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Research Report

Intracerebroventricular administration of creatine protects against damage by global cerebral ischemia in rat

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ABSTRACT

Although a large body of evidence shows that pretreatment of brain tissue with creatine protects against anoxic injury in vitro, only a couple of papers have investigated creatine protection in vivo, and they yielded conflicting results. We attempted to clarify how creatine may be protective in an in vivo model of global cerebral ischemia (GCI). We administered creatine either before of after GCI. We decided to administer it by intracerebroventricular infusion, to maximize its bioavailability to the brain. Our findings show that creatine is clearly protective in vivo when administered before ischemia. In that case, histological evaluation of damage was consistently improved in all regions examined, and neurological score was better in creatine-treated rats than in controls. When administered after ischemia, histology was improved in the hippocampus, while only a not significant trend toward improvement was observed in the cerebral cortex and in the caudo-putamen. Neurological score was not improved by creatine administration after GCI. Our findings show that creatine administration is protective in vivo. Such protection was clear in the case of pretreatment, and was present, to a lesser degree, when treatment was started after ischemia. Our results should encourage further research in the possible role of creatine therapy in neuroprotection.

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1. Introduction

One of the most important determinants of irreversible brain ischemic damage is decreased ATP (Lipton and Whittingham, 1982; Obrenovitch et al., 1988; Yager et al., 1994). Phosphocreatine can to some extent prevent the dangerous fall of ATP levels by donating its phosphate group to ADP to resynthesize ATP even in the absence of oxygen and glucose (Clarke and Sokoloff, 1999; Wyss and Kaddurah-Daouk, 2000).

However, the tissue content of phosphocreatine is limited, and its depletion during anoxia or ischemia is unavoidable. Brain PCr can be substantially increased by treatment with creatine. In fact, when excess Cr is available, the creatine kinase reaction ($Cr + ATP \rightarrow PCr + ADP + H^+$) is shifted toward the synthesis of PCr. Twenty years ago, it was shown (Whittingham and Lipton, 1981) that by incubating in vitro rat hippocampal slices with a high Cr concentration (25 mM), their PCr content increased four-fold, from 40 μmol/g protein

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to approximately 150 μmol/g protein. Accordingly, this treatment was able to prevent the irreversible loss of synaptic transmission in rat hippocampal slices after a 10 min' deprivation of oxygen (Kass and Lipton, 1982). This protection was accompanied by a partial conservation of ATP in anoxic slices: in Cr-treated slices ATP after anoxia fell to 7.9 μmol/g protein, while in control slices it fell to 3.6 μmol/g protein (control preanoxic content: 13.9 μmol/g protein) (Kass and Lipton, 1982). Subsequently, it was shown that pretreatment with creatine also prevented anoxia-induced decrease in protein synthesis in rat hippocampal slices (Carter et al., 1995). In the following years, it has been repeatedly demonstrated in "in vitro" models that administration of creatine increases the neural content of phosphocreatine, delays ATP depletion and protects against harmful effects of anoxia (Balestrino et al., 1999a; Balestrino et al., 2002; Carter et al., 1995; Kass and Lipton, 1982; Wilken et al., 1998; Yoneda et al., 1983; Zapara et al., 2004). Although the data from in vitro models have been repeatedly confirmed and appear quite solid, of the only existing 2 studies of in vivo oral creatine administration one has shown effectiveness (Zhu et al., 2004) while the other one has shown negative results (Wick et al., 1999).

Moreover, an antioxidant action by creatine has also been reported (Lawler et al., 2002), and this, too, could in theory participate in neuroprotection by creatine.

