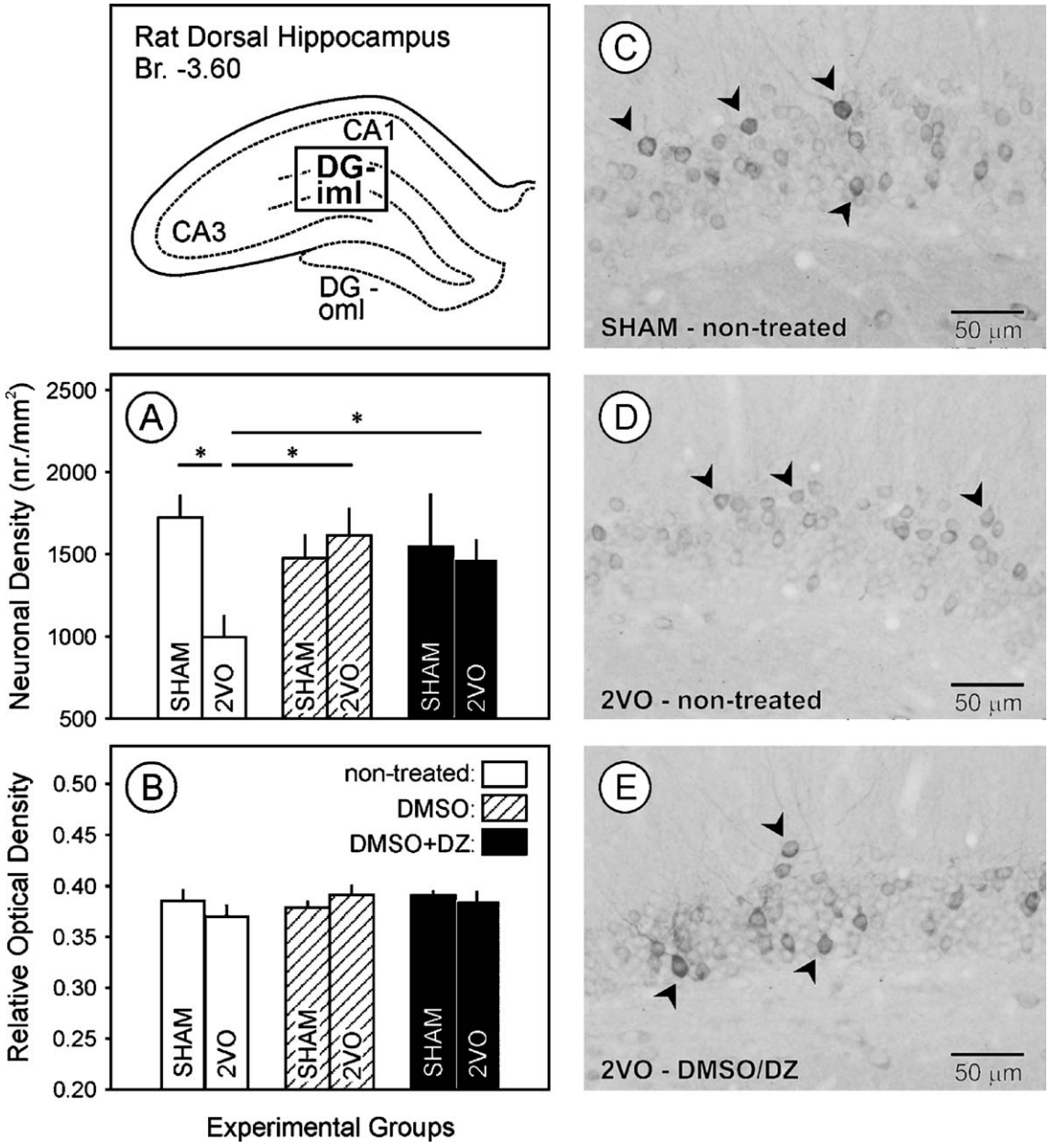


Fig. 2. Cyclooxygenase-2 enzyme (COX-2) immunocytochemistry in rat hippocampus. Panels A and B show neuronal density and COX-2 expression of preserved neurons, respectively. Significance values were obtained by Student *T*-test (*$p < 0.05$). Panels C, D and E demonstrate COX-2-positive neurons in the dentate gyrus inner granular cells (arrowheads are pointing at a few labeled neurons). Abbreviations: bilateral carotid artery occlusion (2VO), dimethyl sulphoxide (DMSO), diazoxide (DZ), sham operation (SHAM).

sections per animal were averaged, and the average was used for further statistical analysis.

The optical density of COX-2-positive neurons was measured by using a Quantimet Q-600HR computerized image analysis system (Leica). The same sections used for COX-2-labeled cell counting were analyzed again, at 10× magnification. Following background subtraction and gray scale threshold determination, COX-2-positive neurons were automatically delineated by the program, and relative optical density was computed based on a standard gray scale. As done previously, values of three sections per animal were averaged, and this value was used for further statistical analysis.

Quantification of the surface covered by OX-42 immunoreactive microglia was performed in a similar manner to GFAP-labeled sections on a computerized image analysis system (Olympus BX50, DP50; software: ImagePro Plus, Media Cybernetics).

The Morris maze test results were statistically analyzed by a two-way repeated measurement model followed by LSD post hoc test of the program SPSS. Individual day comparisons were performed by a univariate model and LSD post hoc analysis of SPSS. GFAP and OX-42 immunocytochemical results were statistically analyzed with two-way ANOVA followed by LSD post hoc test.

## 3. Results

Spatial learning curves obtained in the Morris maze test (Fig. 1) demonstrated that experimental cerebral hypoperfusion induced a marked decrease in learning performance (**$p < 0.002$). Moreover, hardly any day-to-day improvement was observed in the nontreated 2VO group over the 5-day training period. Daily comparison of groups revealed that the nontreated 2VO animals' memory capacity was significantly worse than their respective SHAM controls specifically on days 2, 3, and 5. Conversely, either DMSO or DMSO + DZ treatment improved learning of 2VO rats compared to the nontreated 2VO group, (*$p < 0.040$ and **$p < 0.007$, respectively). On the other hand, the agents did not affect the performance of SHAM animals. Furthermore, statistical analysis of the daily performance showed significant improvement only in the DMSO-DZ treated 2VO rats compared to the nontreated 2VO group on days 2, and 3.

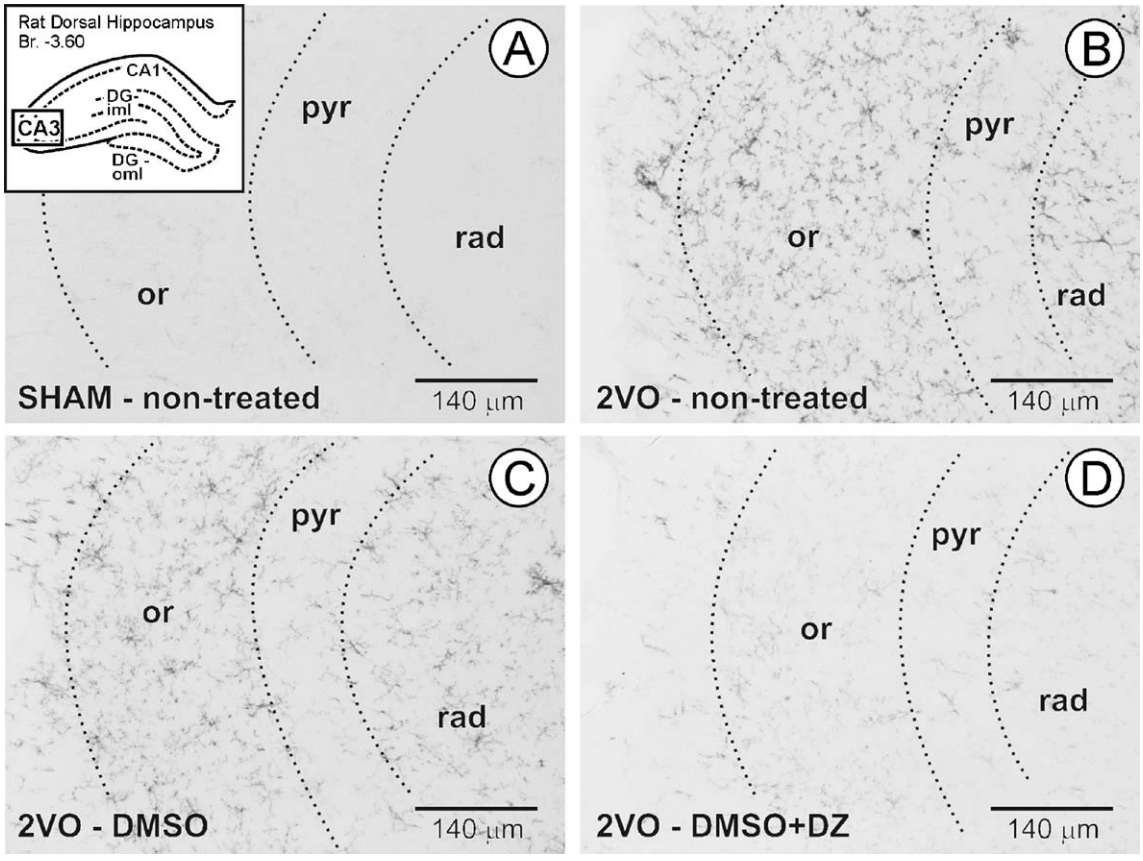


Fig. 3. Microglial activation in the rat hippocampus CA3 region labeled with OX-42 immunocytochemistry. A: image from a SHAM non-treated animal. B: image from a 2VO non-treated animal. C: image from a 2VO, DMSO-treated animal. D: image from a 2VO, DMSO+DZ-treated animal. Images were taken at 10× magnification. Abbreviations: 2VO: bilateral carotid artery occlusion, DMSO: dimethyl sulphoxide, DZ: diazoxide, or: str. oriens, pyr: str. pyramidale, rad: str. radiatum, SHAM: sham-operation.

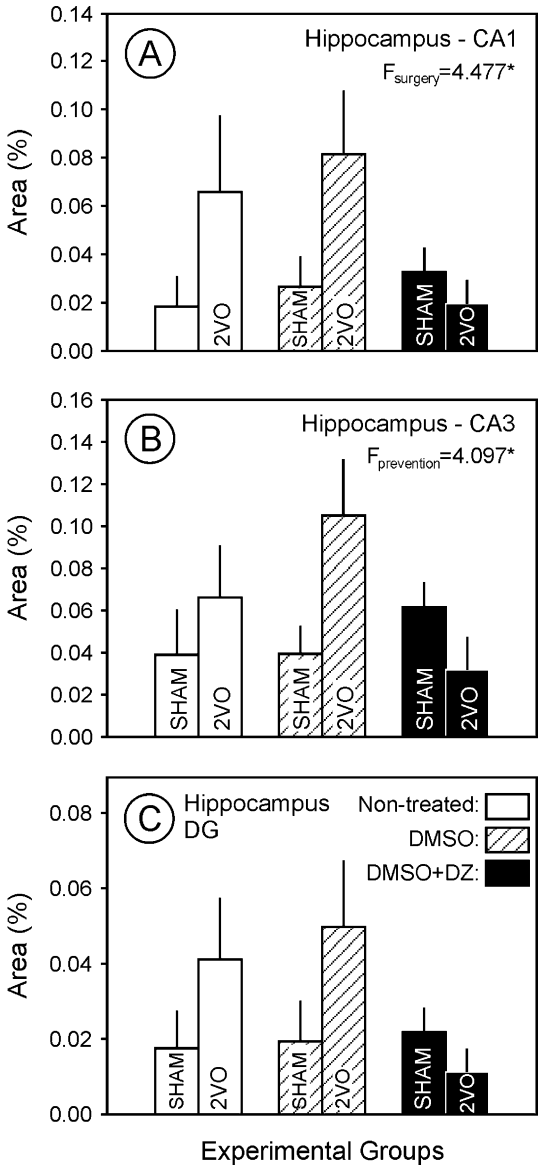


Fig. 4. Quantitative analysis of OX-42-positive microglial activation. A: hippocampus CA1 region. B: hippocampus CA3 region. C: hippocampus dentate gyrus. Statistic $F$ values are based on a two-way ANOVA model (*$p$<0.05). Abbreviations: 2VO: bilateral carotid artery occlusion, DMSO: dimethyl sulphoxide, DZ: diazoxide, SHAM: sham-operation.

Macroscopic evaluation of rat brains demonstrated that 2VO without postoperative treatment induced hippocampal and cortical lesions (in 66.67% and 33.34% of the animals, respectively). DMSO application reduced, while DMSO + DZ completely abolished the occurrence of the lesions (Table 1).

COX-2 immunocytochemistry in the dorsal hippocampus labeled neurons in the stratum pyramidale, the hilus and granular cells of the inner stratum moleculare of the dentate gyrus (DG-iml) (Fig. 2). The outer stratum moleculare of the DG contained only very few, scattered labeled neurons. Cell counting revealed loss of about 50% of COX-2-positive neurons in the nontreated 2VO group specifically in the DG-iml. Both DMSO and DMSO + DZ treatments prevented neuronal loss in the region (Fig. 2A). Optical density measurements showed that the surviving cells expressed an unaltered amount of COX-2 (Fig. 2B).

Glial fibrillary acidic protein (GFAP) immunoreactive astrocytes were present in all hippocampal regions and in all experimental groups. The area covered by astrocytes showed a similar tendency to COX-2-positive neuron counting in that astrocytic proliferation was reduced in 2VO compared to SHAM. However, no clear effect of the treatments could be established (data not shown).

OX-42 positive activated microglia were scarce in the hippocampus of SHAM animals (Fig. 3A), while a dense staining could be observed in all hippocampal areas in the nontreated and DMSO-treated 2VO groups (Fig. 3B and C). On the other hand, DMSO + DZ but not DMSO alone diminished OX-42-positive microglia in 2VO rats (Fig. 3D). Quantitative analysis demonstrated that 2VO increased microglia activation from two to three times in the CA1, CA3 and DG molecular layers in the nontreated and DMSO-treated 2VO animals. The increase was statistically significant in the CA1 region (*$F$ = 4.477). Application of DMSO + DZ but not DMSO alone reduced microglia activation to SHAM control level in all investigated regions, but the preventive effect of DMSO + DZ was statistically significant only in the CA3 area (*$F$ = 4.097) (Fig. 4).

## 4. Discussion

The novel findings of this study are the following. Chronic administration of DMSO following 2VO can moderate ischemia-related memory failure, and diazoxide in concert with DMSO can virtually prevent memory dysfunction. In a similar way, DMSO can confine, and diazoxide applied in DMSO can completely diminish macroscopic hippocampal and cortical lesions. Furthermore, DMSO alone is sufficient to prevent COX-2-positive neuron loss in the DG. Finally, diazoxide, but not DMSO alone can attenuate microglia activation in the hippocampus.

Cerebral hypoperfusion created by 2VO has been repeatedly described to cause spatial memory dysfunction [8,11,15,39,45], and apoptotic neuronal death in the hippocampus of rats [6,19,40]. Similarly, our Morris maze experiment reproduced earlier observations that chronic 2VO causes spatial memory impairment [8,15,39,45]. On the other hand, DMSO, and particularly DMSO + DZ restored learning skills to nearly SHAM level. To the best of our knowledge, the effects of diazoxide on learning and memory have not yet been tested. In addition, only one prior study attempted to identify the potentially beneficial effect of DMSO on spatial learning skills, but in that study DMSO was not administered alone but was combined with fructose 1,6-diphosphate [10].

In addition, our examination of rat brains following 3 months 2VO revealed macroscopic hippocampal and corti-

cal lesions in four out of six untreated animals. The lesions can very well arise from acute ischemic strokes as reported by early studies on the 2VO model, and also correlate with the regional distribution (hippocampus and neocortex), and ratio of affected animals (35–65%) in previous experiments [21,34,50]. Chronic treatment with DMSO reduced, while DMSO + DZ abolished, such ischemic lesions. Because DMSO can improve hemodynamic variables and CBF [23,28,59] in addition to its known free radical scavenger properties [2,51], an increased flow or reduced concentration of free radicals may underlie the preventive effect of the agent. Our observation also supports the data that DMSO could reduce infarct volume in focal cerebral ischemia [47,54]. Diazoxide was also found to be neuroprotective in ischemia–reperfusion brain injury [55]. Therefore, the cumulative effect of DMSO and diazoxide could be responsible for the complete prevention of lesions. Furthermore, such a protective action accomplished by the drugs corresponds with the treatment-related improvement in spatial learning skills demonstrated in our study.

The distribution and temporal aspects of COX-2 expression have been well characterized in the hippocampus after transient but not permanent forebrain ischemia [25,37,44]. The enzyme was suggested to promote neuronal death because selective COX-2 inhibitors could prevent ischemic injury in the hippocampus and other brain areas [13,37,42]. In our permanent cerebral hypoperfusion model, the pattern of COX-2-positive neurons in the hippocampal CA3 and DG appeared to be very similar to that seen with in situ hybridization 3–4 days postischemia, following 5–20 min global ischemia–reperfusion [25,37]. However, the labeling was also present in SHAM animals in our studies as well as other studies [37]. Thus, the data here strongly support the assumption that the acute onset of ischemia rather than chronic cerebral hypoperfusion or neuroinflammation is the condition that induce neuronal COX-2 expression. Furthermore, we detected a drop in COX-2-positive neuron density in the DG inner granular cells in untreated 2VO rats, which was not accompanied by altered COX-2 expression in surviving cells. Because COX-2 had been expected to be upregulated in ischemia, the lower number of labeled neurons compared to SHAM control might indicate neuronal death in the COX-2 producing cell population. Both DMSO and DMSO + DZ treatments prevented neuronal loss to an equal degree, which suggests that DMSO administration was sufficient to preserve neuronal integrity in the DG.

We found reduced astrocytic proliferation in 2VO rats, which corresponds with a previously described dynamics of astrocytic reaction in the 2VO model. In transient 2VO, no change was detected in astrocyte number up to 2 weeks in case of a 5-min occlusion, but a gradual degeneration and loss of GFAP-positive astrocytes were seen with the longer duration of the ischemic period or survival time [57]. Because our samples were obtained 3 months after the onset of permanent 2VO, the decline of GFAP signal could be due to a similar, progressive loss of astrocytes. Such degeneration of astrocytes can have serious functional consequences because an interaction between astrocytes, neurons and the cerebral microcirculation is essential to maintain neural energy metabolism and synaptic plasticity [3–5].

Finally, microglial activation was remarkably augmented by 2VO, which could be completely prevented only with DMSO + DZ, and not with DMSO alone. Microglia activation is an early response in the neuroinflammatory reaction to ischemia and was repeatedly detected with OX-42 immunocytochemistry in the hippocampus during the acute phase of ischemia [1,38,57]. However, there is no clear data on microglial reaction in chronic cerebral hypoperfusion models. Our results here show that microglial activation persists over a long period of time after the onset of chronic ischemia, which may suggest an ongoing neuroinflammatory process that accompanies chronic cerebral hypoperfusion.

