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Effect of Carnosine on Rats under Experimental Brain Ischemia

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Zoetic Neurosciences Ltd., England, UK, [1]Laboratory of Neurochemistry, Institute of Neurology, Moscow, Russia, and [2]International Biotechnological Center and Center of Molecular Medicine, M.V. Lomonosov Moscow State University, Moscow, Russia

GALLANT, S., KUKLEY, M., STVOLINSKY, S., BULYGINA, E. and BOLDYREV, A. *Effect of Carnosine on Rats under Experimental Brain Ischemia*. Tohoku J. Exp. Med., 2000, **191** (2), 85-99 —— The effect of dietary carnosine on the behavioral and biochemical characteristics of rats under experimental ischemia was studied. Carnosine was shown to improve the animals orientation and learning in "Open Field" and "T-Maze" tests, and this effect was accompanied with an increase in glutamate binding to N-methyl-D-aspartate (NMDA) receptors in brain synaptosomes. Long-term brain ischemia induced by both sides' occlusion of common carotid arteries resulted in 55% mortality of experimental rats, and those who survived were characterized by partial suppression of orientation in T-maze. In the group of rats treated with carnosine, mortality after ischemic attack was decreased (from 55% to 17%) and most of the learning parameters were kept at the pre-ischemic level. Monoamine oxidase B (MAO B) activity in brain of the carnosine treated rats was not changed by ischemia significantly (compared to that of ischemic untreated rats) but NMDA binding to brain synaptosomal membranes being increased by ischemic attack was significantly suppressed and reached the level characteristic of normal brain. The suggestion was made that carnosine possesses a dual effect on NMDA receptors resulting in increase in their amount after long-term treatment but decrease the capacity to bind NMDA after ischemic attack. ——— ischemia; learning; carnosine; anti-ischemic drug © 2000 Tohoku University Medical Press

Ischemic brain damage is characterized by increased production of reactive oxygen species (ROS) resulting in neuronal cell death (Coyle and Puttfarcken 1993; Olanow 1993). The ability of synthetic antioxidants like idebenone or

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Authors dedicate this work to the memory of Prof. Kineshiro Nagai (Nihon University, Tokyo) developing modern ideology to studying biological function of carnosine.

N-*tert*-butyl-α-phenylnitrone (PBN), both being appropriate hydroxide radical traps to protect brain against ischemic attack can prove this fact (Folbergova and Zhao 1995; Grieb et al. 1998). These data have initiated the study of the ability of natural antioxidants to provide the defense of brain against ischemia. The neuropeptide carnosine can be called such a probable protector. This is a histidine containing dipeptide, characterized by antioxidant features. It is accumulated in the excitable tissues such as muscles and brain, of vertebrates in concentrations higher than that of ATP (Boldyrev and Severin 1990). Concentration of carnosine is proportional to functional activity of the tissue and is known to decrease during myodistrophy or neurodegeneration (Boldyrev 1999). Addition of carnosine to food was found to improve antioxidant status of the organism and wound healing following surgery (Nagai and Suda 1988; Chan and Decker 1994; Roberts et al. 1998).

Carnosine is able to suppress ROS production within the cells (Boldyrev et al. 1995, 1999a, b), to decrease the level of lipid peroxides (Aruoma et al. 1989), to protect proteins from oxidative modifications in vitro (Boldyrev et al. 1997; Hipkiss et al. 1998) and to diminish the in vivo ROS production by leukocytes (Stvolinsky et al. 1997), to prevent the consequences of tissue radiodamage (Boldyrev and Severin 1990) and to increase the survival of animals during experimental hypoxia (Boldyrev et al. 1997). Suggestion was made that carnosine and its derivatives serve as modulators of intracellular homeostasis increasing resistance of organisms toward unfavorable conditions (Nagai and Suda 1986, 1988). The aim of the present study was to characterize the effect of carnosine on rats during experimental rat brain ischemia, caused by prolonged occlusion of common carotid arteries.

Materials and Methods

Characteristics of the animals used

Male Wistar rats of the age of 2-2, 5 months (160-180 g) were randomly divided into two groups, 30 animals each. Animals of both groups were maintained for 7 months on a standard diet with no additions (Group A) or with addition of carnosine into the drinking water (Group B) at a dose of 100 mg/kg of body weight daily. This concentration was found to be within the physiological range (Boldyrev and Severin 1990). To the end of the period observed, the weight of rats of both groups was raised to 300-330 g (equal in both experimental groups).