One of the limiting problems that are encountered when transferring "in vitro" findings into "in vivo" protec-tion is the poor brain bioavailability of systemically administered creatine. Creatine is a very polar molecule, thus it crosses with difficulty the blood–brain barrier. It is carried across the barrier by a transporter (Snow and Murphy, 2001), but its brain accumulation after systemic administration is nevertheless very slow and limited, even after administration of high parenteral doses (Perasso et al., 2003). By contrast, "in vitro" protection by creatine requires high concentrations of the compound, about 1 mM for a 3-h incubation period (Balestrino et al., 1999a). Thus, it is little surprising that the dramatic protection by creatine treatment in vitro has been only inconsistently reproduced by oral administration of creatine "in vivo" (Wick et al., 1999; Zhu et al., 2004) (one should also add a single report showing in vivo protection of biochemical parameters by systemically administered phosphocreatine, a compound analog to but different from creatine; Rauchovà et al., 2002).

In the present paper, we investigated whether or not creatine affords protection against an in vivo model of cerebral ischemia when administered directly to the brain by way of an osmotic pump implanted in the lateral ventricle of a rat, a method that some of us have previously used to successfully increase brain creatine and phosphocreatine in vivo (Rebaudo et al., 2000b). While this way of administration may be impractical in humans, it offers the advantage of bypassing the blood–brain barrier, thus permitting us to investigate whether or
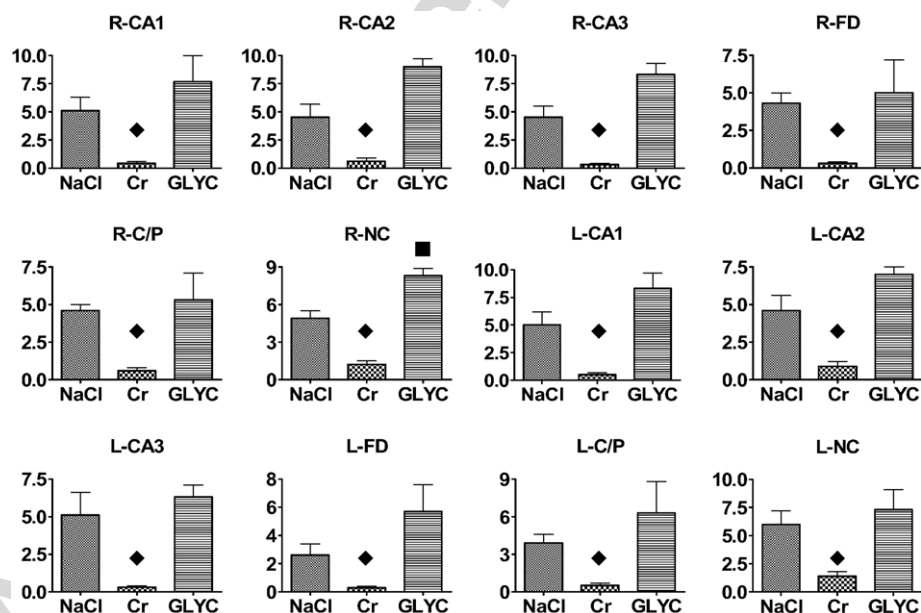

Fig. 1 – Pretreatment with creatine, but not with glycerol, significantly improves ischemic damage as evaluated by histology. All treatments were administered ICV through an Alzet pump implanted into the left lateral ventricle before, during and after ischemia (see text). Bars show mean and SEM. NaCl=animals treated with saline solution (*N*=8); Cr=animals treated with 50 mM creatine (*N*=11); Glyc=animals treated with 50 mM glycerol (*N*=3). On the ordinate axis, percentage of picnotic neurons in the various regions. R=right, L=left. CA1, CA2 and CA3=Cornu Ammonis region 1, 2 and 3 of the hippocampus. FD=Fascia Dentata. C/P=Caudato/Putamen. NC=NeoCortex. Analysis of variance showed a highly significant effect of treatment in all cases (probability values between 0.0001 and 0.0008). Diamonds show significant difference of creatine treatment in comparison with NaCl (*p*<0.005, Bonferroni post hoc test). The black square in the R-NC graph shows the only case where glycerol treatment was different (worse) than NaCl (*p*<0.005, Bonferroni post hoc test).

not the protection that has been consistently reported by creatine in vitro can be reproduced in vivo. We investigated the neuroprotective effect of creatine when administered either before ischemia (in a "prophylactic" protocol) or shortly after it (in a "therapeutic" protocol). We chose the global cerebral ischemia model because it reproduces some of the pathophysiological determinants of stroke and is a model of the global cerebral ischemia that often occurs in open heart surgery (Herczeg et al., 1996; Johansson et al., 1995; Shaaban et al., 2000), a clinical condition where pretreatment is realistically feasible and can be expected to be effective.