Microglia may serve as part of regenerative processes by scavenging necrotic tissue, but can also promote delayed neuronal damage by generating cytotoxic agents, such as proinflammatory cytokines [27,56]. Consequently, the lower activation state of microglia due to DMSO + DZ in our study may be interpreted in two alternative ways. The treatment may have confined neuronal damage that would recruit microglia to clean up necrotic debris. On the other hand, microglia activation itself could have been directly inhibited, which would implicate delayed neuroprotection via the restricted production of cytotoxic compounds. We believe the latter is more probable because neuronal damage that recruits microglia is an early event in stroke, and our samples were obtained 3 months after the onset of ischemia.

Because DMSO + DZ limited microglia activation but DMSO alone did not, this effect can be attributed specifically to diazoxide. The beneficial action of diazoxide on neurons and astrocytes has been studied in detail [12,31,48], but no such data is available on microglia. Therefore, these findings may trigger further experiments to identify through which pathways diazoxide can alter microglial activation, and how exactly it may contribute to the outcome of ischemic insults.

Based on our data, we have no direct evidence as to the exact mechanism of DMSO and diazoxide that was responsible for the improvement of behavior and neuronal integrity. Yet, we speculate that the two agents achieved neuroprotection through different, but to some extent complementary pathways. DMSO has long been proposed to be protective against ischemia by antagonizing platelet aggregation and platelet-related vasoconstriction, or by neutralizing hydroxyl radicals [9,22]. Conversely, diazoxide has recently come to the focus for mimicking ischemic preconditioning by selectively opening mitochondrial $K_{ATP}$ channels [58]. Therefore, the target of DMSO could be the cerebral microcirculation, while diazoxide probably acted directly on neural compartments.

In spite of its known pharmacological actions, DMSO is frequently used as a solvent for diazoxide [17,29]. Based on

our results, and because DMSO itself is a potent neuroprotective chemical, an alternative method (e.g., NaOH and saline) is recommended to dissolve diazoxide in future studies designed for the investigation of neuroprotection through $K_{ATP}$ channel opening.

Finally, diazoxide in this study was used unconventionally as a posttraumatic agent. Diazoxide is usually applied as pretreatment before CNS insults because the drug is known to mimic the effects of ischemic preconditioning [12,31,35,53]. Our data that diazoxide (dissolved in DMSO) can be potentially neuroprotective given in a posttraumatic manner may open up new possibilities as to the therapeutic application of the drug.

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# Diazoxide and dimethyl sulphoxide alleviate experimental cerebral hypoperfusion-induced white matter injury in the rat brain

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**Abstract**

Aging and dementia are accompanied by cerebral white matter (WM) injury, which is considered to be of ischemic origin. A causal link between cerebral ischemia and WM damage has been demonstrated in rats; however, few attempts appear to have been made to test potential drugs for the alleviation of ischemia-related WM injury.

We induced cerebral hypoperfusion via permanent, bilateral occlusion of the common carotid arteries of rats. A mitochondrial ATP-sensitive potassium channel opener diazoxide (5 mg/kg) or its solvent dimethyl sulphoxide (DMSO) was administered i.p. (0.25 ml) on 5 consecutive days after surgery. Sham-operated animals served as control for surgery, and non-treated rats as controls for treatments. Thirteen weeks after surgery, the animals were sacrificed and astrocytes and microglia were labeled immunocytochemically in the internal capsule, the corpus callosum and the optic tract.

The astrocytic proliferation was enhanced by cerebral hypoperfusion in the optic tract, and reduced by diazoxide in DMSO, but not by DMSO alone in the corpus callosum. After carotid artery occlusion, microglial activation was enhanced two-fold in the corpus callosum and four-fold in the optic tract. DMSO decreased microglial activation in the optic tract, while diazoxide in DMSO, but not DMSO alone, restored microglial activation to the control level in the corpus callosum.

In summary, the rat optic tract appeared to be particularly vulnerable to ischemia, while the effect of diazoxide was restricted to the corpus callosum. We conclude that diazoxide dissolved in DMSO can moderate ischemia-related neuroinflammation by suppressing glial reaction in selective cerebral WM areas.


*Keywords:* Astrocyte; Cerebral hypoperfusion; Diazoxide; Dimethyl sulphoxide; Microglia; White matter

Cerebral white matter lesions have been associated with the progression of aging and cognitive impairment [2,4]. Further, these lesions have been suggested to originate from a variety of vascular causes ranging from hypertension to cerebral microinfarcts and ischemia [5,7]. To support the ischemic theory of white matter injury, experimental animal models have been employed, such as bilateral occlusion of the common carotid arteries of rats [8,17,18]. The findings of such studies have compellingly demonstrated that chronic cerebral hypoperfusion can initiate a wide array of neuropathological white matter changes. For instance, axonal degeneration, myelin and oligodendrocyte damage, astrogliosis and microglial activation have been identified in the optic tract and the corpus callosum of rats with occluded carotid arteries [8,17,18]. Our own results emphasized the specific involvement of the optic tract in ischemic white matter damage in the rat brain, and pointed to the marked proliferation of the astrocytes and to the activation of the microglia in the region [8].

A number of pharmacological compounds have been considered to alleviate neuronal damage after ischemic insults in the brain, but not too much is known about the pos-

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sible means of limiting ischemia-related white matter injury. A couple of studies have demonstrated that nimesulide, a cyclooxygenase-2 inhibitor, and ibudilast, a phosphodiesterase inhibitor succesfully limited white matter injury in the ischemic rat brain shortly after carotid artery occlusion [19,20].

We have recently demonstrated the neuroprotective effect of diazoxide and its solvent dimethyl sulphoxide (DMSO) in chronic experimental cerebral hypoperfusion [9]. Diazoxide is a putative, mitochondrial ATP-sensitive potassium channel opener [1] that has proved to be neuroprotective in several ischemia models. For example, pretreatment with diazoxide reduced infarct size after middle cerebral artery occlusion in experimental animals [12,16], and preserved the neuronal viability in cell cultures after glucose–oxygen deprivation [11]. Our previous study has predominantly demonstrated that diazoxide cannot only act on neurons but also prevents ischemia-induced, long-term microglial activation [9]. On the other hand, its solvent DMSO is itself also known to possess vascular and neuroprotective properties [9,14].

In the present study, we set out to evaluate the potential protective effects of diazoxide and DMSO on the glial compartments of the cerebral white matter in a rat model of chronic cerebral hypoperfusion.

The experiments were approved by the ethical committee of the University of Szeged, Hungary. Fifty-one male Wistar rats ($210 \pm 10$ g) were anesthetized with 400 mg/kg chloral hydrate i.p., followed by 0.05 ml atropine i.m. Experimental cerebral hypoperfusion was induced by permanent, bilateral occlusion of the common carotid arteries (2VO) in 26 animals [9]. The same surgical procedure was performed in the control group (SHAM), but without the actual ligation. The survival rate was 78.4%.

Both groups were divided into three subgroups ($n = 6$) on the basis of the postsurgical treatment, as described earlier [9]. The first set of animals was treated with 5 mg/kg diazoxide given in 0.25 ml DMSO, i.p. (SHAM-DMSO + DZ, 2VO-DMSO + DZ). The second set of animals of both SHAM and 2VO groups received 0.25 ml DMSO, i.p. (SHAM-DMSO, 2VO-DMSO, respectively). The last set of animals received no postoperative treatment and served as controls (SHAM-non-treated, 2VO-non-treated). The animals were injected on 5 consecutive days in the initial phase of cerebral hypoperfusion. The first injection was applied directly after surgery.

Thirteen weeks later, the animals were anesthetized with an overdose of pentobarbital, and perfused transcardially with 100 ml saline, followed by 400 ml 3.5% paraformaldehyde and 0.5% picric acid in 0.1 M phosphate buffer (PB, pH 7.4). The brains were removed, and one hemisphere was post-fixed in the fixative solution for up to 1 h.

Immunocytochemical staining was performed as described earlier [8]. Briefly, free-floating coronal sections at Bregma −3.14 mm were cut at 20 μm thickness on a cryostat microtome. Slices were stained immunocytochemically for glial fibrillary acidic protein (GFAP) to visualize astrocytic proliferation. The samples were incubated in a primary antibody solution containing mouse anti-GFAP antibody (Sigma), 1:200, 1% normal sheep serum, and 0.3% Triton X-100 in 0.01 M PBS. The secondary antibody solution consisted of sheep anti-mouse biotinylated IgG (Jackson), 1:200, and 0.3% Triton X-100 in 0.01 M PBS. Finally, the sections were incubated in HRP-Streptavidine (Zymed), 1:200, and the color reaction was conventionally developed with diaminobenzidine (DAB) and $H_2O_2$.

Microglial activation was visualized over the white matter areas with the cell surface marker CD11b (OX-42). The sections were incubated in a primary antibody solution containing biotinylated mouse anti-CD11b antibody (OX-42, Serotec), 1:500, 20% normal swine serum (NSS), and 0.03% merthiolate in 0.01 M PBS. Next, the sections were incubated in a solution of STA-PER (Jackson), 1% NSS, and 0.03% merthiolate in 0.1 M Tris buffer. Finally, the color reaction was developed by nickel-DAB and $H_2O_2$.

The percentage surface area of GFAP-positive astrocytes was quantified in the medial corpus callosum and the internal capsule; in the optic tract, relative optical density was computed instead of the percentage area, since the homogenous labeling did not permit area measurements. As regards the OX-42 labeling, the percentage surface area was measured for all three regions of interest (Olympus BX50, DP50, software: ImagePro Plus, Media Cybernetics). As in our previous protocol [8], three consecutive coronal sections were selected for analysis. Regions of interest were delineated manually at 10× magnification. The area covered by GFAP-or OX-42-positive glia was computed as a percentage of the total area delineated. The measured results on the three sections per animal were averaged and the average values were used for further statistical analysis. The data were analyzed by a two-way ANOVA model of the software SPSS.

The internal capsule was unaffected by either cerebral hypoperfusion or the pharmacological treatment.

In the medial corpus callosum, astrocyte proliferation was not enhanced by cerebral hypoperfusion, but the postsurgical treatment, particularly with diazoxide, reduced the area covered by GFAP-positive processes by 33% in both SHAM and 2VO groups (Fig. 1A–C). The microglial activation in the corpus callosum displayed a tendency to be elevated due to cerebral hypoperfusion; this was manifested as a 56% increase in the non-treated 2VO group compared with the non-treated SHAM group. The solvent DMSO did not change this tendency; however, diazoxide treatment restored the microglial activation in the 2VO animals to the control level (Fig. 1D–F).

The optic tract was the most clearly affected by cerebral hypoperfusion. The astrocytic proliferation was consistently increased by about 20% in the 2VO groups as compared with their respective SHAM controls, regardless of pharmacological treatment (Fig. 2A–C). Similarly, the

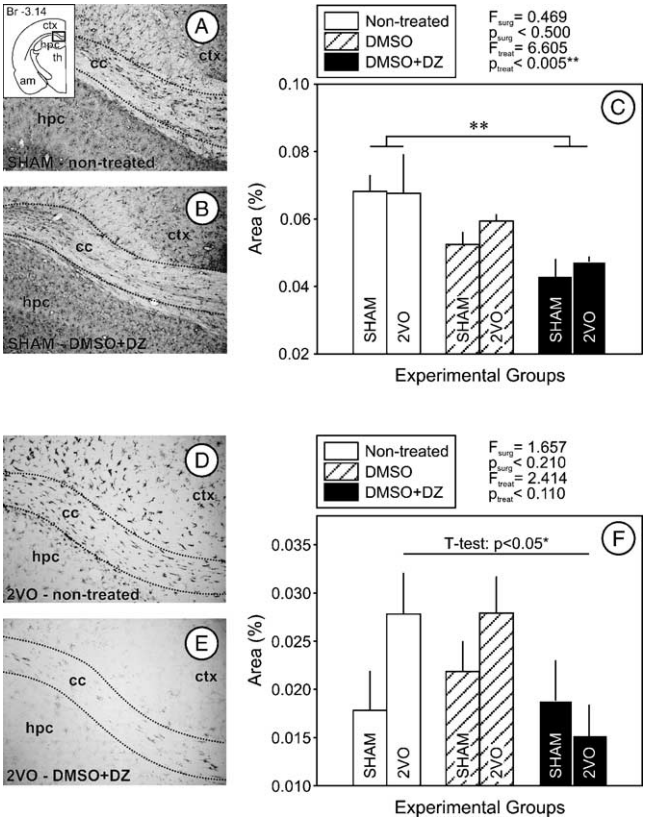


Fig. 1. Corpus callosum: photomicrographs and quantitative data on GFAP-positive astrocytic proliferation and OX-42-positive microglial activation. Panels A and B: Representative microscopic images of GFAP immunolabeling; original magnification: 10×. Panel C: Quantitative data on astrocytic proliferation; $^{**}P < 0.01$. Panels D and E: Representative microscopic images of OX-42 immunolabeling; original magnification: 10×. Panel F: Quantitative data on microglial activation. Abbreviations: 2VO: bilateral carotid artery occlusion, am: amygdala complex, cc: corpus callosum, ctx: cerebral cortex, DMSO: dimethyl sulphoxide, DZ: diazoxide, hpc: hippocampus, SHAM: sham-operated control, th: thalamus.

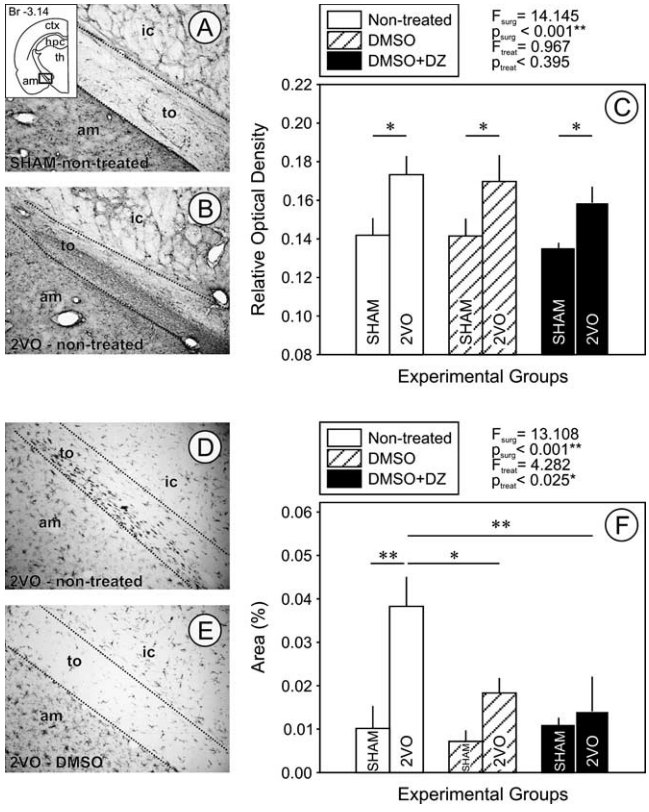


Fig. 2. Optic tract: photomicrographs and quantitative data on GFAP-positive astrocytic proliferation and OX-42-positive microglial activation. Panels A and B: Representative microscopic images of GFAP immunolabeling; original magnification: 10×. Panel C: Quantitative data on astrocytic proliferation; $^{*}P < 0.05$. Panels D and E: Representative microscopic images of OX-42 immunolabeling; original magnification: 10×. Panel F: Quantitative data on microglial activation; $^{*}P < 0.05$, $^{**}P < 0.01$. Abbreviations: 2VO: bilateral carotid artery occlusion, am: amygdala complex, ctx: cerebral cortex, DMSO: dimethyl sulphoxide, DZ: diazoxide, hpc: hippocampus, ic: internal capsule, SHAM: sham-operated control, th: thalamus.

microglial activation was markedly enhanced in the non-treated 2VO group as compared with the corresponding SHAM group, but both DMSO and diazoxide restored the microglial activation in the 2VO animals to the SHAM level (Fig. 2D–F).

Thus, both cerebral hypoperfusion and the pharmacological treatment elicited region-specific changes in astrocyte proliferation and microglial activation. The optic tract was predominantly vulnerable to cerebral hypoperfusion, while diazoxide dissolved in DMSO preferentially exerted an effect in the corpus callosum.

The findings reported here primarily confirm our previous observation that chronic cerebral hypoperfusion leads to astrocytic proliferation and microglial activation, specifically in the optic tract [8]. As noted earlier, the varying degree of blood supply to the different white matter areas in the rat brain may be responsible for the regional specificity, the optic tract receiving a direct branch of the internal carotid artery originating bellow the level of the circle of Willis [15], which makes flow compensation to the optic tract after common carotid artery occlusion improbable.

The pharmacological treatment applied here also appeared to exert region-specific effects. Diazoxide dissolved in DMSO reduced the astrocytic proliferation in the corpus callosum, but not in the optic tract, regardless of the degree of cerebral perfusion. Diazoxide in DMSO also decreased the ischemia-induced microglial activation specifically in the corpus callosum, but not in the optic tract. The pattern of microglial activation in the corpus callosum, with or without drug treatment, appeared to be very similar to that previously seen in the adjacent hippocampus [9].

There can be many reasons why diazoxide detectably attenuated the glial reaction only in the corpus callosum of the three white matter areas investigated. In the case of microglial activation, DMSO could possibly obscure the effect of diazoxide in the optic tract, but not in the corpus callosum, and it could be DMSO rather than diazoxide that differentially affected the various white matter areas. Conversely, the

different reactions of the astrocytes in the examined white matter regions to diazoxide treatment may stem from the region-specific sensitivity/composition of the mitochondrial and surface cation channels of the astrocytes, as detailed below.

The mechanism behind the action of diazoxide on the astrocytes is a matter of debate. When cultured astrocytes were incubated with diazoxide, loss of mitochondrial membrane potential, an elevated free radical production, and protein kinase C activation were observed. These results suggested that mitochondrial ATP-sensitive potassium channels served as the target for diazoxide in the astrocytes [13]. On the other hand, another recent investigation has revealed the possibility that ATP-sensitive non-selective cation channels on the surface of reactive astrocytes could be regulated by sulfonylurea receptor-1, which is the site of action of diazoxide [3]. These data suggest that diazoxide can target both mitochondrial and surface cation channels on the astrocytes, thereby modulating the astrocytic function, which could explain our present data.

Very little is known about the effect of diazoxide on the microglia. We published the first report that diazoxide dissolved in DMSO may suppress ischemia-related microglial activation in the rat hippocampus [9]. Our present data reveal a similar tendency in the corpus callosum. Although the resting/active state of the microglia is defined by the cell surface potassium, proton and chloride channels [6,10,21], there is no experimental evidence that demonstrates whether sulfonylurea receptors (the target of diazoxide) play a role in the regulation of these ion channels. It is not clear either how the binding of diazoxide to the mitochondrial ATP-sensitive potassium channels can alter the microglial activity, since no direct causal link has been established between mitochondrial function and microglial activity. The intracellular pathways of the effects of diazoxide on the microglial functions therefore remain to be clarified.

In summary, the present results indicate that pharmacological intervention can attenuate ischemia-related white matter injury. The diazoxide and DMSO treatment applied here was specifically effective in suppressing glial reactivity. Such attenuation of astrocytic proliferation and microglial activation tends to weaken the ischemia-induced neuroinflammatory responses, and prevent the progression of white matter lesions.