We evaluated the effects of creatine administration using not only histology but also a functional examination. The latter is increasingly recommended in the evaluation of neuroprotection in animal models (Hunter et al., 1998; Zausinger et al., 2000). To the best of our knowledge, there are still no data regarding the effects of creatine treatment on the functional outcome after brain ischemia.

2. Results

2.1. Histology

2.1.1. "Prophylactic" protocol of Cr administration (pretreatment)

Fig. 1 (left and middle bars of each small graph) shows the results of histological examination in control rats (i.e., infused i.c.v. with saline solution before ischemia), and in rats infused i.c.v. with 50 mM creatine before ischemia ("prophylactic" protocol). In all regions examined, creatine-pretreated rats showed a lower degree of damage. This difference was always statistically significant compared to controls.

Fig. 2 shows sample of histological findings from CA1 and, respectively, CA3 hippocampal regions.

We checked whether or not the protective effect of creatine infusion could be due to a specific effect of the high osmolarity of the solution. To this aim, we studied a group in which 50 mM glycerol was infused i.c.v. through Alzet pumps for
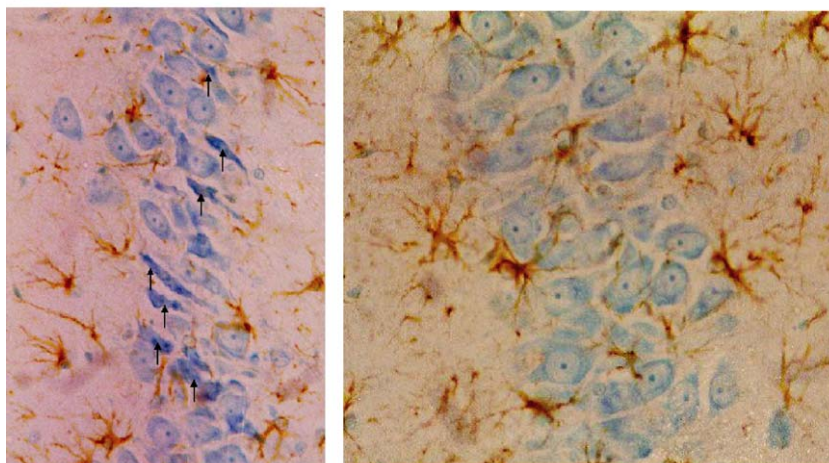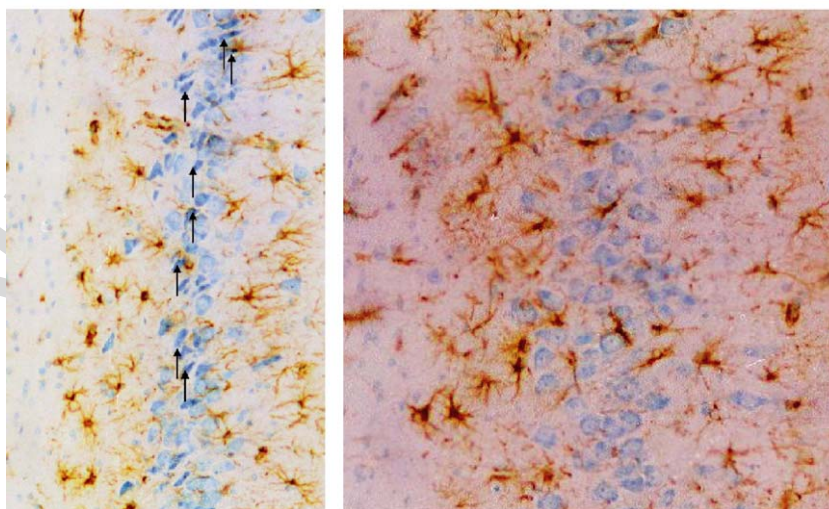

Fig. 2 – Microphotographs of CA1 hyppocampal region of rats subjected to global cerebral ischemia after pretratment with saline (left) or creatine (right) i.c.v. infusion. Shrunken neurons are indicated by arrows.

13 days (5 before and 7 after GCI). Results are reported in Fig. 1 (rightmost bar of each small graph). High-osmolarity glycerol infusion caused in all regions a worse damage than saline solution. Even considering the small numbers of glycerol-treated rats, it is clear that high-osmolarity alone does not justify the benefit observed in creatine administration. The glycerol-induced worsening was statistically significant in the right neocortex (R-NC, Fig. 1) despite the low numbers involved. Thus, the effect of creatine pretreatment is not due to the high osmolarity of the solution.

2.1.2. *"Treatment" protocol of Cr administration (administration after ischemia)*

Fig. 3 shows the histology results in rats treated with either saline solution or 50 mM creatine starting half an hour after ischemia ("therapeutic" protocol). A non-significant trend towards lesser damage in creatine-treated rats was seen, but a statistically significant difference was observed only in both hippocampi with the exceptions of the right CA3 region ($p = 0.08$, t test) and of the left fascia dentata ($p = 0.14$, t test).

2.2. *Neurological function*

2.2.1. *"Prophylactic" protocol of Cr administration (pretreatment)*

Neurological scores are reported in Fig. 4 (top graph). As it can be seen, at baseline (before ischemia) there was no significant difference between saline- and creatine-infused rats. Two days after ischemia, the score of saline-treated rats was

significantly deteriorated, while this deterioration was not seen in creatine-treated rats.

Again, to test that this protective effect of creatine was not due to the very high osmotic concentration (50 mM) of the creatine solution we used, we carried out experiments where rats were pretreated according to the same "Prophylaxis" protocol with a 50 mM glycerol in saline solution. Results are shown in Fig. 5. It is clear that glycerol could not duplicate the protective effect of creatine.

2.2.2. *"Treatment" protocol of Cr administration (administration after ischemia)*

Neurological scores are reported in Fig. 4 (bottom graph). Deterioration is observed in both groups, with no significant difference between controls and creatine-treated rats.

3. Discussion

This paper shows that creatine is able to protect neuronal tissue in global cerebral ischemia when administered before ischemia or, to a lesser extent, after it. For pretreatment ("Prophylaxis" protocol) both histology (Fig. 1) and neurological score (Fig. 4) were improved by pretreatment with creatine.