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# Neuroprotection by Diazoxide in Animal Models for Cerebrovascular Disorders

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**Abstract:** Diazoxide, a mitochondrial ATP-dependent $K^+$ channel opener has been investigated as a potential anti-ischemic agent in the brain. The neuroprotective effect of diazoxide has emerged from *in vitro* experiments employing brain slices and neuronal cell cultures. The intracellular mechanisms that are responsible for the neuroprotective properties of diazoxide have also been identified in cell cultures and isolated mitochondria. Thus, diazoxide has been shown to depolarize mitochondria, preserve mitochondrial matrix volume, block cytochrome C release and Bax translocation, activate protein kinase C and facilitate the production of reactive oxygen species, which lead to enhanced cell viability and preserve electrophysiological properties of neurons after oxygen and glucose deprivation.

Recently, diazoxide has been administered to experimental animals to examine the drug's effect on the ischemic brain. Various animal models of ischemic cerebrovascular disorders such as ischemia/reperfusion injury, stroke and chronic cerebral hypoperfusion were used to evaluate the neuroprotective properties of diazoxide *in vivo*. The results of numerous studies demonstrate that diazoxide limits infarct size after middle cerebral artery or unilateral carotid artery occlusion in rodents, preserves cerebrovascular function in newborn piglets, and reduces the activation of microglia after permanent, bilateral common carotid artery occlusion in rats.

This review provides a comprehensive summary of the experimental data on the neuroprotective effects of diazoxide in animal models. This overview may facilitate drug development in this field.

**Keywords:** Brain, cerebral hypoperfusion, diazoxide, ischemia, $K^+$-channel, mitochondria, stroke.

## INTRODUCTION

Cerebrovascular disorders represent a major group of neurological diseases, which considerably impair the quality of life and pose a substantial burden to society and to the health care system. Ischemia/reperfusion injury, stroke, vascular dementia and chronic cerebral hypoperfusion are among the most common cerebrovascular disorders, which are often accompanied by the degeneration of the nervous tissue, resulting in the loss of executive function and cognitive deficits. Although intensive research has focused on the development of drugs to prevent the progression of cerebrovascular diseases and to alleviate the associated symptoms, the medications at our disposal are often not potent enough to limit neuronal damage successfully and to restore health.

Medical treatments for chronic cerebral hypoperfusion have targeted the vasoregulatory circuits in the brain (cholinergic transmission, nitric oxide-cGMP mediated vasodilation) and hemorheological parameters (blood viscosity, platelet aggregation) in order to improve cerebral blood flow [1]. In the management of stroke, L-type calcium channel antagonists, for example nimodipine emerged as promising chemicals to prevent intracellular calcium overload and consequent neuronal death [2,3], but the results of the clinical trials were inconclusive [4]. Recently, mitochondria have received increasing attention as a potential target of therapy that aims to preserve neural integrity and function after an ischemic insult or under chronic ischemic conditions.

Mitochondria play a pivotal role in the energy metabolism (ATP production) and in the regulation of cellular $Ca^{2+}$ homeostasis in the central nervous system [5]. Under cerebral ischemic conditions, mitochondrial dysfunction is considered a central event in neuronal and astrocytic injury [6, 7, 8]. Glucose and oxygen deprivation of the nervous tissue leads to the disruption of the mitochondrial respiratory chain, the rapid depletion of ATP, the elevated concentration of $Ca^{2+}$ in the mitochondrial matrix, the release of cytochrome C from the mitochondria and the generation of reactive oxygen species [8,9]. These mitochondria-related pathogenic processes initiate necrotic or apoptotic cell death in the ischemic brain [10]. Therefore mitochondria emerge as a potential target for the prevention of ischemic neural injury.

In order to protect mitochondria in cerebral ischemia, various approaches have been considered. For example, blocking the mitochondrial permeability transition pore can inhibit apoptotic pathways. The use of specific drugs such as cyclosporin-A (and its analogs) and rasagiline (N-propargyl-

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1R-aminoindan) exploits this possibility [11, 12]. Another target for mitochondrial protection is the activation of ion channels, such as the mitochondrial, ATP-sensitive potassium channels (mitoK$_{ATP}$ channels). We present an overview of the efforts to protect mitochondrial function in cerebral ischemia by activation of the mitoK$_{ATP}$ channels.

## MITOCHONDRIAL ATP-SENSITIVE POTASSIUM CHANNELS AND THEIR ROLE IN ISCHEMIA

MitoK$_{ATP}$ channels are located in the inner mitochondrial membrane. Although the exact molecular composition of mitoK$_{ATP}$ channels has not been definitively determined, mitoK$_{ATP}$ channels in the brain are presently known to constitute four pore-forming subunits (probably the inwardly rectifying potassium channel subunits K$_{IR}$6.1 and K$_{IR}$6.2), and four regulatory subunits (members of the sulphonylurea receptor family, supposedly the SUR2 type) (Fig. **1**) [8, 13, 14, 15].

In general, the primary, endogenous regulator of K$_{ATP}$ channels on the cell surface appears to be the ratio of ATP/ADP, which can stabilize the closed state of the channel if the ATP concentration is high, or to enable the opening of the channel if ADP concentration increases [16,17]. However, the opening of mitoK$_{ATP}$ channels may be subjected to other or additional regulatory mechanisms. Under physiological circumstances, the mitoK$_{ATP}$ channels are closed due to the high concentration of ATP, which also requires the presence of $Mg^{2+}$. In addition to ATP and ADP, long-chain acyl-CoA esters have been demonstrated to inhibit mitoK$_{ATP}$ channels [18,19]. In turn, the inhibited mitoK$_{ATP}$ channels can be opened by other nucleotide phosphates, such as GTP and GDP [19]. Finally, protein kinase C and G have also been suggested as regulators or modulators of mitoK$_{ATP}$ channels [20, 21, 22].

During ischemia, potassium influx through the mitoK$_{ATP}$ channels primarily prevents contraction of the matrix and preserves the intermembrane space, which is essential for the proper function of the respiratory chain (Fig. **2**) [23]. However, opening of mitoK$_{ATP}$ channels under physiological conditions leads to mitochondrial matrix alkalization and the generation of reactive oxygen species. Therefore, mitoK$_{ATP}$ channels play a significant role in maintaining mitochondrial function under ischemic conditions and, consequently, protect cells against ischemic injury. These properties serve as the basis for ischemic preconditioning that can be achieved by the activation of the mitoK$_{ATP}$ channels [8, 24].

Ischemic preconditioning by mitoK$_{ATP}$ channel activation has been widely investigated as a cardioprotective strategy [23, 25, 26]. Interestingly, mitochondria extracted from the brain have been found to contain six to seven times more mitoK$_{ATP}$ channels than cardiomyocytes [21]. This finding indicates that the mitoK$_{ATP}$ channels may have an accentuated functional significance in the brain, and may serve as an appropriate target of neuroprotective strategies in cerebrovascular disorders.

## DIAZOXIDE, A MITOCHONDRIAL ATP-SENSITIVE POTASSIUM CHANNEL OPENER

At present, several exogenous ligands have been identified that activate K$_{ATP}$ channels. A group of these agents is non-selective, and can open both surface K$_{ATP}$ channels and mitoK$_{ATP}$ channels. The most prominent, vasorelaxant members of this group are cromakalim and its analogs [27], nicorandil, which also has a nitrate-like effect [28] and iptaka-

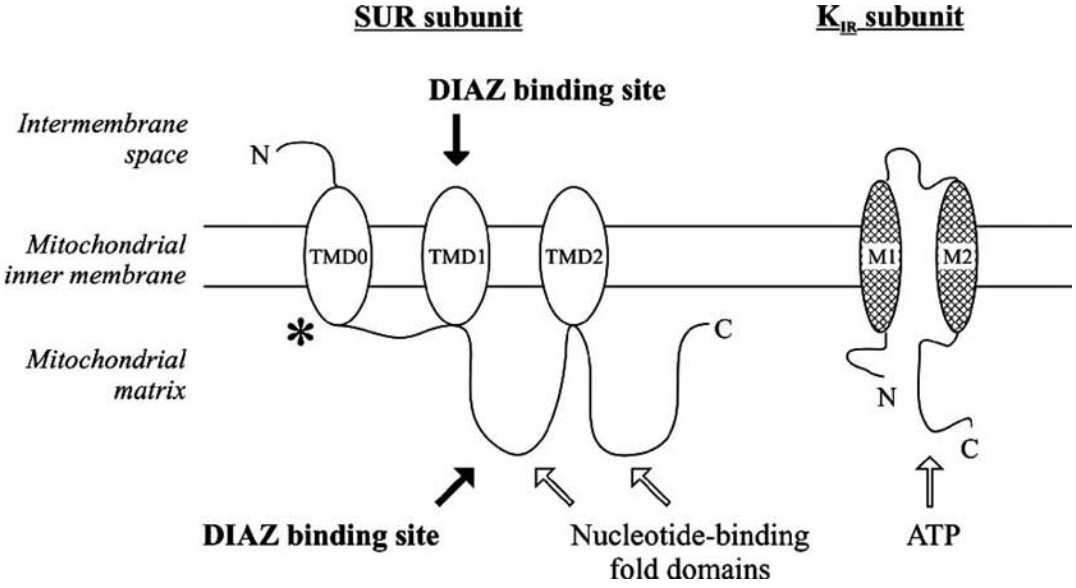


**Fig. (1).** The hypothetical, structural organization of the mitoK$_{ATP}$ channels in the inner mitochondrial membrane, and the putative binding sites of diazoxide. One regulatory subunit is presented to the left, while a pore-forming subunit is indicated to the right. Four regulatory and four pore-forming subunits constitute a functional, heteromeric, octameric complex [14,16]. Indicated by an asterisk in the schematic image, succinate dehydrogenase has recently been suggested as a possible component of mitoK$_{ATP}$ channels [24]. Abbreviations: C: C-terminus, DIAZ: diazoxide, K$_{IR}$: inwardly rectifying pore-forming channel subunit, M1 & M2: transmembrane helices, N: N-terminus, SUR: sulphonylurea regulatory subunit of the ABC family, TMD: transmembrane domain.

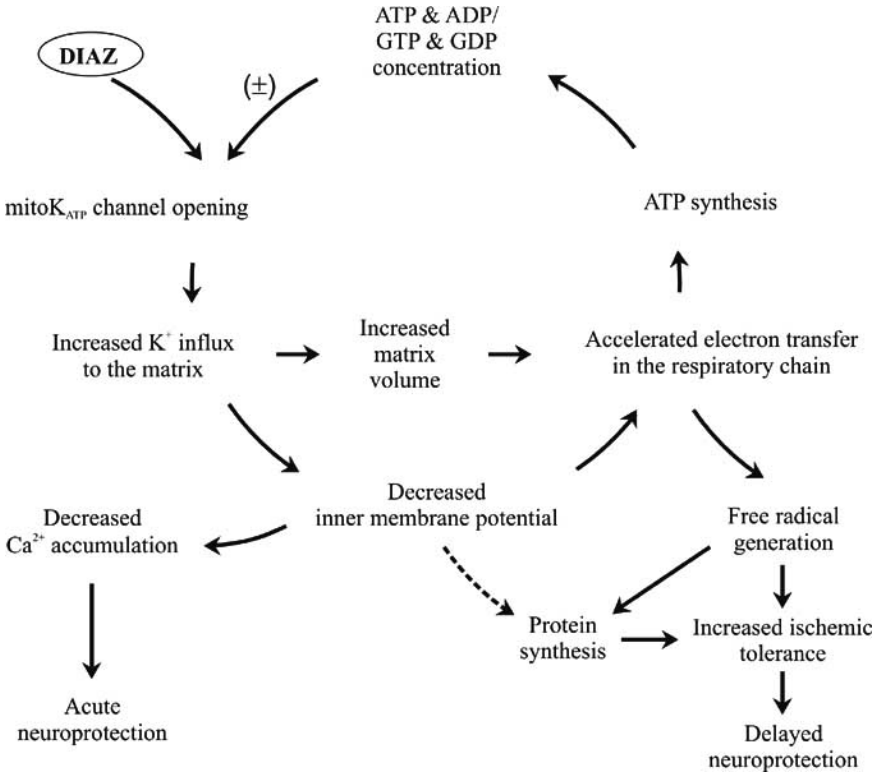


**Fig. (2).** The theoretical regulation and the consequences of the opening of the mitoK $_{ATP}$ channels; pathways leading to ischemic preconditioning. Abbreviations: DIAZ: diazoxide, mitoK$_{ATP}$ channel: mitochondrial, ATP-sensitive potassium channel.

lim, which preferably acts on small arteries and produces neuroprotection in ischemic stroke [29,30]. Currently, the most widely-accepted K $_{ATP}$ channel opener regarded to be specific for the mitoK $_{ATP}$ channels is the benzothiadiazine diazoxide (though a novel drug designated as BMS-191095 appears at least as potent as diazoxide [31, 32]).

As a consequence of their different subunit composition, diazoxide applied at a low concentration has been reported to have a greater affinity for the mitoK $_{ATP}$ channels than to the cell surface K$_{ATP}$ channels, [8, 16]. Diazoxide opens the mitoK$_{ATP}$ channels probably by binding to the SUR regulatory subunit of the channel (Fig. **1**), which is facilitated by ADP [16]. The action of diazoxide can be abolished by the putative mitoK$_{ATP}$ channel inhibitor 5-hydroxydecanoate (5-HD) [33]. This pharmacological property is considered as evidence that diazoxide is a specific mitoK $_{ATP}$ channel opener. However, some other studies have challenged the view that diazoxide acts exclusively on mitoK $_{ATP}$ channels (e.g. diazoxide opens surface K$_{ATP}$ channels at sufficiently high concentration, and can inhibit succinate dehydrogenase) [34, 35, 36, 37], which complicates the interpretation of the physiological responses obtained with the compound.

Diazoxide was first used in clinical practice in hypertensive emergencies [38, 39]. At high concentrations, the drug acts on cell surface K$_{ATP}$ channels of vascular smooth muscle cells and causes vasodilation by reducing the intracellular $Ca^{2+}$ concentration [40]. Another important field of application of diazoxide has been the treatment of hyperinsulinemia. Diazoxide has been shown to inhibit insulin release from pancreatic beta cells and therefore to exert a beneficial effect as an anti-hypoglycemic agent [41, 42, 43]. In addition, rapidly progressing research has been conducted on the role of diazoxide in cardiac ischemic preconditioning [33, 37]. These studies demonstrated that diazoxide protects cardiac muscle in ischemia by acting on the mitoK $_{ATP}$ channels and consequently, preserves mitochondrial function and cell integrity [25, 26, 44]. For its well-characterized, preventive action in cardiac ischemia, diazoxide has become the focus of recent investigations as a potential anti-ischemic agent in the brain.

## THE NEUROPROTECTIVE EFFECTS OF DIAZOXIDE DEMONSTRATED *IN VITRO*

The use of neuronal and astrocytic cell cultures and brain slices provided evidence that diazoxide can effectively protect nervous tissue against ischemic insults. In cell cultures, unfavorable conditions that cause cell death were created by either glutamate exposure or oxygen-glucose deprivation. Diazoxide preserved the viability of cells in both of these models of ischemic cell injury [36, 45, 46, 47]. Another approach examined the effect of diazoxide on oxidative neuron injury, a pathological component of cerebral ischemia. In this study, diazoxide successfully increased hippocampal neuronal survival in a toxic oxidative environment created by exposure to $FeSO_4$ and β-amyloid peptide [48].

Similarly, in hippocampal and cortical slices exposed to hypoxic/ischemic conditions, diazoxide prevented cell damage. A major advantage of experimenting with brain slices is that the neural circuits are partially intact, which enables electrophysiological recordings. In such an experimental setup, diazoxide prolonged the latency of ischemic depolarization and depressed hypoxia-induced depolarization [49,

50, 51, 52]. Furthermore, diazoxide blocked the anoxic depolarization in rat CA3 hippocampal neurons exposed to anoxic episodes [53].

The neuroprotective effect of diazoxide has been associated with the state of the mitochondria and certain intracellular signaling pathways. For example, diazoxide depolarized mitochondria, abolished mitochondrial swelling, and prevented $Ca^{2+}$ accumulation in the mitochondrial matrix in neurons under ischemic conditions, which maintain the proper function of the respiratory chain (Fig. **2**) [54, 55]. Furthermore, diazoxide inhibited cytochrome C release from the mitochondria and increased the production of reactive oxygen species in neuronal cell cultures [46, 54]. Others observed the activation of protein kinase C due to treatment of cultured neurons with diazoxide [36]. Diazoxide has also been shown to induce anti-apoptotic signaling such as the association of Bcl-2 protein with mitochondria and the prevention of Bax translocation [54]. Finally, the inhibition of succinate dehydrogenase, a mito$K_{ATP}$ channel-independent action of diazoxide was identified in cultured neurons [36].

A few of these intracellular effects of diazoxide, for example the generation of reactive oxygen species may seem controversial, since reactive oxygen species are known to attack phospholipid membranes. Yet, in healthy cells, a moderately enhanced production of free radicals can play an important role in ischemic preconditioning by possibly increasing the levels or the capacity of scavenging enzymes. Therefore a transient or moderate elevation of the production of reactive oxygen species by diazoxide is considered as pharmacological preconditioning [8].

In summary, the experimental evidence obtained *in vitro* support the concept of the neuroprotective capacity of diazoxide, and encourage the testing of diazoxide in *in vivo* models of cerebral ischemia.

**Table 1. Experimental Models for Cerebrovascular Disorders to Assess the Neuroprotective Effect of Diazoxide**

| Cerebrovascular Disorder | Animal Model | Species | Age | Reference |
|---|---|---|---|---|
| Chronic, global cerebral hypoperfusion | Permanent, bilateral common carotid artery occlusion | Rat | Adult | Farkas *et al. Brain Res.,* 2004 [63] |
| | | Rat | Adult | Farkas *et al. Neurosci. Lett.,* 2005 [64] |
| | | Rat | Adult | Farkas *et al. Neurosci. Lett.,* 2005 [65] |
| Transient, global ischemia/reprefusion | Transient, bilateral common carotid artery occlusion | Mouse | Adult | Munoz *et al. Stroke,* 2003 [62] |
| | Increased intracranial pressure | Piglet | Newborn | Domoki *et al. Stroke,* 1999 [56] |
| | | Piglet | Newborn | Domoki *et al. Brain Res.,* 2004 [57] |
| | | Piglet | Newborn | Domoki *et al. Am. J. Physiol. Heart Circ. Physiol.,* 2005 [58] |
| Transient forebrain ischemia/reperfusion | Transient, unilateral middle cerebral artery occlusion | Mouse | Adult | Liu *et al. J. Cereb. Blood Flow Metab.,* 2002 [54] |
| | | Rat | Adult | Shimizu *et al. Am. J. Physiol. Heart Circ. Physiol.,* 2002 [61] |
| Transient, global ischemia/reperfusion + arterial hypotension | Transient, bilateral common carotid artery occlusion + blood withdrawal through the femoral vein | Rat | Adult | Lenzsér *et al. Brain Res.,* 2005 [67] |
| Hypoxic-ischemic brain injury | Permanent, unilateral common carotid artery occlusion + hypoxia | Rat | 7 days old | Rajapakse *et al. Neurosci. Lett.,* 2002 [55] |
| | | Rat | 7 days old | Jiang *et al. Mol. Brain Res.,* 2005 [59] |
| Hypoxic brain injury | Hypothermic circulatory arrest | Dog | Adult | Shake *et al. Ann. Thorac. Surg.,* 2001 [60] |
| Venous ischemia | Cerebrocortical vein occlusion + KCl-induced spreading depression | Rat | Adult | Nakagawa *et al. Neurosurg.,* 2005 [71] |
| Transient spinal cord ischemia | Transient aorta occlusion near the renal artery takeoff | Rabbit | Adult | Caparrelli *et al. Ann. Thorac. Surg.,* 2002 [84] |

## THE NEUROPROTECTIVE EFFECTS OF DIAZOXIDE IN EXPERIMENTAL ANIMAL MODELS

So far, 16 reports have presented data on the neuroprotective capacity of diazoxide in animal models of cerebrovascular disorders. The comparison and summary of these studies requires careful inspection, because the experiments were performed with different species, at different ages of the animals, and with different routes of drug administration (Tables **1** and **2**). Furthermore, several cerebrovascular disorder models were used, and various end points were examined (Tables **1** and **3**). Still, the demonstration of the neuroprotective property of diazoxide is common in these studies, and the diversity of models strengthens the conclusion that diazoxide limits the neurodegenerative processes of cerebrovascular origin.