So far, only 2 papers have investigated protection by creatine in in vivo ischemia, yielding conflicting results (Wick et al., 1999; Zhu et al., 2004). A third paper has
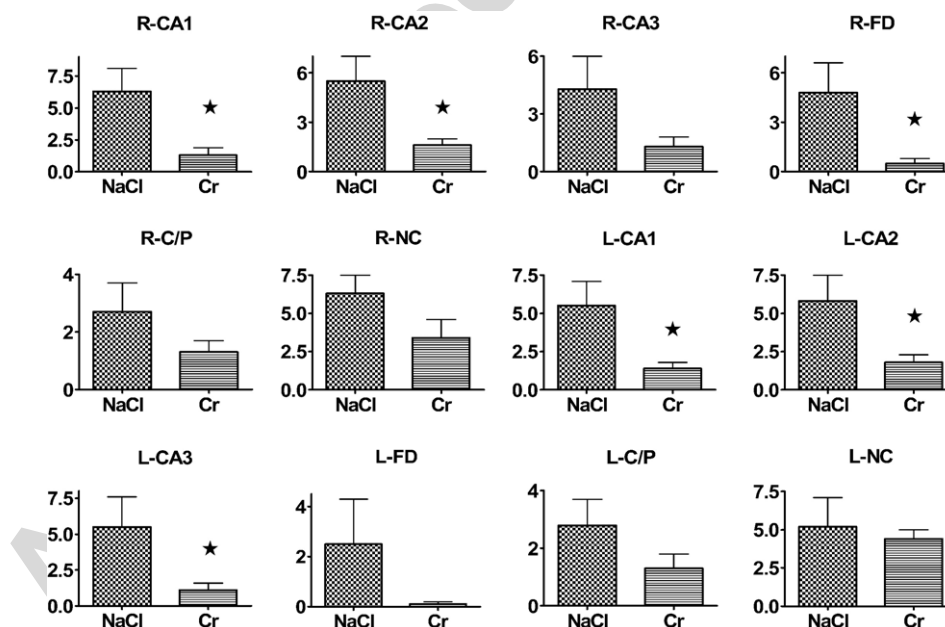
Fig. 3 – Treatment with creatine after ischemia significantly improves ischemic damage, as evaluated by histology, in some but not all the regions of interest. All treatments were administered ICV through an Alzet pump implanted into the left lateral ventricle, beginning 30 min after ischemia (see text). Bars show mean and SEM. NaCl = animals treated with saline solution (N = 6); Cr = animals treated with 50 mM creatine (N = 8). On the ordinate axis, percentage of picnotic neurons in the various regions. R = right, L = left. CA1, CA2 and CA3 = Cornu Ammonis region 1, 2 and 3 of the hippocampus. FD = Fascia Dentata. C/P = Caudato/Putamen. NC = NeoCortex. Stars show significant differences (probability values between 0.01 and 0.04 in all cases, *t* test).

A - "Prophylactic" protocol
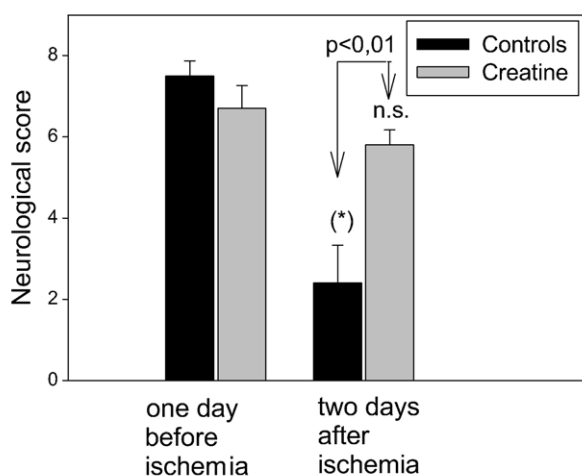
B - "Treatment" protocol

Fig. 4 – Neurological scores of rats subjected to ischemia and infused i.c.v. with saline solution (controls) and rats subjected to ischemia infused i.c.v. with 50 mM creatine. The lowe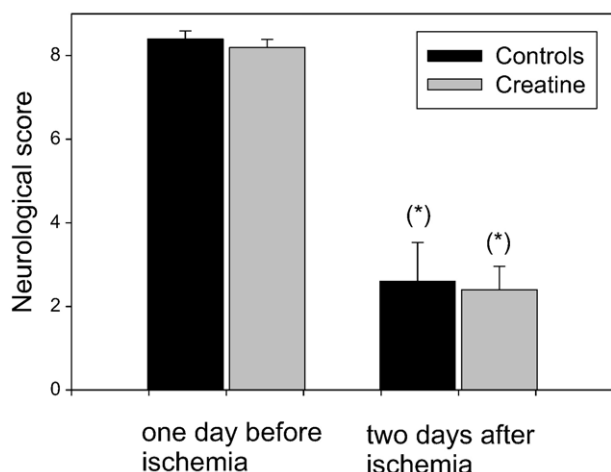r the score, the more severe the impairment. Values are expressed as means and SEM. Asterisks show statistically significant difference as compared to one day before ischemia ($p < 0.05$, Wilcoxon test); n.s.: not significant as compared to one day before ischemia. A significant difference was observed between the two groups at 2 days after ischemia in the "prophylaxis" protocol (bracketed arrows, Mann–Whitney test). Number of rats was as follows: (A) "Prophylaxis" protocol: $N = 18$ in the controls group, $N = 9$ in the creatine group. (B) "Treatment" protocol: $N = 13$ in the controls group, $N = 27$ in creatine group.