The studies follow two lines of investigation based on the clinical condition they reproduce. In newborn or neonatal animals, diazoxide was administered with the intent to prevent the neural consequences of perinatal ischemia/hypoxia [55, 56, 57, 58, 59]. In other investigations, the neuroprotective actions of diazoxide were examined in adult animals with age-associated cerebral ischemic conditions, such as ischemic stroke or chronic cerebral hypoperfusion [54,60,61,62,63,64,65,66,67].

### Perinatal Hypoxia/Ischemia

Perinatal hypoxia/asphyxia occurs frequently during complicated deliveries, and can directly lead to ischemic brain damage. However, the subsequent impairment of cerebrovascular function (e.g. impaired regulation of cerebral blood flow) can indirectly contribute to a poor neurological outcome, as well [68]. The neuroprotective strategies involving diazoxide focused on two aspects of perinatal ischemic brain injury. One group of studies examined cerebrovascular reactivity (the regulation of cerebral blood flow), while others aimed to investigate the nervous tissue itself.

**Table 2. The Various Applications of Diazoxide in Experimental Animal Models**

| Pre- or Post-Treatment | Way of Administration | Concentration | Solvent | Reference |
|---|---|---|---|---|
| Pre | i.p., bolus | 1.9 or 3.8 mg/kg, 100 µl | buffered saline | Rajapakse *et al. Neurosci. Lett.,* 2002 [55] |
| | i.p., repeated | 6, 20 or 40 mg/kg | n.d. | Lenzsér *et al. Brain Res.,* 2005 [67] |
| | i.v., bolus | 5 mg/kg | saline | Caparrelli *et al. Ann. Thorac. Surg.,* 2002 [84] |
| | | 5 mg/kg | n.d. | Liu *et al. J. Cereb. Blood Flow Metab.,* 2002 [54] |
| | | 3 mg/kg | saline | Domoki *et al. Brain Res.,* 2004 [57] |
| | | 3 mg/kg | saline | Domoki *et al. Am. J. Physiol. Heart Circ. Physiol.,* 2005 [58] |
| | i.c.v., bolus | 0.4 or 2.0 mM; 30µl | n.d. | Shimizu *et al. Am. J. Physiol. Heart Circ. Physiol.,* 2002 [61] |
| | | 0.1 mM/l; 4 µl | aCSF | Munoz *et al. Stroke,* 2003 [62] |
| | | 2 mmol/l, 15 µl | 0.1 mol/l NaOH | Nakagawa *et al. Neurosurg.,* 2005 [71] |
| | brain superfusion | 1, 5 or 10 µM/l | aCSF | Domoki *et al. Stroke,* 1999 [56] |
| Pre- or post | i.c.v., bolus | 1 mg/ml; 5 µl | aCSF | Jiang *et al. Mol. Brain Res.,* 2005 [59] |
| | i.p., bolus and repeated | 5 or 0.5 mg/kg | 1N NaOH | Farkas *et al.* unpublished ms, 2005 [66] |
| Pre- and post | i.v., bolus + continuous infusion | 15 mg/kg + 0.5 mg/min | | Shake *et al., Ann. Thorac. Surg.,* 2001 [60] |
| Post | i.p., repeated | 5 mg/kg; 0.25 ml | DMSO | Farkas *et al. Brain Res.,* 2004 [63] |
| | | 5 mg/kg; 0.25 ml | DMSO | Farkas *et al. Neurosci. Lett.,* 2005 [64] |
| | | 0.5 mg/kg; 0.25 ml | 1N NaOH | Farkas *et al. Neurosci. Lett.,* 2005 [65] |

Abbreviations: aCSF: artificial cerebrospinal fluid; DMSO: dimethyl sulphoxide; i.c.v.: intracerebroventricular; i.p.: intraperitoneal; i.v.: intravenous; n.d.: no data

In order to characterize cerebrovascular reactivity, changes in pial arteriolar diameter in response to hypercapnia and *N*-methyl-D-aspartate (NMDA) stimulation were recorded in newborn piglets with the help of a closed cranial window and intravital microscopy. In piglets, $CO_2$-induced vasodilation requires intact endothelial function [69]. Both hypercapnia and NMDA elicit vasodilation under physiological circumstances, but these vasodilator responses are considerably impaired after ischemia/reperfusion (I/R). When diazoxide was applied before the initiation of I/R, the vasodilator response to hypercapnia and NMDA stimulation was preserved [56, 58]. In the case of hypercapnia-induced vasodilation, a direct effect of diazoxide on endothelial mitochondria was suggested to account for the preserved function. In support of this view, endothelial cells in pial arterioles have been strongly implicated in vasodilation [69]. Furthermore, diazoxide was shown to prevent endothelial dysfunction after I/R in the human forearm [70], and to reduce the mitochondrial membrane potential in cultured piglet cerebral endothelial cells [58]. In the NMDA model, diazoxide preserved neuronal-vascular function probably via a reduced production of reactive oxygen species from the mitochondria [56]. These results lead to the conclusion that diazoxide exerts a beneficial effect on the cerebrovascular component of perinatal ischemic brain injury.

In addition to cerebrovascular reactivity, treatment with diazoxide also protects nervous tissue in perinatal hypoxia/ischemia. The most conspicuous evidence for the neuroprotective effect of diazoxide was provided by the measurement of infarct volume. The 2,3,5-triphenyltetrazolium chloride (TTC) staining of slices prepared from 7-day old pup brains demonstrated that diazoxide reduced infarct volume by about 20%, 24 h after the onset of ischemia [55]. Meanwhile, the activation of an enzymatic mediator of neuronal death, calpain was significantly down-regulated in the cortex and the hippocampus, together with a decrease in DNA fragmentation [59]. In addition, the expression of the immediate-early genes c-Fos and c-Jun, which is highly up-regulated after ischemia, was depressed by diazoxide [59]. These studies proposed that diazoxide mediated its neuroprotective effect by acting on the mitoK $_{ATP}$ channels and by preserving mitochondrial function. This assumption gains support from the finding that hippocampal mitochondria in newborn piglets undergo extensive swelling and $Ca^{2+}$ accumulation after I/R, which can be completely blocked by diazoxide [57]. Also, in order to prove that diazoxide acted on the mitoK $_{ATP}$ channels, 5-HD (a mitoK $_{ATP}$ channel antagonist) was co-applied with diazoxide in the previously mentioned experiments; it abolished the beneficial effect of diazoxide (i.e. infarct volume, mitochondrial swelling) [55,57].

In summary, treatment with diazoxide appears to be a promising strategy for the management of perinatal ischemic brain injury with some restrictions (concerning dose, the route and time of application) discussed below.

## Age-Associated Conditions - Transient, Ischemic Stroke

Stroke is a leading cause of mortality and neurological dysfunction in Western societies, therefore the development of preventive strategies and effective therapy is very important. Diazoxide has been tested in experimental animal models of transient cerebral ischemia for the last five years. In these stroke models, I/R was created by the transient occlusion of the common carotid or the middle cerebral arteries of adult rats and mice. Similar to the studies on perinatal hypoxia/ischemia, diazoxide reduced infarct volume assessed with the TTC staining [54, 61, 67, 71]. Furthermore, diazoxide also decreased the number of TUNEL-positive apoptotic cells in the infracted hemisphere and prevented cell death in the hippocampus CA1 area [54,62]. In addition, a higher number of astrocytes appeared in the peri-infarct area of diazoxide-treated animals as compared with vehicle-treated controls [54]. Finally, diazoxide limited the ischemia-induced dramatic increase in blood-brain barrier permeability indicated by the leakage of Evan's blue and sodium fluorescin into the brain parenchyma, and reduced the water content of the brain tissue [67].

The co-application of 5-HD with diazoxide abolished the protective effect of diazoxide in these models [54,61], which indicates that the mitochondrial mitoK $_{ATP}$ channels were most probably targeted. Yet, beyond this indication, direct, *in vivo* evidence to explain the exact intracellular mechanisms that lead to neuroprotection in the animal studies is scarce. Therefore, we must rely on the results of the *in vitro* experiments presented above.

Studies on ischemic stroke have emphasized the preventive approach; diazoxide was invariably administered before the onset of ischemia. The design of the experiments made use of the widely accepted concept that diazoxide mediates pharmacological preconditioning. For this reason, particular clinical conditions or patients at high risk for ischemic brain damage/stroke should be identified, where preconditioning the brain with diazoxide would afford prevention against a later ischemic insult.

## Age-Associated Conditions - Chronic Cerebral Hypoperfusion

Chronic cerebral hypoperfusion has been recognized as a cerebrovascular condition that accompanies aging and dementia. Chronically reduced cerebral blood flow has been associated with cognitive decline and a slow degeneration of brain tissue [72, 73, 74]. To improve brain perfusion and to alleviate associated cognitive symptoms, acetylcholinesterase inhibitors and acetylcholine release enhancers were applied to increase the availability of acetylcholine in the synapses of the cerebral vasodilatory circuits [1]. Also, dietary supplements such as long-chain polyunsaturated fatty acids and antioxidants that are involved in the constitution and protection of phospholipid membranes decreased spatial learning dysfunction and augmented the state of the blood-brain barrier in chronic cerebral hypoperfusion [75]. Recently, diazoxide has also been considered as a new candidate to moderate the consequences of chronic cerebral hypoperfusion.

A widely accepted model of chronic cerebral hypoperfusion is the permanent, bilateral occlusion of the common carotid arteries in rats (2VO). This model creates a reproducible and consistent spatial learning defect that can be determined from one week up to a year after surgery [72]. When diazoxide was applied before the onset of 2VO, learning capacity measured one week after the surgery was

restored almost to control level [66]. However, post-treatment with diazoxide failed to improve spatial learning measured either one week or 13 weeks after 2VO surgery [65, 66]. These data are in agreement with the known pharmacological preconditioning effect of diazoxide. According to the working hypothesis, diazoxide given as pretreatment most probably caused a transient increase in the level of reactive oxygen species, which activated their scavenging enzymes in the brain. A higher level of the scavenging enzymes protected the neural tissue against a subsequent ischemic insult, which was reflected in the cognitive improvement.

In the same experiments, treatment with diazoxide yielded a mismatched pattern of microglial activation. Pretreatment did not affect microglial activation in the hippocampus one week after 2VO [66]. In post-treatments, however, diazoxide delivered paradoxical results. One week after 2VO, cerebral hypoperfusion did not alter microglial activation as compared with the sham-operated control animals, but post-treatment with diazoxide given directly after sham and 2VO surgery augmented the basal level of microglial activation selectively in the 2VO animals [66]. These findings may demonstrate an adverse effect of the post-treatment by diazoxide in the 2VO model.

In contrast, 13 weeks after 2VO, cerebral hypoperfusion enhanced microglial activation compared with the sham-operated controls, which was prevented by treatment with diazoxide also administered directly after 2VO surgery [63, 65]. Such a reduction of microglial activation by diazoxide is considered beneficial. Nevertheless, the interpretation of these results would be premature at this point, since the existence and function of mito$K_{ATP}$ channels in microglia, or an alternative binding site of diazoxide on these cells is yet to be determined. Still, a few, possible links between microglial activation and the pharmacological effects of diazoxide may be contemplated as follows.

Microglial activation involves a distinct pattern of inwardly rectifying potassium channels and altered potassium currents [76, 77], but there is no evidence whether diazoxide can influence microglial potassium currents. Furthermore, microglial activation entails distinct mitochondrial changes reflected in the increased number and elongated shape of mitochondria [78], but a potential role of mito$K_{ATP}$ channels in this process has not been identified, yet. Finally, since the experiments were performed *in vivo,* it is difficult to determine whether the effect of diazoxide on microglia was direct or secondary to that achieved on neurons in these studies. The issues raised here may stimulate more experimental work to elucidate the action of diazoxide on microglial activation and to describe its temporal pattern. The use of microglial cell cultures appears to be an appropriate and promising tool to this end.

## CONSIDERATIONS FOR FUTURE DRUG DEVELOPMENT

Based on the presented experimental data, treatment with diazoxide appears to be a potential strategy for the management of ischemic brain injury. For future drug development, however, a few issues must be considered. First, since the target of the neuroprotective treatment is the central nervous system, it is essential that the drug is able to cross the blood-brain barrier. It is also of interest from the point of view of perinatal ischemia, whether diazoxide can cross the blood-placenta barrier. According to early results, diazoxide readily crosses the blood-placenta barrier and appears in fetal blood

**Table 3. Read-Outs Taken to Demonstrate the Neuroprotective Effect of Diazoxide**

| Read-Out | Reference |
|---|---|
| Cognitive performance | Farkas *et al. Brain Res.,* 2004 [63] |
| | Farkas *et al. Neurosci. Lett.,* 2005 [65] |
| | Farkas *et al.* unpublished ms, 2005 [66] |
| Infarct volume | Liu *et al. J. Cereb. Blood Flow Metab.,* 2002 [54] |
| | Rajapakse *et al. Neurosci. Lett.,* 2002 [55] |
| | Shimizu *et al. Am. J. Physiol. Heart Circ. Physiol.,* 2002 [61] |
| | Lenzsér *et al. Brain Res.,* 2005 [67] |
| | Nakagawa *et al. Neurosurg.,* 2005 [71] |
| Histology | Shake *et al. Ann. Thorac. Surg.,* 2001 [60] |
| | Caparrelli *et al. Ann. Thorac. Surg.,* 2002 [84] |
| | Liu *et al. J. Cereb. Blood Flow Metab.,* 2002 [54] |
| | Munoz *et al. Stroke,* 2003 [62] |
| | Farkas *et al. Brain Res.,* 2004 [63] |
| | Farkas *et al. Neurosci. Lett.,* 2005 [64] |
| | Farkas *et al. Neurosci. Lett.,* 2005 [65] |
| | Farkas *et al.* unpublished ms, 2005 [66] |
| Mitochondrial morphology | Domoki *et al. Brain Res.,* 2004 [57] |
| c-Fos/c-Jun expression | Jiang *et al. Mol. Brain Res.,* 2005 [59] |
| DNA fragmentation | Jiang *et al. Mol. Brain Res.,* 2005 [59] |
| m-calpain activation | Jiang *et al. Mol. Brain Res.,* 2005 [59] |
| Pial arteriolar diameter | Domoki *et al. Stroke,* 1999 [56] |
| | Domoki *et al. Am. J. Physiol. Heart Circ. Physiol.,* 2005 [58] |
| Blood-brain barrier permeability | Lenzsér *et al. Brain Res.,* 2005 [67] |
| Cerebral blood flow | Liu *et al. J. Cereb. Blood Flow Metab.,* 2002 [54] |
| | Lenzsér *et al. Brain Res.,* 2005 [67] |
| | Nakagawa *et al. Neurosurg.,* 2005 [71] |

[79], but the blood-brain barrier permeability of diazoxide has been largely ignored. Only indirect evidence (namely that diazoxide is neuroprotective *in vivo*) suggests that diazoxide can probably penetrate the brain parenchyma, but further studies with a focus on the blood-brain barrier passage of diazoxide must be conducted to confirm this assumption.

Some of the animal studies discussed above successfully demonstrated that diazoxide can be administered peripherally (i.v. or i.p., though *per os* data is not yet available) in order to exert a beneficial effect in the brain (Table **2**). Nevertheless, the optimal concentration of the drug has yet to be determined for human patients. The determination of effective concentration is crucial, since diazoxide at relatively high concentration exerts pronounced effects on blood pressure and pancreatic β-cell function. The observation that diazoxide can reduce cerebral blood flow received much attention, when diazoxide was under development as a blood pressure reducing agent [80,81]. Therefore, the optimal therapeutic dose to treat cerebral ischemia should be low enough not to harm peripheral organs and not to reduce cerebral blood flow, but sufficiently high enough to achieve neuroprotection. The results of the animal studies are scarce but promising in this respect. A recent study identified neuroprotective doses of diazoxide (20 mg/kg, i.p.), which did not have a long-term effect on blood pressure and blood glucose level in rats [67].

The reduced cerebral blood flow may pose a particular problem when diazoxide is applied in a post-ischemic manner. Application of the drug at this point might decrease the blood flow to the ischemic site even further, and possibly enhance neuronal damage. Data available on the action of diazoxide on cerebral blood flow appears to be conflicting. Diazoxide given at a concentration of 5 mg/kg (i.v.) with the purpose to control hypertension was found to reduce cerebral blood flow in hypertensive patients and rats [80,81]. Nevertheless, the unwanted reduction in cerebral blood flow was suggested not to be a direct effect of diazoxide, but secondary to a sudden drop in blood pressure, and occurred when blood pressure decreased below the lower limit of the autoregulatory capacity of the brain [82]. In contrast, another set of experiments found no effect of diazoxide on cerebral blood flow in a model of venous ischemia, in which diazoxide (2 mmol/l,intracerebroventricular, i.c.v.) reduced the infarct volume [71]. Further, diazoxide administered at a concentration of 3-5 mg/kg (i.v.) increased cerebral blood flow in goats [83] and preserved the I/R-impaired vasodilatory response in piglets [56, 58]. Nevertheless, mean arterial blood pressure was briefly lowered from 5 to 15 min after the administration of the drug in the latter experiments [58]. The mechanism behind the cerebral vasodilatory effects of diazoxide has not yet been definitively determined, but a direct action on the endothelium as well as on the neural-vascular unit may play a mediating role [56, 58, 70]. Taken together, these experiments indicate that care must be taken to choose the appropriate concentration of the drug, so that a drop in blood pressure will not challenge the autoregulatory capacity of the brain. The route of application may also modify the drug's effect; in rats, 5 mg/kg diazoxide i.v. reduced blood pressure and cerebral blood flow [81], while a neuroprotective dose of 6-20 mg/kg given i.p. did not [67].

Another consideration is the timing of treatment. Most studies applied diazoxide as a pretreatment (i.e. before the onset of cerebral ischemia), because diazoxide has been known to mediate pharmacological preconditioning. This approach can be useful in cases of planned surgical procedures (e.g. open heart surgery) that can disturb cerebral blood flow. In such cases, preconditioning the brain with diazoxide could prevent or limit neurological insults during recovery from such surgery.