reported protection against biochemical parameters in vivo by treatment with phosphocreatine, a compound similar to albeit different from creatine (Rauchovà et al., 2002). At variance with these previous investigations, we chose to administer creatine intraventricularly because some of us previously showed that intracerebroventricular creatine causes a large (67%) increase in cerebral phosphocreatine

(Rebaudo et al., 2000a), comparable to that after in vitro administration (Balestrino et al., 1999b). By contrast, oral administration of creatine increases its brain levels to a much lower extent (Dechent et al., 1999; Ipsiroglu et al., 2001), due to poor penetration across the blood–brain barrier. Intracerebral administration is not easily feasible in human patients, but we recently found that a modified creatine molecule is capable to reproduce the biological effects of creatine while more easily crossing biological membranes (Balestrino et al., 2005).

Still at variance with previous investigations, we chose to research the effects of creatine against global (not focal) cerebral ischemia. Global cerebral ischemia is closer to some of the conditions where pretreatment with creatine could be feasible. Such conditions may include, for example: coronary heart bypass surgery, that is often followed by cognitive decline due to intraoperative brain oligemia (Newman et al., 2001); transient ischemic attacks (TIA), that are attended by a 7.27% stroke risk within the first year after the event (Lisabeth et al., 2004); carotid endarterectomy, where decrease of brain flow during interventions often causes subtle neurological deficit (Connolly et al., 2001). These are examples of the conditions where pretreatment with creatine or creatine-derived molecules (Balestrino et al., 2005) could in principle afford protection against brain damage.

As for the mechanisms of the protection we observed, it has been shown that global cerebral ischemia causes a decrease of high-energy phosphates (Lanier et al., 1996), thus we believe that creatine-induced sparing of high-energy phosphates is the first hypothesis to be made (see also Introduction, above). Other mechanisms of neuroprotection by creatine could be at work, including its reported antioxidant action (Lawler et al., 2002) and interaction with the benzodiazepine receptor (Kawasaki et al., 2001). A
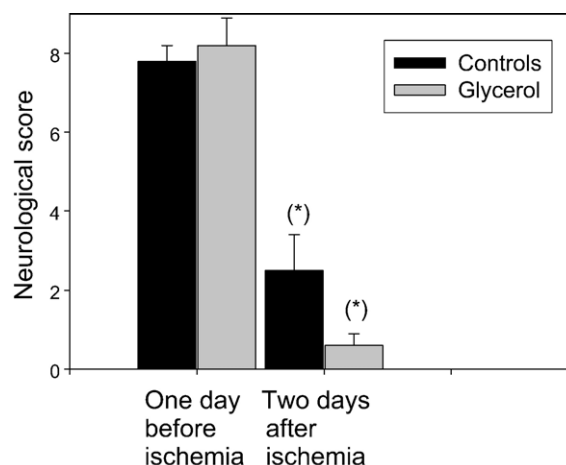
Fig. 5 – Neurological scores of rats treated with saline ("controls") and with glycerol (50 mM). Values are expressed as means and SEM. The rats in the control group are the same ones as in Fig. 4A ("prophylaxis" protocol). Number of glycerol-treated rats was 9 before ischemia and 4 two days after ischemia, due to mortality. Statistically significant differences between groups are marked with asterisks ($p < 0.05$, Wilcoxon matched pairs test).

detailed investigation of these mechanisms should be the object of further research.

Finally, we investigated creatine administration not only as a pretreatment, but also as a possible therapy after ischemia ("treatment" protocol). So far, either creatine or phosphocreatine has been investigated in vivo only in a "pretreatment" protocol (Rauchovà et al., 2002; Wick et al., 1999; Zhu et al., 2004). When we investigated creatine treatment after ischemia, histology showed a decrease of ischemic damage in about half of the brain regions studied (Fig. 3), but no effect on neurological score was detected (Fig. 4). Thus, administration of creatine after ischemia is much less protective than administration before ischemia; however, it still shows some benefit. It should not be forgotten that ICV infusion is an invasive treatment, one that per se causes some damage to the brain. It is conceivable that better drugs, that share the effects of creatine but can be administered by a simple i.v. infusion, will have a better therapeutic effect. While clinical trials of neuroprotective therapies have so far been disappointing (Wahlgren and Ahmed, 2004), treatment with creatine is an entirely novel approach, and could have useful effects if suitable compounds were available.

4.   Experimental procedures

4.1.   Global cerebral ischemia

Adult male Sprague–Dawley rats weighing 270–320 g were used. The animals were fasted during the night preceding the operation with free access to tap water.

Anesthesia was induced with pentobarbital sodium solution (Nembutal Sodium Solution, 50 mg per ml, Abbot Laboratories North Chicago, USA, at initial dose of 50 mg/kg, i.p., followed by supplemental dose of 10 mg/kg i.v., at every 40–60 min of experiment; the absence of foot withdrawal when pinched was used as index of adequate anesthesia).

Animals were intubated with plastic tubing (o.d. 2.5 mm) to facilitate spontaneous breathing. Both femoral arteries and femoral vein were catheterized with Teflon catheters for blood withdrawal/re-infusion, measuring of blood pressure and supplementation of anesthesia.

Following isolation of the common carotid arteries, the animals were allowed a steady state period of 15–20 min; body temperature was continuously monitored via rectal probe and maintained at 37–38 °C with thermostatically controlled heating table and heating lamp.

The reversible forebrain ischemia was induced by combination of bilateral carotid artery clamping and reduction of mean arterial blood pressure, the latter being achieved by blood withdrawal (Smith et al., 1984). The carotid arteries were occluded with silicone-coated microvascular clamps when blood pressure had reached 60–50 mm Hg. The pressure was then adjusted to and maintained at 45 mm Hg by blood withdrawal or reinfusion. Prior to sampling of blood 50 IU Heparine was given i.v.

For measuring of blood pressure, one of the arterial catheters was connected to the pressure transducer (PDP-300K, Institute of Experimental Medical Systems, Moscow, Russia) and standard amplifier (Institute of Experimental Medical Systems, Moscow, Russia). The blood pressure, together with electrocardiogram was monitored on the computer connected to the amplifier through the ADC (PC LPM-16, National Instruments, USA).

Ischemia was terminated after 12 min by removal of carotid clamps and by reinfusion of shed blood. When the blood pressure was restored to the preischemic levels, 0.3 ml/100 g body weight of 0.6 M sodium bicarbonate solution was given i.v. to minimize systemic acidosis, the vascular catheters were removed, all incisions were sutured and animals were allowed to recover from anesthesia on the heating pad. When animals regained sing of normal motility, tracheal tube was removed and animals were placed to their home cages with access to tap water and pellet food.

To bypass blood–brain barrier, all experimental solutions were continuously infused with osmotic minipumps Alzet® model 1002 (Charles River Laboratories, Wilmington, MA, USA) through a cannula implanted into left lateral brain ventricle stereotaxically according to atlas of Pellegrino and Cushman (1967); the coordinates used were 1.7 mm lateral to the midline, 0.1 mm caudal to bregma and 4.5–4.9 mm ventral to dura.

The reservoir of the Alzet pump was lodged between the shoulder blades. The latter was filled with either Creatine 50 mM dissolved in saline solution, or with saline solution alone, or with 50 mM glycerol in saline solution. The latter solution was chosen to imitate the osmolarity of 50 mm creatine, and was chosen because available data show that 40 mM glycerol do not alter neuronal function (Andrew and MacVicar, 1994). Infusion rate was 0.25 μl/h. There were two protocols of i.c.v. infusion: (a) beginning 5 days before ischemia and continuing with no interruption for 7 days after the ischemia ("prophylactic" protocol); (b) beginning 30 min after ischemia and continuing without interruption for 7 days ("treatment" protocol).