Nevertheless, post-administration of drugs in general may have a higher therapeutic value, especially in cases of perinatal ischemic brain injury, where the prediction of the incidence and the prevention of ischemia are quite difficult to realize. Therefore, a few attempts have been taken to characterize the neuroprotective properties of a post-treatment with diazoxide. These experiments delivered various results. In a study of perinatal ischemia, the application of diazoxide immediately after the ischemic insult appeared as effective as pretreatment with the drug [59]. However, in another set of experiments of chronic cerebral hypoperfusion in adult rats, only the pretreatment reduced cognitive dysfunction; the post-treatment did not [66]. These conflicting findings encourage further research to characterize the potential, neuroprotective effects of a post-treatment with diazoxide, and to identify conditions where such approach can be recommended.

In summary, though the application of diazoxide as a neuroprotective strategy has only recently been considered, treatment of cerebrovascular disorders by targeting the mito$K_{ATP}$ channels offers broad prospects. The animal studies gathered consistent data that diazoxide is particularly effective in models of stroke and I/R, both in newborn and adult animals. These findings may stimulate further research for drug development in this field.

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## ABBREVIATIONS

| | | |
|---|---|---|
| 2VO | = | Permanent, bilateral common carotid artery occlusion (two-vessel occlusion) |
| 5-HD | = | 5-Hydroxydecanoate |
| aCSF | = | Artificial cerebrospinal fluid |
| ADP | = | Adenosine diphosphate |
| ATP | = | Adenosine triphosphate |
| DIAZ | = | Diazoxide |
| DMSO | = | Dimethyl sulphoxide |
| GDP | = | Guanosine diphosphate |
| GTP | = | Guanosine triphosphate |
| i.c.v. | = | Intracerebroventricular |

i.p. = Intraperitoneal

i.v. = Intravenous

I/R = Ischemia/reperfusion

$K_{ATP}$ channels = ATP-sensitive potassium channels

$K_{IR}$ = Inwardly rectifying potassium channel subunit

mito$K_{ATP}$ channels = Mitochondrial ATP-sensitive potassium channels

NMDA = *N*-methyl-D-aspartate

SUR = Sulphonylurea receptor

TMD = Transmembrane domain

TTC = Triphenyltetrazolium chloride

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Research Report

# The effect of pre- and posttreatment with diazoxide on the early phase of chronic cerebral hypoperfusion in the rat

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*Abbreviations:*
2VO, permanent, bilateral common carotid artery occlusion (two-vessel occlusion)
ANOVA, analysis of variance
DAB, diaminobenzidine
DIAZ, diazoxide
GFAP, glial fibrillary acidic protein
$mK_{ATP}$ channel, mitochondrial, ATP-dependent, $K^+$ channel
$NC_{Ca\text{-}ATP}$ channel, $Ca^{2+}$-activated, ATP-sensitive, nonselective, cation channel
NPS, normal pig serum
PB, phosphate buffer
RT, room temperature
SHAM, sham-operated control
SUR, sulphonylurea

ABSTRACT

Diazoxide has been identified as a mitochondrial, ATP-dependent $K^+$ channel opener, and a potentially neuroprotective compound under ischemic conditions. We set out to characterize the consequences of various treatment strategies with diazoxide in a rat model of chronic cerebral hypoperfusion. Cerebral hypoperfusion was induced by permanent, bilateral occlusion of the common carotid arteries (2VO, $n$ = 36), sham-operated rats serving as controls (SHAM, $n$ = 29). Diazoxide or its vehicle was administered i.p. daily (5 × 0.5 mg/kg/0.25 ml) or as a bolus injection (5 mg/kg/0.25 ml) before surgery or daily after surgery (5 × 0.5 mg/kg/0.25 ml). Spatial learning performance was assessed 1 week after 2VO in the Morris maze. Hippocampal pyramidal cell loss was assessed on cresyl violet-stained sections, while glial reactivity was labeled immunocytochemically. Daily or bolus pretreatment with diazoxide significantly improved 2VO-related learning impairment, whereas posttreatment was ineffective. The number of CA1 pyramidal neurons was reduced by 2VO, which was prevented by repeated or bolus pretreatment with diazoxide. Astrocyte proliferation and microglial activation were enhanced by posttreatment with diazoxide in the hippocampus CA1 area of 2VO animals as compared with SHAM. These data demonstrate that the neuroprotective effect exerted by diazoxide depends on the time of administration with respect to the onset of ischemia; pretreatment but not posttreatment with the compound has proved to be neuroprotective in chronic cerebral hypoperfusion. Thus, pretreatment with diazoxide offers therapeutical prospects for the treatment of cerebral ischemia.



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## 1. Introduction

Diazoxide, a mitochondrial, ATP-dependent $K^+$ ($mK_{ATP}$) channel opener, has originally been used in clinical practice in hypertensive emergencies (Goodman Gilman et al., 1990). By now, an accumulating body of experimental data has demonstrated that diazoxide can be used for pharmacological preconditioning, attenuating ischemic heart damage (O'Rourke, 2004). In view of its preventive action in cardiac ischemia, diazoxide has become the focus of recent investigation as a potential anti-ischemic agent in the brain. The growing interest in the pharmacological action of diazoxide on the central nervous system was encouraged by the finding that the brain contains a considerably higher concentration of $mK_{ATP}$ channels (the target of diazoxide) than other peripheral organs (Bajgar et al., 2001).

Compelling evidence of the neuroprotective effect of diazoxide has emerged from experiments on brain slices or neuronal cell cultures, where diazoxide applied before oxygen and glucose deprivation enhanced the cell viability and preserved the electrophysiological properties of the hippocampal neurons (Zawar and Neumcke, 2000; Kis et al., 2003). Although neuroprotection on cell cultures and the intracellular pathways initiated by diazoxide have been documented in detail (Busija et al., 2004), little is known about the neuroprotective potential of diazoxide at the level of the brain. To date, only a few studies have administered diazoxide to experimental animals with the aim of examining the effect of the drug on the ischemic brain. In this way, diazoxide has been shown to limit the infarct size after middle cerebral artery occlusion or unilateral carotid artery occlusion combined with hypoxia in rodents (Liu et al., 2002; Rajapakse et al., 2002; Shimizu et al., 2002). Despite these preliminary data, no clear evidence has been gathered as to whether diazoxide can actually prevent the cognitive dysfunction after an ischemic insult, and whether the cognitive status correlates with histologic neural markers. Furthermore, most studies have applied diazoxide as pretreatment because of its known effects of acute and delayed pharmacologic preconditioning (Busija et al., 2004). Although diazoxide pretreatment has thus appeared to be a neuroprotective strategy of promise in cerebral ischemia, it is of interest from a therapeutic point of view to establish whether postischemic administration of the drug can also exert beneficial effects on the central nervous system.

In the present study, we followed previously devised methods (Farkas et al., 2004; 2005a,b) to identify the potentially neuroprotective effects of diazoxide in a rat model of chronic cerebral hypoperfusion. Since our previous work had focused on the effect of diazoxide at a late phase of chronic cerebral ischemia, our present objective was to investigate the action of diazoxide at an early phase of cerebral hypoperfusion. Further, as diazoxide had been administered only as posttreatment in our previous experiments (while others had used it only as pretreatment), in the present study, we also set out to compare the neural effects of pretreatment and posttreatment with diazoxide.

## 2. Results

The spatial learning curves obtained with the Morris maze test demonstrated that 2 weeks of experimental cerebral hypoperfusion induced a marked decrease in learning performance in the non-treated 2VO group as compared with the non-treated SHAM group (Fig. 1A). Both repeated and bolus pretreatment with diazoxide improved the spatial memory. The 2VO animals that received either repeated or bolus diazoxide pretreatment exhibited the same performance as concerns the average swimming distance as compared with their respective SHAM controls, specifically on days 2, 3, and 5 (Figs. 1B and C). Conversely, the 2VO animals that received diazoxide posttreatment did not succeed in learning the Morris maze paradigm. On days 2 and 5, the 2VO rats posttreated with diazoxide achieved significantly worse results as compared with the corresponding SHAM posttreated group (Fig. 1D).

Corresponding with the results of the learning test, cerebral hypoperfusion induced a moderate but constant loss of hippocampal CA1 pyramidal neurons (Fig. 2). Although massive necrotic cell death was not detected, the CA1 str. pyramidale in the 2VO animals displayed a loose cellular arrangement as compared with the compact structure of the pyramidal layer in the SHAM animals (Figs. 2A and B). Cell counting revealed that 2VO decreased pyramidal cell number with 6%. Both the repeated and the bolus pretreatment with diazoxide preserved the compact structure of the CA1 str. pyramidale (Fig. 2C) and prevented pyramidal cell loss (Fig. 2D).

GFAP-immunoreactive astrocytes were present in all the hippocampal regions and in all the experimental groups. Neither 2VO nor pretreatment with diazoxide exerted a detectable influence on the GFAP immunoreactivity in the investigated hippocampal areas. However, posttreatment with diazoxide doubled the GFAP signal in the CA1 str. radiatum and str. oriens of the 2VO rats (Fig. 3).

Activated microglia labeled with OX-42 were scarce not only in the SHAM animals but also in the 2VO non-treated rats in all regions of the hippocampus. In contrast, dense staining was detected in the CA1 str. oriens and str. radiatum of the 2VO animals posttreated with diazoxide (Figs. 4A–C). Quantitative analysis demonstrated that the application of diazoxide after 2VO increased the OX-42 signal approximately 2.5-fold in the CA1 str. oriens and 7-fold in the CA1 str. radiatum as compared with the SHAM groups (Figs. 4D and E).

## 3. Discussion

The present study has revealed that pretreatment with diazoxide at the dose of 5 × 0.5 mg/kg or 1 × 5 mg/kg, given i.p. is neuroprotective as regards chronic cerebral hypoperfusion in rats. The neuroprotective action of the pretreatment with diazoxide was reflected in an improvement in spatial learning performance and the inhibition of pyramidal cell loss in the hippocampus CA1 region.

Our line of investigation is novel in the sense that the action of diazoxide on learning and memory has not previously been

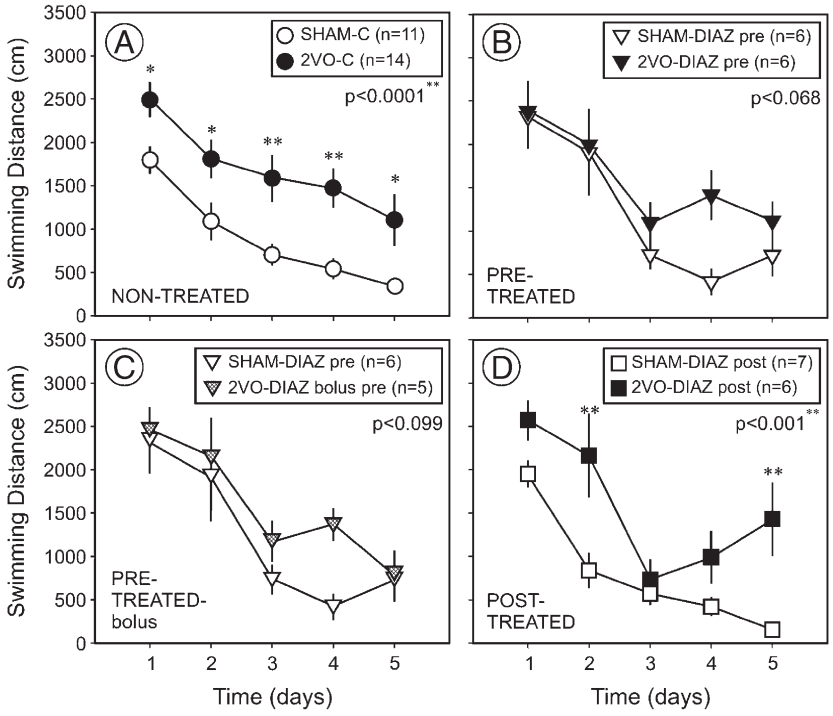


**Fig. 1 – The results of the Morris water maze spatial orientation test. Data are presented as means ± SEM; *$P < 0.05$, **$P < 0.01$. Abbreviations: 2VO: permanent, bilateral common carotid artery occlusion (two-vessel occlusion); C: control for treatment; DIAZ: diazoxide; post: posttreatment; pre: pretreatment; SHAM: sham-operated control.**

characterized in cerebral hypoperfusion or ischemia. The present results demonstrate that diazoxide prevents the cerebral hypoperfusion-related learning dysfunction when administered as pretreatment. However, posttreatment with diazoxide exerted no clear effect on the learning performance, similarly as we earlier observed at a later time point in cerebral hypoperfusion (Farkas et al., 2005a). The finding that pretreatment with diazoxide has a beneficial effect on learning, and the integrity of the hippocampal pyramidal cells is in accord with the pharmacological action of diazoxide revealed in studies on cultured neurons. The pretreatment of neurons with diazoxide before oxygen-glucose deprivation increased their viability as compared with that of non-treated cultures (Nagy et al., 2004). Diazoxide is known to depolarize the mitochondria and to simultaneously elevate the production of reactive oxygen species, which is assumed to play a significant role in the preconditioning effect of diazoxide (Busija et al., 2004). The transient, moderate elevation of the concentration of reactive oxygen species by diazoxide is suggested to condition the cells to be able to tolerate a later, robust ischemic event better. Such preconditioning may account for the preserved learning performance and enhanced hippocampal neuronal viability of 2VO rats following pretreatment with diazoxide.

The immunocytochemical signals for both astrocytes and microglia were markedly enhanced by posttreatment with diazoxide in the 2VO animals, while 2VO or diazoxide alone had no effect on the glial cells. This suggests that, given the sequence of 2VO and treatment, diazoxide was able to stimulate the glial cells, provided that the glia were already made sensitive by ischemia.

Although the lack of evidence means that it is difficult to establish a causal link between treatment with diazoxide and a glial reaction, the intriguing observation was made that a subpopulation of reactive astrocytes expressed a $Ca^{2+}$-activated, ATP-sensitive nonselective cation channel ($NC_{Ca\text{-}ATP}$) under hypoxic conditions. Interestingly, these channels are controlled by a sulfonylurea regulatory subunit, SUR1, which is the target of diazoxide. It has been shown that the treatment of such astrocytes with diazoxide results in activation of the $NC_{Ca\text{-}ATP}$ channels, which are suggested to control the swelling and migration of astrocytes in hypoxic nervous tissue (Chen et al., 2003). Even though a strict correlation cannot be drawn with our data, a similar mechanism may play a role in the diazoxide-induced astrocytic proliferation shown here.

The background of diazoxide-related microglial activation is even more obscure. Although the activity of the microglia is dependent on the opening state of the surface ion channels (Eder, 1998), regulatory SUR subunits (the potential sites of the action of diazoxide) have not been associated with these channels. Further, microglial activation is accompanied by an increased number and elongated shape of mitochondria (Banati et al., 2004), but a potential contribution of mitochondrial $K^+$ currents to these processes has not been identified, yet. Finally, the existing data on the effect of diazoxide on microglial activation in cerebral hypoperfusion are inconsistent: posttreatment with diazoxide prevents microglial activation at a late time point in cerebral hypoperfusion (Farkas et

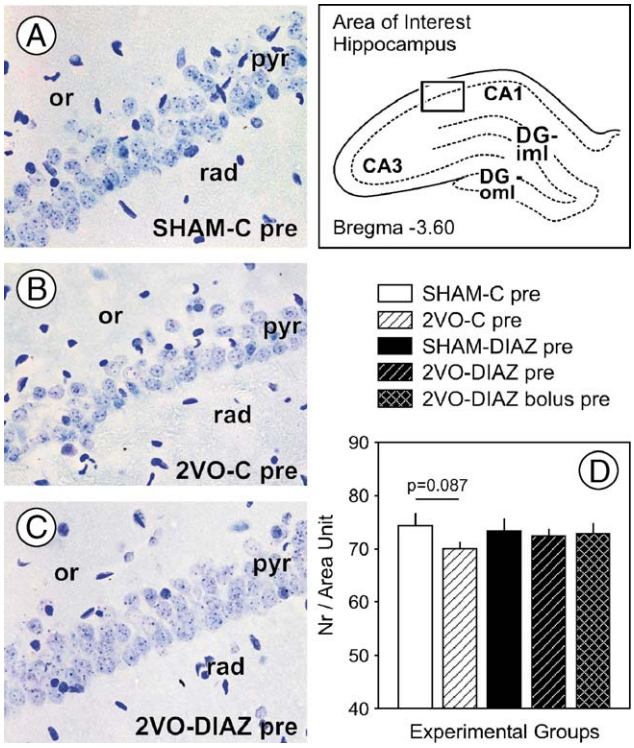


**Fig. 2 – Cresyl violet staining and cell counting in the hippocampus CA1 str. pyramidale. (A–C) Representative photomicrographs of the hippocampal CA1 area from pretreated animals. (D) Quantitative data on pyramidal cell viability in the hippocampus CA1 str. pyramidale; area unit is 0.024 mm$^2$ (3 × 10 grid holes at 40× magnification). Data are presented as means ± SEM; $^*P < 0.05$. The *P* value indicated in the graph was obtained with a Student's *t* test. Abbreviations: 2VO: permanent, bilateral common carotid artery occlusion (two-vessel occlusion); C: control for treatment; DIAZ: diazoxide; pre: pretreatment; SHAM: sham-operated control.**

al., 2004, 2005a,b), while the same treatment enhances microglial activation at an early time point, as shown here. This may suggest that the effect of diazoxide on ischemia-related microglial activation is defined by the time of sampling with respect to the treatment and the onset of cerebral hypoperfusion.

The concentration of diazoxide was chosen to be low enough to exclude potentially harmful side effects such as hypotension, reduced cerebral blood flow or hyperglycemia, which are known to potentiate ischemic injury. Recent results have demonstrated that the plasma glucose level and mean arterial pressure in rats are not significantly affected by even 6 mg/kg diazoxide i.p. (Lenzsér et al., 2005). A selective effect of a low dose of diazoxide on the brain is probably due to the distribution and molecular composition of the $mK_{ATP}$ channels. The brain contains a 6–7 times higher concentration of $mK_{ATP}$ channels than the liver or the heart (Bajgar et al., 2001). Our present observation that a low concentration of diazoxide attenuates ischemic hypoperfusion-induced brain damage, yet does not alter the vascular and metabolic parameters, may be of therapeutic potential in cerebral ischemia.

In conclusion, we have found that a low dose of diazoxide that is ineffective on the mean arterial pressure or blood glucose concentration (Lenzsér et al., 2005) is potent on the central nervous system. Further, diazoxide prevents an ischemia-induced learning impairment when the drug is administered as pretreatment, but not as posttreatment. Finally, diazoxide potentiates glial reactivity when given as posttreatment, but not as pretreatment. The present findings on the glial reaction call for further research on the mechanisms of action of diazoxide on glia cells. Our results may additionally provoke investigations on the neuroprotective properties of diazoxide with therapeutical prospects in ischemia.