4.2.   Rat sacrifice, histological preparation and morphological evaluation of the damage

Seven days after ischemia, rats were decapitated. Their brains were fixed in ethanol-formaldehyde (Korzhevskii et al., 2005), embedded in Histoplast (Shandon, U.S.A) or Paraplast X-tra (Sigma, U.S.A). Some of serial sections were Nissl-stained with cresyl violet (Merck, Germany). These sections were used for study of general cytoarchitectonics of the lesions and proportion of shrunken neurons was estimated in hippocampus, parietal neocortex and nucleus caudatus/putamen in both hemispheres. Counting was carried out by scanning the preparation along a zigzag line and counting all cells that crossed that line. When comparing two groups, their differences were statistically analyzed using parametric (t test) or non-parametric (Wilcoxon or Mann–Whitney test). When more than 2 groups had to be compared, analysis of variance (ANOVA) was used. If ANOVA showed a significant difference, possible differences between individual pairs of groups were further analyzed using Bonferroni post hoc test. Significance level was always set a $p < 0.05$.

4.3. *Neurological state evaluation*

Preliminary experiments showed no effect of either cannula implantation *per se* (i.e., without subsequent infusion) or of sham-implantation surgery on neurological testing (not shown). Therefore, intact rats, rats implanted with cannula only (no reservoir nor infusion) and rats receiving sham cannula implantation surgery were pooled as a control group for statistical analysis.

For the neurological state evaluation, we used a test battery composed of three tests of muscular strength and senso-motory coordination originally developed by Combs and D'Alecy (1987), with minor modifications. These tests were:

1. *The screen test*, evaluating general muscle strength. Rats were placed on a horizontal screen (60×38 cm with grid size 1×1 cm) rotating from 0° (horizontal) to 90° (vertical). After 15 s period of rat habituation, the screen was manually rotated to vertical plane. A rat scored 3 points after spending 11–15 (or more) s on the board, 2 points for 5–10 s, 1 point for up to 5 s and zero if unable to stay at all.
2. *The balance beam test*, evaluating muscle strength and equilibrium maintenance capacity. Rats were placed on center of a horizontal wooden beam (2.5 cm diameter and 70 cm long, positioned 70 cm above a sponge pad). The time the animal remained balancing on the beam was scored as following: 3 points for 11 or more seconds on the beam, 2 points for 6 to 10 seconds, 1 point for up to 5 s and zero points if unable to stay on the beam at all.
2. *The prehensile traction test*, evaluating muscle strength and equilibrium maintenance capacity. Rats were handled and allowed to grab a rope (0.25 cm stainless wire sleeved cable, 70 cm long stretched horizontally 70 cm above a sponge pad) with forefoot pads, and then hang on when released. The rat was assigned a score of 3 when clinging 4 or more seconds on the rope, 2 for 2 to 3 seconds, 1 for 1 to 2 s and 0 for no rope clinging. Bringing the rear limb up to the rope brought 1 additional point when the test score was lower than 3.

Rats were given 10–15 min resting time between tests. The final minimum overall score was 0 and the maximum was 9. The lower the score, the more severe was the injury.

5. Note added in proofs

While correcting the proofs of this paper we read the advance online publication of the paper "Improved reperfusion and neuroprotection by creatine in a mouse model of stroke" (*J Cereb Blood Flow Metab*, 2006, Jun 14; [Epub ahead of print]) by K. Prass, G. Royl, U. Lindauer, D. Freyer, D. Megow, U. Dirnagl, G. Stockler-Ipsiroglu, T. Wallimann, and J. Priller. This paper confirms that orally administered creatine does not increase the brain content of creatine, yet it reports that oral creatine administration decreases brain damage after transient middle cerebral artery occlusion. Prass et al. show evidence that this is probably due to a protection by creatine on the cerebral vessels, a novel effect of creatine not previously described. In our present paper, we administered creatine intracerebroventricularly, so there is little doubt that the protective effect was

on the parenchima itself; however, we would like to underline how the paper by Prass et al. and our present paper seem to complement each other by showing neuroprotection by creatine with at least two different mechanisms of action.

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