## 4. Experimental procedures

Sixty-five male Wistar rats (302 ± 18 g) were used for the study. All animal experiments were approved by the ethical committee of the University of Szeged. Chronic, experimental cerebral hypoperfusion was induced in half of the animals by permanent bilateral occlusion of the common carotid arteries (2VO), the other half serving as sham-operated controls (SHAM) (Farkas et al., 2004). Prior to surgery, the animals were anesthetized with 400 mg/kg chloralhydrate i.p., followed by 0.05 ml 0.1% atropine i.m. The common carotid arteries were exposed via a ventral cervical incision, carefully separated from their sheaths and vagal nerves, and permanently ligated with surgical sutures. Lidocaine (1%) was applied as local anesthetic. The same procedure was performed on the SHAM group but without the actual ligation.

The animals were treated with diazoxide or its vehicle (0.25 ml 0.1 N NaOH), i.p. In the first group, diazoxide (0.5 mg/kg/0.25 ml) or its vehicle was injected on 5 consecutive days immediately before 2VO surgery. In the second group, diazoxide (5 mg/kg/0.25 ml) was administered once (bolus) the day before 2VO surgery. In the third group, diazoxide (0.5 mg/kg/0.25 ml) or its vehicle was injected on 5 consecutive days after 2VO surgery, the first injection given within 1 h following surgery. The final compositions of the experimental groups and the survival rates are presented in Table 1.

One week following 2VO surgery, the animals were trained in the Morris water maze (Farkas et al., 2004), which consisted of a circular pool (diameter: 160 cm, depth: 35 cm) filled with water (22 °C), made opaque with milk so that the rats were unable to see an underwater platform 2 cm below the water surface. Invariant visual cues were placed on the wall of the testing room, and an auditory source with a fixed location was switched on throughout the testing. All the rats performed two trials per day, with a constant inter-trial interval of 4 h, for 5 consecutive days. The starting positions were selected at random from four standard entry points. The rats were given 2 min to find the platform and sit on it for 15 s. Rats that failed to find the location within the given time were gently guided to the platform and allowed to stay on it for 15 s. Swimming paths were recorded with a computerized video imaging analysis system (EthoVision, Noldus Information Technology BV, Wageningen,

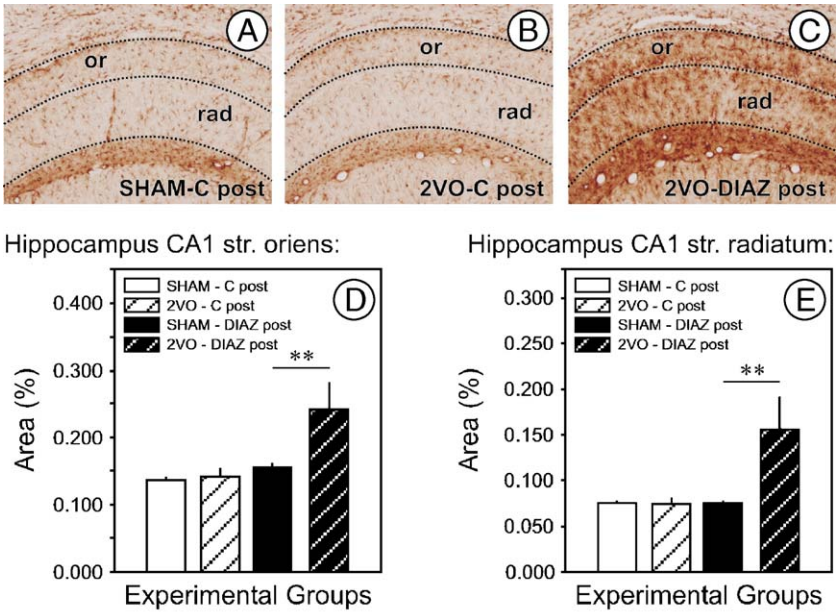


**Fig. 3 – GFAP immunocytochemistry for astrocyte proliferation. (A–C) Representative photomicrographs of the hippocampal CA1 area from posttreated animals. (D) Quantitative data on GFAP immunocytochemistry in the hippocampal CA1 str. oriens of the posttreated animals. (E) Quantitative data on GFAP immunocytochemistry in the hippocampal CA1 str. radiatum of the posttreated animals. Data are presented as means ± SEM; *$P < 0.05$, **$P < 0.01$. Abbreviations: 2VO: permanent, bilateral common carotid artery occlusion (two-vessel occlusion); C: control for treatment; DIAZ: diazoxide; post: posttreatment; SHAM: sham-operated control.**

The Netherlands). In each trial, the escape latency and the swimming distance traveled before the rats reached the platform were analyzed.

Seven days after the beginning of the Morris water maze training, and 14 days after the performance of 2VO, the animals were reanesthetized with an overdose of chloralhydrate (i.p.)

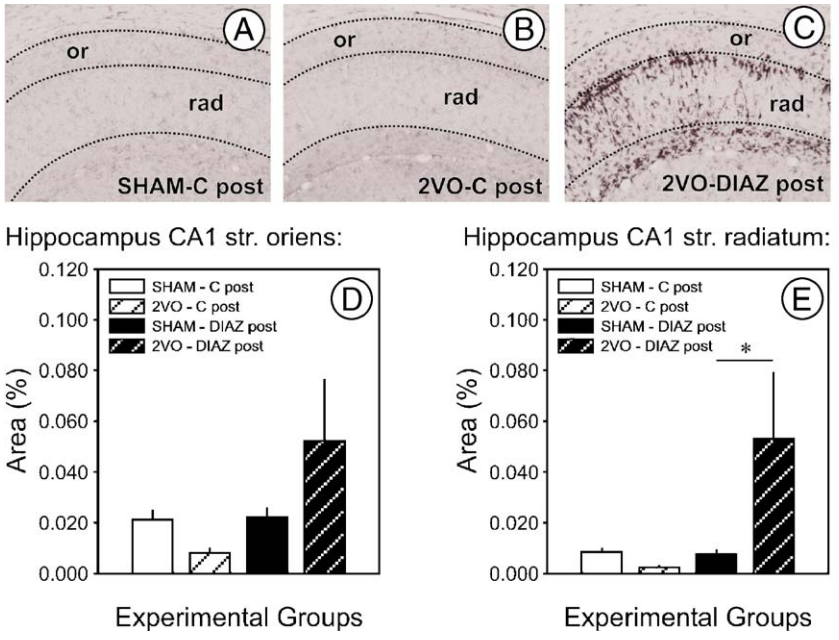


**Fig. 4 – OX-42 immunocytochemistry relating to microglia activation. (A–C) Representative photomicrographs of the hippocampal CA1 area from posttreated animals. (D) Quantitative data on OX-42 immunocytochemistry in the hippocampal CA1 str. oriens of the posttreated animals. (E) Quantitative data on OX-42 immunocytochemistry in the hippocampal CA1 str. radiatum of the posttreated animals. Data are presented as means ± SEM; *$P < 0.05$, **$P < 0.01$. Abbreviations: 2VO: permanent, bilateral common carotid artery occlusion (two-vessel occlusion); C: control for treatment; DIAZ: diazoxide; post: posttreatment; SHAM: sham-operated control.**

**Table 1 – Experimental groups and survival rate**

| Type of treatment | Experimental group | No. of operated animals | No. of survived animals | Survival rate (%) |
|---|---|---|---|---|
| Pretreatment | SHAM-C | 6 | 5 | 83 |
| | 2VO-C | 9 | 8 | 89 |
| | SHAM-DIAZ | 8 | 6 | 75 |
| | 2VO-DIAZ | 6 | 6 | 100 |
| | 2VO-DIAZ bolus | 6 | 5 | 83 |
| Posttreatment | SHAM-C | 7 | 6 | 86 |
| | 2VO-C | 7 | 6 | 86 |
| | SHAM-DIAZ | 8 | 7 | 88 |
| | 2VO-DIAZ | 8 | 6 | 75 |

Abbreviations: 2VO: permanent, bilateral common carotid artery occlusion (two-vessel occlusion), C: control for treatment, DIAZ: diazoxide, SHAM: sham-operated control.

and perfused transcardially with 100 ml saline, followed by 400 ml 3.5% paraformaldehyde and 0.5% picric acid in 0.1 M phosphate buffer (PB, pH 7.4). The brains were removed and postfixed in the same solution for up to 1 h and then stored in 0.1 M PB containing 0.1% sodium azide.

Free-floating coronal sections at the level of the dorsal hippocampus were cut at 20-μm thickness on a cryostat. Three sections per animal that contained the dorsal hippocampus (Bregma –3.60, Paxinos and Watson, 1986) were mounted on gelatine-coated microscopic slides and stained with cresyl violet.

A second set of sections was stained immunocytochemically for glial fibrillary acidic protein (GFAP) to visualize the astrocytic proliferation. Briefly, sections were treated with 3% $H_2O_2$ and 0.5 Triton X-100 in 0.01 M PBS and preincubated in 20% normal pig serum (NPS). The samples were then incubated overnight at RT in a primary antibody solution containing mouse anti-GFAP antibody (Sigma), 1:40 000, 20% NPS, and 0.03% merthiolate in 0.01 M PBS. The secondary antibody solution consisted of goat anti-mouse biotinylated IgG (Jackson), 1:400, 10% NPS, 5% normal rabbit serum and 0.03% merthiolate in 0.01 M PBS. Finally, the sections were incubated in STA-PER (Jackson), 1% NPS, and 0.03% merthiolate in 0.1 M Tris buffer, and the color reaction was conventionally developed with diaminobenzidine (DAB) and $H_2O_2$.

To detect and analyze the microglial activation over the hippocampal areas, OX-42 antibody was used on a third set of sections. The procedure started with rinsing and pretreating the sections with 0.5% Triton X-100 and 3% $H_2O_2$ in 0.01 M PBS, followed by preincubation in 20% normal NPS and 0.5% Triton X-100 in 0.01 M PBS for 1 h. The sections were incubated overnight in a primary antibody solution containing biotinylated mouse anti-CD11b antibody (OX-42, Serotec), 1:500, 20% NPS, and 0.03% merthiolate in 0.01 M PBS at RT. Next, the sections were rinsed and incubated in a solution of STA-PER (Jackson), 1% NPS, and 0.03% merthiolate in 0.1 M Tris buffer for 1 h at RT. Finally, the color reaction was developed with Ni-DAB and $H_2O_2$. All the sections were mounted on gelatin-coated microscopic slides, air-dried, dehydrated, and coverslipped with DPX.

The number of pyramidal neurons in the hippocampus CA1 region was counted with the help of an ocular grid at 40× magnification on a surface of 0.024 $mm^2$ (3 × 10 grid holes) that covered the entire diameter of the CA1 str. pyramidale. Cell counting was performed bilaterally on three consecutive brain sections. The six values were averaged, and the average was used for further statistical analysis.

The percentage surface areas of GFAP-positive astrocytes and OX-42-immunoreactive microglia in the dorsal hippocampus were quantified by using a computerized image analysis system (Olympus BX50, DP50, software: ImagePro Plus, Media Cybernetics, U.S.A.). Briefly, three consecutive coronal sections at Br. –3.60 mm (Paxinos and Watson, 1986) were selected for the analysis. Hippocampal regions of interest were delineated manually at 10× magnification, after background subtraction and gray scale threshold determination. The area covered by immunoreactive material was computed as a percentage of the total area delineated. Measurements were carried out on both sides of the hippocampus. Six values per animal per area were averaged, and the average was used for further statistical analysis. The following areas were analyzed separately: the CA1 str. radiatum, the CA1 str. oriens, the CA3 str. radiatum, the CA3 str. oriens, the inner and outer molecular layers of the dentate gyrus, and the hilus.

The Morris maze test results were analyzed statistically via the repeated measures of the general linear model of the software SPSS. Individual daily comparisons were performed by analysis of variance (ANOVA) followed by the LSD post hoc test. The immunocytochemical results were analyzed statistically with two-way ANOVA for surgery and treatment, followed by the LSD post hoc test.

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# Effects of cyclooxygenase (COX) inhibition on memory impairment and hippocampal damage in the early period of cerebral hypoperfusion in rats

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**Abstract**

Chronic cerebral hypoperfusion is related to neurological disorders and contributes to a cognitive decline. Its experimental model in rats is permanent, bilateral common carotid artery occlusion. The cyclooxygenase (COX) system plays a pivotal role in the evolution of ischemic brain damage. Several COX inhibitors have proved to be neuroprotective in stroke models. We set out to characterize the effects of COX inhibitors in rats with permanent cerebral hypoperfusion. Some of the animals were exposed to two-vessel occlusion ($n=72$), while the others served as sham-operated controls ($n=54$). This was followed by a 3-day post-treatment with the nonselective COX inhibitor indomethacin (3 mg/kg) or with the selective COX-2 inhibitor NS-398 (15 mg/kg) or with the solvent. Some groups of the animals were sacrificed after 3 days, while the remainder were tested in the Morris watermaze for 5 days, and were sacrificed after 2 weeks. Neurons in the hippocampus were subjected to immunocytochemical labeling with cresyl violet, the dendrites with microtubule-associated protein-2, astrocytes with glial fibrillary acidic protein and microglia activation with OX-42 antibody. Two-vessel occlusion induced a learning impairment, mild neuronal damage, marked dendritic injury and moderate astrocytic reaction in the hippocampus. NS-398, but not indomethacin improved the survival rate and abolished the learning disability. However, both drugs increased the proportion of animals displaying neuronal damage. Glial markers revealed a time-dependent elevation in both the sham and the two-vessel occluded group, and were unaffected by the treatments. In summary, NS-398 prevented the hypoperfusion-induced memory impairment, but not by protecting the hippocampal neurons.


*Keywords:* Carotid artery occlusion; Cerebral ischemia; Cyclooxygenase-2; Hippocampus; Spatial learning; Neurodegeneration

## 1. Introduction

Various clinical observations support the hypothesis that chronic cerebral hypoperfusion is a major contributor to a cognitive decline, both in aging and in age-related neurological disorders (de la Torre, 2006; Kalaria, 2000; Sopala and Danysz, 2001). The persistent decrease in the cerebral blood flow in Alzheimer's disease, vascular dementia and post-stroke hypoperfusion correlates with the severity of memory disturbances (Komatani et al., 1988). Permanent occlusion of both common carotid arteries in the rat (two-vessel occlusion) has emerged as a suitable experimental model for chronic cerebral hypoperfusion; it allows simultaneous investigation of the cerebral blood flow, the learning ability and histopathological changes (Farkas et al., 2007). Studies involving use of the two-vessel occlusion model have demonstrated that an impaired spatial learning function coincides with hippocampal damage (Farkas et al., 2007; Otori et al., 2003; Sarti et al., 2002).

Tissue ischemia leads to the development of inflammatory reactions, such as reactive gliosis, the migration into and accumulation of leukocytes in the ischemic brain tissue, and the production of inflammatory cytokines and reactive oxygen species due to microglia activation or the release of arachidonic acid metabolites (Dewar et al., 2003; Wang et al., 2007). These changes result in a more severe evolution of tissue damage. Inflammation sets in rapidly after transient ischemia or ischemia/reperfusion, while a slowly-developing and persistent reaction occurs in chronic hypoperfusion. Thus, reduction of the neuronal damage and inflammation is a possible target via which to alleviate the cognitive symptoms.

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The cyclooxygenase (COX) system plays a critical role in the progression of ischemic brain injury. Three isoforms of the enzyme COX have been described: COX-1, COX-2 and COX-3, the latter being a splice variant of COX-1. COX-1 is expressed constitutively, and participates in various homeostatic processes, such as the maintenance of tissue perfusion and synaptic plasticity (Iadecola et al., 2001; Phillis et al., 2006). COX-2 production is induced globally in response to brain infarction in humans (Sairanen et al., 1998) and in various stroke models in animals (Candelario-Jalil et al., 2003; Collaço-Moraes et al., 1996; Degi et al., 1998; Nogawa et al., 1997). Nonsteroid anti-inflammatory drugs (NSAIDs) and selective COX-2 inhibitors have proved to be neuroprotective in stroke and ischemia/reperfusion injury. For example, pretreatment with indomethacin (a nonselective COX inhibitor) reduced the infarct size following middle cerebral artery occlusion (MCAO)-induced transient focal ischemia in rats (Buccellati et al., 1998) and diminished the ischemia-evoked CA1 hippocampal injury in gerbils (Sasaki et al., 1998). NS-398 (*N*-[2-(cyclohexyloxy)-4-nitrophenyl]-methanesulphonamide), a selective COX-2 inhibitor, reduced infarcts following MCAO-induced focal cerebral ischemia (Nogawa et al., 1997). In the majority of these experiments, the drugs were applied as pretreatment to prevent subsequent ischemic damage. However, post-treatment strategies may be more desirable, as the occurrence of a cerebral ischemic event is most often unpredictable. Further, the long-term application of NSAIDs or selective COX-2 inhibitors results in cardiovascular side-effects, such as stroke and myocardial infarction (Topol, 2004), and acute therapy therefore appears preferable.

Since the inhibition of COXs had proved neuroprotective in various stroke models, but had not been tested in a mild ischemic condition, the aim of our present study was to characterize the effects of COX inhibition after two-vessel occlusion in rats. In our experimental setup, the treatment was chosen to correspond to clinical treatment strategies; thus, indomethacin and NS-398 were administered as acute 3-day post-treatment after two-vessel occlusion. We analyzed the changes in the learning performance and in histological markers such as neuronal damage (labeled with cresyl violet staining), dendritic integrity (labeled with microtubule-associated protein-2 (MAP-2) antibody), astrocytic activation and microglia activation (labeled with glial fibrillary acidic protein (GFAP) and OX-42 (CD-11b receptor) antibody immunocytochemistry).

## 2. Materials and methods

### 2.1. Treatment and surgery

All animal experiments were approved by the Ethical Committee of the University of Szeged. One hundred twenty-six male Wistar rats (263±37 g) were used for the study. The animals were anesthetized with 400 mg/kg chloral hydrate intraperitoneally (i.p.), followed by 0.05 ml atropine intramuscularly (i.m.). Subsequently, experimental cerebral hypoperfusion was induced in some of the animals by two-vessel occlusion ($n=72$), while the others served as sham-operated controls ($n=54$). The common carotid arteries were exposed via a ventral cervical incision, and separated from their sheaths and vagal nerves. Silk sutures (5.0) were used for the ligation. The same surgical procedure was performed on the sham-occluded group, but without the actual ligation.

Immediately after surgery, the rats were divided into 12 groups: Groups 1–3 and 7–9 involved sham-occluded animals, while those in Groups 4–6 and 10–12 had undergone two-vessel occlusion.

Groups 1–3 served as controls for Groups 4–6. Group 1 ($n=7$) received 0.5 ml 5% sodiumhydrogencarbonate i.p. on 3 consecutive days, the first injection being applied directly after surgery.

The Group 2 rats were treated with 3 mg/kg indomethacin ($n=8$) dissolved in 0.5 ml 0.9% saline i.p., while 15 mg/kg NS-398 ($n=9$) in 0.5 ml 5% sodiumhydrogencarbonate was administered i.p. to Group 3, likewise for 3 days.

The two-vessel occluded Groups 4–6 received solvent ($n=8$) or indomethacin ($n=10$) or NS-398 ($n=8$) treatment for 3 days, respectively, as described for Groups 1–3.

Group 7 ($n=9$), Group 8 ($n=8$), Group 9 ($n=7$) were treated analogously to Groups 1–3, respectively, with solvent, indomethacin and NS-398, as were the two-vessel occluded Groups 10 ($n=5$), 11 ($n=5$) and 12 ($n=8$).

Postoperatively, not all of the rats survived for a sufficiently long time to receive the third injection on postsurgery day 3. The data relating to the survival rates in the various groups are presented in Table 1.

Following completion of the 3-day course of injections, the surviving animals in Groups 1-6 were sacrificed and subjected to comparative immunocytochemical examinations, while the survivors in Groups 7–12 underwent spatial learning tests.

### 2.2. Spatial learning test

The animals in Groups 7–12 were trained in the Morris watermaze spatial learning test, starting 7 days after surgery. The test was carried out in accordance with a previously established protocol (Farkas et al., 2006). All rats performed 2 trials per day, with a constant interval of 4 h between the trials, for 5 consecutive days. The water tank ($d=160$ cm), containing a hidden platform submerged 2 cm below the water surface,

Table 1
Survival rates in the various experimental groups after 2VO or Sham surgery

| Experimental group | | | Survival rate |
|---|---|---|---|
| Groups 1 and 7 | Solvent | Sham | 16/17 (94%) |
| Groups 4 and 10 | | 2VO | 13/26 (50%) |
| Groups 2 and 8 | Indomethacin | Sham | 16/20 (80%) |
| Groups 5 and 11 | | 2VO | 15/24 (62%) |
| Groups 3 and 9 | NS-398 | Sham | 16/17 (94%) |
| Groups 6 and 12 | | 2VO | 16/22 (73%) |

The various experimental groups were injected with solvent, indomethacin or NS-398 immediately following the surgical procedure. The survival rate relates to the animals that survived up to and including the third injection. Abbreviations: 2VO: permanent, bilateral common carotid artery occlusion (two-vessel occlusion); Sham: sham-operated control. Survival rate was expressed as percentage of survived animals to total.

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was located in an experimental room equipped with various spatial and auditory extra maze cues. The animals were placed in the water at one of four starting quadrant points, which was varied randomly over the trials. The rats were given 2 min to find the platform and sit on it for 15 s. Swimming paths were recorded by a computerized video imaging analysis system (EthoVision, Noldus Information Technology BV, Wageningen, The Netherlands). For each of trials 2–10, the distance swam before reaching the platform, and the proportion of failed trials to the total number were analyzed. The distance swam in the 2 daily trials was calculated as a daily average for further data analysis.

### 2.3. Histology

The animals in Groups 1–6 were sacrificed 3 days after the operation, while those in Groups 7–12 were sacrificed 2 days after the Morris watermaze experiments (2 weeks after surgery). The procedures for the preparation of sections followed previously reported protocols (Farkas et al., 2006). Briefly, after deep pentobarbital anesthesia, the rats were perfused transcardially with 100 ml saline followed by 400 ml 4% paraformaldehyde. The brains were removed, postfixed, and cut to 20 μm thick, free-floating coronal sections on a cryostat. The first set of sections was stained with cresyl violet. For each animal 3 sections containing the dorsal hippocampus (bregma −3.60, Paxinos and Watson, 1986) were mounted on gelatin-coated microscopic slides and stained with cresyl violet in order to allow examination of the integrity of the CA1 and CA3 pyramidal cell layers and the DG granule cell layers. The proportion of animals displaying neuronal damage was determined with the help of the cresyl violet-stained sections (Annaházi et al., 2007). Hippocampal neuronal damage was evaluated at 40X magnification with a light microscope by an investigator blind to the experimental groups. Damage of a hippocampal region was considered when neurons showed typical signs of necrosis (dark staining, shrinkage and a dysmorphic shape). The number of animals showing necrotic neuronal injury was counted and expressed as percentage for each hippocampal region and each group.

The second set of sections was immunocytochemically stained for MAP-2 to visualize the dendritic integrity. Endogenous peroxidase activity was blocked with 0.3% $H_2O_2$, nonspecific binding sites were covered with 20% normal swine serum (NSS), and the membrane permeability was enhanced with 0.3% Triton X-100. The samples were then incubated overnight in a primary antibody solution containing mouse anti-MAP-2 antibody (Sigma), 1:10000, 20% NSS, and 0.03% merthiolate in 0.01 M PBS. The secondary antibody solution consisted of goat anti-mouse biotinylated IgG (Jackson) 1:400, 10% NSS, 0.03% merthiolate, 5% normal rabbit serum (NRS) in PBS. Finally, the sections were incubated in HRP-Streptavidin (Jackson) 1:1000, 1% NSS, 0.03% merthiolate in Tris–buffer solution (TBS). The color reaction was developed with Ni-diaminobenzidine (Ni-DAB) and $H_2O_2$.

In the third set of sections astrocytic reaction was immunocytochemically labeled with GFAP antibody. After pretreatment with 5% normal sheep serum (NShS), 0.3% $H_2O_2$ and 0.3% Triton X-100, the sections were incubated overnight in a solution containing the mouse anti-GFAP primary antibody (Sigma), 1:200, 1% NShS and 0.3% Triton-X in 0.01 M PBS. The secondary antibody solution consisted of goat anti-mouse biotinylated IgG (Jackson), 1:400, 10% NShS, 5% NRS and 0.03% merthiolate in 0.01 M PBS. Finally, the sections were incubated in HRP-Streptavidine (Zymed), 1:200, and the color reaction was developed conventionally with DAB and $H_2O_2$.

For the last set of sections, OX-42 antibody was used to detect and analyze microglia activation over the hippocampal area. After pretreatments similar to those for GFAP staining, the sections were incubated overnight in a primary antibody solution containing a biotinylated mouse anti-CD11b antibody (OX-42, Serotec), 1:500, 20% NSS and 0.03% merthiolate in 0.01 M PBS. Next, the sections were rinsed, and incubated in a solution of HRP-Streptavidin (Jackson), 1% NSS and 0.03% merthiolate in 0.1 M TBS for 1 h. The color reaction was developed with Ni-DAB and $H_2O_2$. All sections were mounted on gelatin-coated microscopic slides, air-dried, dehydrated and coverslipped with Distrene 80 (dibutyl phthalate with xylene).

The percentage surface areas of MAP-2 antibody-positive microtubules, GFAP-positive astrocytes and OX-42-positive immunoreactive microglia in the dorsal hippocampus were quantified by using a computerized image analysis system (Olympus BX50, P50, software: ImagePro Plus, Media Cybernetics, U.S.A.) as previously described (Farkas et al., 2004, 2006). Briefly, 3 consecutive coronal sections with a standard distance of 160 μm, starting at br. −3.60 mm were examined. After background subtraction and gray scale threshold determination, hippocampal regions of interest were delineated manually at 10X magnification. The area covered by immunopositive structures was computed as a percentage of the total area delineated. The measurements on the 3 sections per animal were averaged and the values were used for further statistical analysis.

### 2.4. Statistical analysis

The Morris maze test results were analyzed statistically by means of a two-way repeated measurement model, followed by the LSD (least significant difference) post hoc test of the program SPSS. Individual day comparisons were performed with a univariate model and LSD post hoc analysis of SPSS. The proportions of failed trials to total and the proportion of animals displaying neuronal damage in the hippocampus were analyzed statistically by a chi square test of the program Statistica. MAP-2, GFAP and OX-42 immunocytochemical results were analyzed statistically with three-way ANOVA, followed by the LSD post hoc test.

## 3. Results

Table 1 presents the survival rates. Two-vessel occlusion resulted in a survival rate of only 50% in the solvent-treated groups (Groups 4 and 10) versus 94% in the sham-operated vehicle-treated groups (Groups 1 and 7). The treatments with

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COX inhibitors did not significantly alter the mortality in the sham-occluded groups (80% [Groups 2 and 8] and 94% [Group 3 and 9] vs 94% [Groups 1 and 7]). However, the indomethacin treatment exerted a slightly beneficial effect on the survival rate of the two-vessel occluded rats 63% (Groups 5 and 11) vs 50% (Group 4 and 10), while NS-398 improved the survival rate of the two-vessel occluded rats markedly (73% [Groups 6 and 12] vs 50% [Groups 4 and 10]).

In the Morris watermaze, the distance swam by the sham-occluded animals gradually decreased during the 5-day learning test (Group 7), while the learning capacity of the solvent-treated two-vessel occluded rats (Group 10) proved significantly worse ($P<0.001$) than that of the sham-occluded controls (Group 7) (Fig. 1A). The administration of either COX inhibitor did not affect the learning ability of the sham-occluded animals. NS-398 treatment significantly improved the performance of the two-vessel occluded rats throughout the period of learning as compared with the two-vessel occluded controls (Group 12) ($P<0.024$). The indomethacin-treated two-vessel occluded group (Group 11) performed poorly on days 3 and 4 as compared with the sham-occluded groups and Group 12, but delivered optimal performance by day 5. A similar tendency could be observed concerning to the proportion of failed trials to the total number (Fig. 1B).The two-vessel occluded solvent-treated group (Group 10) displayed a significantly elevated ratio of failed trials to the total number compared with the sham-operated animals (Group 7, 8 and 9). Treatment with NS-398 resulted in significantly less failed trials as compared with the two-vessel occluded controls (Group 12) ($P<0.0014$).

Following surgery and COX-inhibitor treatments, the proportion of animals with neuronal damage in the CA1 and CA3 pyramidal cell and DG granule cell layers of the hippocampus exhibited different spatial and temporal distributions (Fig. 2). The sham operation caused only mild damage in the CA1, CA3 and DG at either 3 days or 2 weeks postoperatively, affecting 0–22% of the animals (Groups 1 and 7). In contrast, 3 days after two-vessel occlusion (Group 4), neuronal injury was seen particularly in the CA3 and DG regions (38%). At 2 weeks, the presence of damaged neurons was less obvious (20%) in the CA3 and DG, whereas it was more pronounced in the CA1 area (40%) (Group 10). Both treatments altered the incidence of neuronal damage in the hippocampus. Indomethacin aggravated the neuronal injury in the CA1 and CA3 regions at 3 days after two-vessel occlusion (50–60%) (Group 5), compared with its sham-occluded control (0–11%) (Group 2) ($P<0.05$; $P<0.01$), but reduced the proportion of affected animals 2 weeks after the onset of two-vessel occlusion (0%) (Group 11). NS-398 displayed only similar tendencies in the CA1 region, but clearly enhanced the neuronal injury in the DG both 3 days and 2 weeks after two-vessel occlusion surgery in comparison with its respective solvent-treated groups (Group 6 [100%] vs Group 4 [50%], $P<0.01$ and Group 9 [85%] vs Group 7 [22%], $P<0.05$, respectively). However, this finding indicates that the damaged neurons after 3 days have partially been cleared away by microglia after 2 weeks in the CA3 and DG regions.

The changes in MAP-2 antibody signal can be in coherence with cerebral hypoperfusion- and time-related changes in the stratum lucidum of the hippocampal CA3 region. Two-vessel occlusion significantly decreased MAP-2 immunoreactivity (Groups 4 and 10) as compared with the sham-occluded animals (Groups 1 and 7) both 3 days and 2 weeks following surgery (Fig. 3). Additionally, the MAP-2 antibody signal decreased in all the sham and two-vessel occluded groups at 2 weeks (Groups 7–12) as compared with that at 3 days (Groups 1–6) after surgery (Fig. 3). The indomethacin and NS-398 treatments did not prevent the two-vessel occlusion-induced dendritic injury in this region at either time point.

Three days after the onset of two-vessel occlusion, astrocytic activation in the CA1 stratum radiatum labeled with GFAP antibody exhibited a strong basal signal, which increased

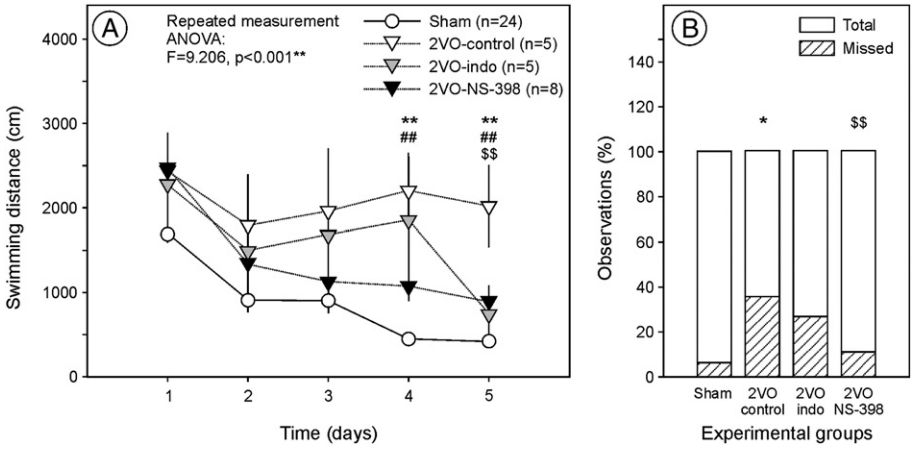


Fig. 1. Performance of the rats ($n=42$) in the Morris watermaze. Panel A: swimming distance (cm) in the Morris maze on each experimental day. Data are presented as means±S.E.M. Statistical analysis of the acquisition trials was performed with a repeated measures ANOVA model followed by an LSD post hoc analysis for group comparison of daily performance. Significance levels of the post hoc test are given as: $^{**}P<0.01$ (Groups 10, 11, 12 vs Sham), $^{\#\#}P<0.01$ (Group 11 vs Group 10), $^{\$\$}P<0.01$ (Group 12 vs Group 10). Panel B: Proportion of missed trials to total between trials 2–10. Data are expressed as percentage averages (%). Statistical analysis was performed with a non-parametric chi-square test. Significance levels of the post hoc test are given as: $^{*}P<0.05$ (Group 10 vs Sham), $^{\$\$}P<0.01$ (Group 12 vs Group 10). Abbreviations: 2VO: permanent, bilateral common carotid artery occlusion (two-vessel occlusion); Sham: sham-operated control.

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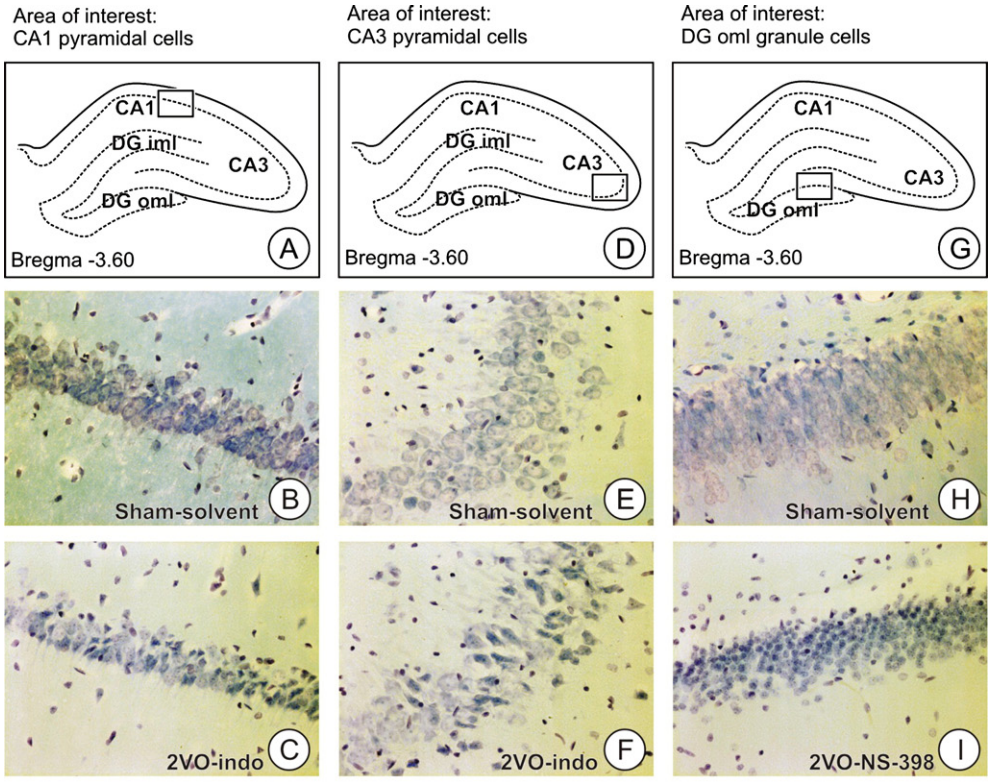


J The number and proportion of animals with neuronal damage in the hippocampal areas:

| | | | CA1 | | CA3 | | DG | |
|---|---|---|---|---|---|---|---|---|
| Time to post-operative sacrifice | Surgery | Treatment | n (damaged/ total) | % | n (damaged/ total) | % | n (damaged/ total) | % |
| 3 days | Sham | solvent | 0/7 | 0.00 | 1/7 | 14.29 | 1/7 | 14.29 |
| | | indo | 0/8 | 0.00 | 0/8 | 0.00 | 1/8 | 12.50 |
| | | NS-398 | 1/9 | 11.11 | 2/9 | 22.22 | 4/9 | 44.44 |
| | 2VO | solvent | 1/8 | 12.50 | 3/8 | 37.50 | 3/8 | 37.50 |
| | | indo | 5/10 | 50.00* | 6/10 | 60.00** | 4/10 | 40.00 |
| | | NS-398 | 3/8 | 37.50 | 3/8 | 37.50 | 8/8 | 100.00## |
| 2 weeks | Sham | solvent | 0/9 | 0.00 | 1/9 | 11.11 | 2/9 | 22.22 |
| | | indo | 0/8 | 0.00 | 1/8 | 12.50 | 2/8 | 25.00 |
| | | NS-398 | 0/7 | 0.00 | 4/7 | 57.14 | 6/7 | 85.71 |
| | 2VO | solvent | 2/5 | 40.00 | 1/5 | 20.00 | 1/5 | 20.00 |
| | | indo | 0/5 | 0.00 | 0/5 | 0.00 | 2/5 | 40.00 |
| | | NS-398 | 1/8 | 12.50 | 0/8 | 0.00$ | 4/8 | 50.00 |

Fig. 2. Cresyl violet staining and the proportions of animals displaying neuronal damage in the dorsal hippocampus (bregma −3.60). Area of interest: CA1 stratum (str.) pyramidale (Panel A), CA3 str. pyramidale (Panel D) and dentate gyrus outer molecular layer (DG oml) (Panel G). Representative photomicrographs of the hippocampal CA1 (Panels B and C), CA3 (Panels E and F) and DG (Panels H and I) at 40× magnification. White arrow heads are pointing at intact pyramidal and granular neurons. Black arrow heads are pointing at damaged pyramidal and granular neurons. Panel J: Semiquantitative data on the proportions of damaged CA1, CA3 and DG in the hippocampus. Statistical analysis was performed with a non-parametric chi-square test. Significance levels of the post hoc test are given as: *$P<0.05$, **$P<0.01$ (Group 5 vs Group 2), $^{\$}P<0.05$ (Group 12 vs Group 10), $^{\#\#}P<0.01$ (Group 6 vs Group 4). Abbreviations: CA: cornu ammonis; DG: dentate gyrus; 2VO; permanent, bilateral common carotid artery occlusion (two-vessel occlusion); Sham: sham-operated controls.

moderately, particularly in the NS-398-treated two-vessel occluded group (Group 6) as compared with its sham control (Group 3) (Fig. 4). The survival time proved to be the most sensitive parameter for the GFAP antibody labeling. The GFAP signal was ~4 times higher at 2 weeks (Groups 7–12) than at 3 days (Groups 1–6) in all experimental groups (Fig. 4). The COX inhibitors exerted no effect on the GFAP levels at either time point.

The changes in OX-42 antibody-labeled microglial activation revealed a pattern similar to that for the GFAP antibody signal (Fig. 5). Two-vessel occlusion tended to increase the microglial activation, although the changes were not significant

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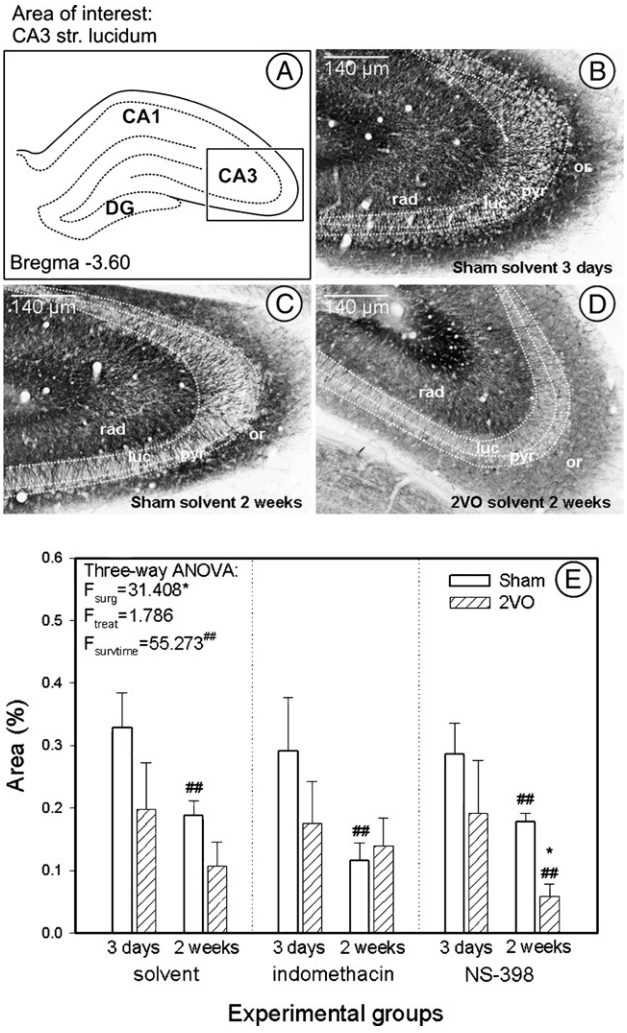


Fig. 3. MAP-2 immunocytochemistry relating to synaptic density. Panel A: Area of interest: CA3 str. lucidum in the dorsal hippocampus (bregma −3.60). Panels B–D: Representative photomicrographs of the CA3 area of the hippocampus. Panel E: Quantitative data on MAP-2 immunocytochemistry in the hippocampal CA3 str. lucidum. Data are presented as means±S.E.M.; $^{*}P<0.05$; $^{\#\#}P<0.01$. The *F* value indicated in the graph was obtained with the LSD (least significant difference) post hoc test. Abbreviations: 2VO: permanent, bilateral common carotid artery occlusion (two-vessel occlusion); Sham: sham-operated controls; CA: cornu ammonis; or: stratum oriens; pyr: stratum pyramidale; rad: stratum radiatum, luc: str. lucidum.

because of the high individual variation (Group 2, 4 and 6). Nevertheless, the OX-42 antibody signal rose significantly from 3 days to 2 weeks after surgery in both the sham and the two-vessel occluded animals (Groups 7–12 vs Groups 1–6). The treatments did not modify the OX-42 antibody-labeled microglial activation in the experimental groups.

## 4. Discussion

The effects of the postoperatively applied nonselective COX inhibitor indomethacin, and the selective COX-2 inhibitor NS-398 on the learning ability and on the neuronal damage in the hippocampus were characterized shortly after the onset of two-vessel occlusion in the rat. The pharmacological effects of the COX inhibitors were assessed at two postoperative time points, which additionally allowed visualization of the time-related, early histological changes in the hippocampus of the two-vessel occluded rats. A few studies have already set out to investigate the progression of neurodegenerative processes via the two-vessel occlusion model, but these focused on late events (i.e. 4 weeks–6 months after the onset of two-vessel occlusion) (Liu et al., 2005; Schmidt-Kastner et al., 2005), while no data are available from the early period of hypoperfusion. Our present experiments permit an analysis of the incidents in the first 2 weeks after the initiation of two-vessel occlusion.

In the first 2 weeks, two-vessel occlusion induced a detectable spatial learning deficit, mild neuronal damage, injury to the dendrites and a slight increase in glial cell proliferation, which correspond with previous observations (Farkas et al., 2006; Liu et al., 2005; Schmidt-Kastner et al., 2005; Watanabe et al., 2006).

NS-398 post-treatment improved the survival rate and the spatial learning ability, but proved detrimental for hippocampal

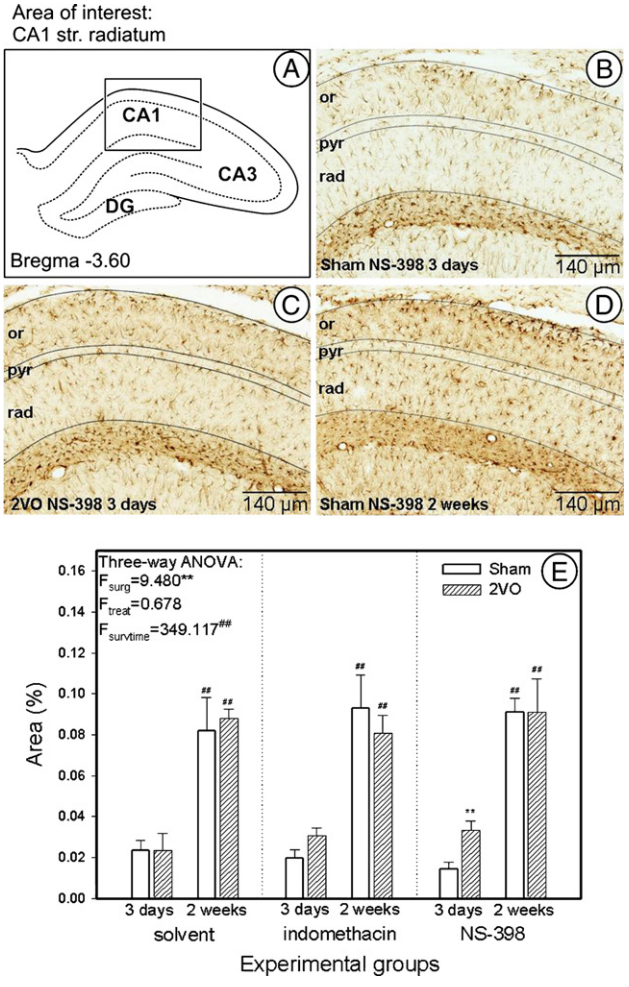


Fig. 4. Astrocytic proliferation in the CA1 str. radiatum of the hippocampus labeled by GFAP immunocytochemistry. Images were taken at 10x magnification (Panel B–E). Data are presented as means±S.E.M.; $^{*}P<0.05$; $^{\#\#}P<0.01$ (Panel E). Abbreviations: 2VO: permanent, bilateral common carotid artery occlusion (two-vessel occlusion); Sham: sham-operated controls; CA: cornu ammonis; or: stratum oriens; pyr: stratum pyramidale; rad: stratum radiatum.

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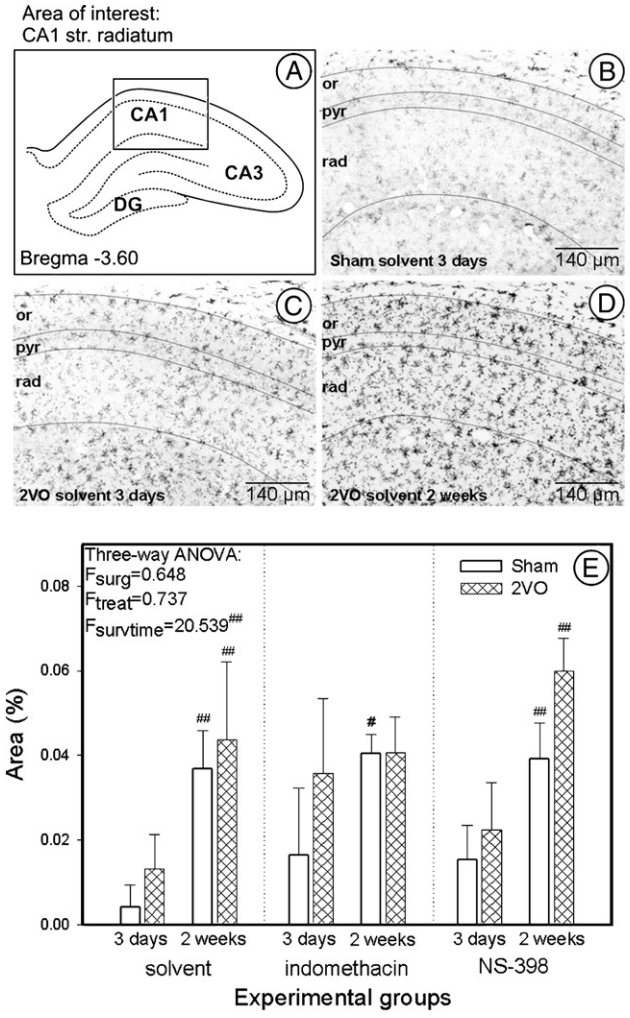


Fig. 5. Quantitative analysis of OX-42 immunocytochemistry indicating microglia activation in the hippocampus CA1 str. radiatum. Significance values were obtained with the LSD post hoc test (**$P<0.01$; ##$P<0.01$). Panels B–D: representative photomicrographs of the CA1 area of the hippocampus (10× magnification). Abbreviations: 2VO: permanent, bilateral common carotid artery occlusion (two-vessel occlusion); Sham: sham-operated controls; CA: cornu ammonis; or: stratum oriens; pyr: stratum pyramidale; rad: stratum radiatum.

neuronal death in the two-vessel occluded rats. This COX inhibition had no influence on the dendritic arborization, astrocytic reaction or microglia activation.

Indomethacin did not improve the survival rate. The probable reason for this is the direct vasoconstriction of the cerebral microvessels by indomethacin, which leads to more severe ischemia in the hypoperfused brain (Hoffmeyer et al., 2007). The beneficial effects of NS-398 on the survival rate might be due to a decrease in the inflammatory reaction of the respiratory tract, arising from vagal nerve manipulation during two-vessel occlusion surgery. Additionally, NS-398 did not affect the cerebral perfusion.

NS-398 significantly alleviated the learning deficit of the two-vessel occluded rats. This finding supposes the role of COX-2 in the ischemia-induced damage in the brain regions to be responsible for spatial learning. However, there is no clear-cut consensus about the regions involved in spatial memory. The area most frequently observed in connection with spatial learning is the CA1 region of the hippocampus. A number of studies have found a direct correlation between a cerebral hypoperfusion-induced memory deficit and CA1 damage (De Jong et al., 1999; Nunn and Hodges, 1994; Pappas et al., 1996). An impaired learning performance 3 weeks after two-vessel occlusion coincided with a significant pyramidal cell loss in the CA1 region (Xiong et al., 2006). Others found no or only a weak correlation between the diminished CA1 neuron number and the Morris maze performance (Jaspers et al., 1990; Lyeth et al., 1990; Olsen et al., 1994). Auer et al. reported that only a neuron loss exceeding 50% in the CA1 pyramidal cell layer resulted in a memory deficit (Auer et al., 1989). We earlier demonstrated that only a mild reduction in neuronal cell number was observed through cresyl violet staining in the hippocampus 2 weeks after two-vessel occlusion (Farkas et al., 2006), when the extent of apoptotic cell death, as indicated by caspase-3 staining in the hippocampus was negligible (unpublished data). In a model of global cerebral ischemia, Henrich-Noack et al. demonstrated that population spike generation in the DG granular cell layer was greatly decreased as early as 1 day post-ischemia, when compared to pre-ischemic values and to sham-operated animals. The functional impairment was detected despite an apparently intact morphology of granular cells as evidenced by Nissl-staining (Henrich-Noack et al., 2005). Therefore it cannot be excluded that at least part of the surviving neurons in the two-vessel occluded animals may be functionally impaired and may not exhibit proper long-term potentiation (LTP). This, in turn, may also limit spatial learning. However, the administration of NS-398 to *in vitro* CA1 pyramidal and DG granular cell culture significantly reduced LTP and long-term depression (LTD) (Murray and O'Connor, 2003). This finding suggests that the memory-preserving effect of the COX-2 inhibitor NS-398 is probably not related directly to the electrical disturbances of the surviving hippocampal cells. The pyramidal and granular cells in the hippocampus are not the exclusive participants in spatial memory; other parts of the hippocampal formation such as the entorhinal cortex, the parahippocampal gyrus, and the rhinal and cingular gyri are also involved (Meunier et al., 1996; Wiig and Bilkey, 1994). Moreover, the memory deficit correlates with the white matter damage in two-vessel occluded rats (Wakita et al., 1994; Watanabe et al., 2006). In summary, a direct link cannot be established between ischemia-induced memory failure and the appearance of neuronal damage in the hippocampus.

Indomethacin or NS-398 treatment alone increased the incidence of damage in the hippocampal pyramidal and granular cell layers in the two-vessel occluded and even in the sham-operated animals. The role of COX in ischemic neuronal damage has long been controversial. The functioning of COX gives rise to the toxic free radical superoxide anion, which can be a source of neuronal damage. On the other hand, a recent finding suggests that COX-2 is not the major source of oxygen radicals after transient cerebral ischemia (Kunz et al., 2007). The detrimental impact of COX-2 in cerebral ischemia is putatively mediated by the production of $PGE_2$ through the prostanoid EP1 receptor (Kawano et al., 2006). Whereas most

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articles are indicative for COX inhibition during ischemia, some references support the neuroprotective effect of COX-derived prostanoids. For example, $PGE_2$, which is highly expressed in the hippocampus, proves neuroprotective by activation of the prostanoid EP2 receptor in neuron cultures and in organotypic hippocampal slices against ischemia and oxygen-glucose deprivation, respectively (de la Torre and Aliev, 2005). Genetic depletion of the prostanoid EP2 receptor in mice resulted in a significantly larger infarct after middle cerebral artery occlusion with reperfusion (McCullough et al., 2004). $PGD_2$, another prostanoid, has been shown to prevent neuron loss in response to glutamate excitotoxicity *in vitro* (Liang et al., 2005). Treatment with iloprost (a stable $PGI_2$ analog) after two-vessel occlusion prevented hypoperfusion-induced lipid peroxidation (Aytac et al., 2006). Since two-vessel occlusion-induced oligemia does not cause a serious neuron loss, these COX derivatives may play an important role in the metabolic adaptation of hippocampal neurons. In this respect, the COX function is more important as concerns the neuronal viability, rather than its free radical production in the oligemic state. Finally, COX-2 inhibitors downregulate $PGI_2$ and upregulate thromboxane $A_2$, resulting in a prothrombotic state (FitzGerald, 2003); selective COX-2 inhibition can therefore induce microvascular thrombosis in the hippocampus. Another aspect of neuronal damage in COX inhibition is the elevated turnover of arachidonic acid to eicosanoids by an enhanced lipoxygenase function. 5-Lipoxygenase-derived eicosanoids cause neuronal damage in the cortex during 14 days after 1 h of transient MCAO (Zhou et al., 2006). Our results do not substantiate the neuroprotective action of selective and nonselective COX inhibitors in mild ischemia, in spite of their beneficial effects on memory.

In our experimental setup, significant time-dependent changes could be observed postoperatively in the cellular markers in the hippocampus. The MAP-2 antibody signaling exhibited a two-vessel occlusion and time-dependent decrease, while the results of GFAP and OX-42 immunocytochemistry in the CA1 str. radiatum indicated extremely enhanced astrocytic and microglia activation 2 weeks vs 3 days after surgery, in both the sham and the two-vessel occluded animals.

It was earlier reported that chronic cerebral hypoperfusion gradually reduces the MAP-2 protein level from 4 weeks to 20 weeks after two-vessel occlusion, this decrease displaying a linear correlation with the learning impairment in the Morris maze (Liu et al., 2005). Our results confirm this relationship and provide evidence of a gradual loss of MAP-2-positive dendrites even at 3 days after two-vessel occlusion. Further, the present data are strongly supported by our previous observations that the density of MAP-2-positive dendrites considerable decreases 2 weeks after the induction of two-vessel occlusion (Annaházi et al., 2007). However, the current data suggests that surgery alone also has a significant impact on MAP-2 staining. Therefore the interaction between treatments and survival time may obscure treatment-related changes.

Astrocytic activation and proliferation which are known to develop under ischemic conditions indicates neuronal injury or affords protection against neurodegeneration (Vibulsreth et al., 1987). The microglia plays a pivotal role in neurodegeneration. During ischemia, it acts as a double-edged weapon. It provides protection by the phagocytosis of cellular detritus, but it exerts cytotoxicity through reactive oxygen species and cytokine production (Gehrmann et al., 1995; Kreutzberg, 1996). Cerebral hypoperfusion alone did not affect these markers significantly in the early period following two-vessel occlusion, but the evolution of astrocytic proliferation and microglia activation can be detected 13 weeks after two-vessel occlusion in various regions of the rat brain (Farkas et al., 2004; 2005). The parallel changes in the GFAP antibody and OX-42 antibody signals in the sham and two-vessel occluded groups are likely to be a consequence of a similar detrimental impact. Probably the most damaging event during the sham operation is the procedure of the separation of carotid artery from the vagal nerve, when the vessel is compressed for several seconds. However, less than 10 min of hypoxia failed to induce microglia activation in hippocampus slice preparations (Abraham et al., 2001). Schmidt-Kastner et al. reported a significantly enhanced astrocytic proliferation from 1 week to 6 months after two-vessel occlusion in the optic tract and in the corpus callosum (Schmidt-Kastner et al., 2005), which supposes a region-specific distribution of glial activation in this model.

In conclusion, we have provided a description of the changes in the most important morphological markers in the hippocampus in the first 2 weeks of experimental cerebral hypoperfusion. Two-vessel occlusion induced a protracted period of neuronal death, progressive dendritic lesions and astrocytic activation in the hippocampus. The selective inhibition of COX-2 exerts a more beneficial effect on the cognitive impairment than that of the nonselective COX inhibition by indomethacin in the hypoperfused brain. No relationship could be discerned between the advantageous effects of NS-398 on the memory capacity and the histological changes in the early period of cerebral hypoperfusion. Thus, the presence of hippocampal neuronal damage alone does not correlate with the spatial memory impairment in the two-vessel occlusion model. To elucidate the mechanism of action of NS-398 on spatial learning, and to detect the differences between NS-398 and indomethacin treatment, further investigations are necessary.

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