T-Maze and Open Field tests

Ten days before the experimental brain ischemia animals of both groups were carried through the every day training in T-Maze and in the Open Field tests (Bureš et al. 1983; Ashmarin et al. 1994). When the T-Maze test was used, each animal has been individually tested 5 times with 3 minutes period each trial. In

each case, the animal was placed in starting box of T-Maze which contained a food stimulus in one of the wings (the same in each experimental series). One minute after, the starting box was open and rat began the food search. During training the following parameters were measured: lag period before beginning the search (seconds, Parameter I); time which is necessary to recognize a food supply (seconds, Parameter II); number of mistakes made by an animal (inability to solve tasks during training, going into the wrong wing of T-Maze, returning to starting box instead of looking for food supply, etc., Parameter III); and the number of defecations (boluses) during the training period (Parameter IV).

The training was considered to be successful if the following features were observed: A reduce in time when an animal exited from the starting box (Parameter I) and recognized a food supply (Parameter II) as well as a decrease in the number of mistakes made (Parameter III) and in the number of boluses during the search (Parameter IV). Successful making up each parameter was estimated as one point and the sum of the points was used to characterize the efficiency of learning. Ten days after training the animals were estimated for successfulness and distributed into three sub-groups according to following range: non-satisfactory (0-1 points), satisfactory (2-3 points), and successful (4 points) rats.

The training of rats in the Open Field test was carried out every day independently from the above procedure. Each animal was put for 5 minute into a circular well-lighted arena of 1 m in diameter divided onto 36 sectors (18 in peripheral area and 18 in the center of the arena). The animals' behavior was estimated according to the following parameters: number of sectors crossed by the animal within the whole arena characterizing the total horizontal activity, (Parameter I); number of sectors crossed by the animal in the peripheral sectors of the area (Parameter II); the total number of stands during the trial (vertical activity, Parameter III); time for exits into the center of the arena (seconds, Parameter IV): and the number of boluses for the trial period (Parameter V). Decrease in time characteristics of parameters I-IV as well as reduction of the amount of boluses (parameter V) was estimated as successful feature and the results were calculated as in the case with T-Maze test. After training, all animals were distributed into three sub-groups according to the following range: non-satisfactory (0-1 points), satisfactory (2-3 points), and successful (4-5 points) rats.

Experimental brain ischemia

Brain ischemia was caused by both sides' occlusion of common carotid arteries for 120 hour period (Boldyrev et al. 1996; Kukley and Gannushkina 1997), using 50% of animals from each group. The rest of the animals was used to carry out shame operations and other controls.

Physiological activity of the animals of both groups was daily estimated during following 5 days after surgical operation using both tests; during this

period group B rats were continuously treated with carnosine in the same dose while group A animals were control to them. At the end of the experiment, the learning ability was calculated and neuronal symptomatics of the animals were estimated, using McGrow Stroke Index modified for the rats under model of experimental ischemia used (occlusion of common carotid arteries) (McGrow 1977; Boldyrev et al. 1996).

Laboratory methods

Preparation of synaptosomes and mitochondria. After decapitation of animals, brain cortex was minced in cool (0–2°C) and homogenized in 10 volumes of solution containing 0.32 M sucrose, 2 mM ethylendiaminetetraacetic acid (EDTA), 10 mM N-[2-hydroxyethyl]piperazine-N′-[2-ethanesulfonic acid] (HEPES) (pH 7.4 at 0°C). Homogenate was used to prepare the synaptosomal and mitochondrial fractions according to Hajos (1975). Supernatant obtained after 10 minutes centrifugation at 1500 g was stored in the cold and the pellet was resuspended in the same volume and repeatedly centrifuged as previously. Combined supernatants were centrifuged for 20 minutes at 9000 g. The final supernatant was used to prepare a mitochondrial fraction and the pellet was resuspended in 0.32 M sucrose, layered onto 4 volumes of 0.8 M sucrose and centrifuged for 20 minutes at 9000 g. The 2/3 of the whole volume was taken from the middle of the tube and diluted by the same volume of double-distilled water and centrifuged for 20 minutes at 10 000 g. The pellet was resuspended in a solution containing 30 mM histidine, 1 mM Na_2EDTA, 0.25 M sucrose (pH 7.4 at 4°C), divided into portions and frozen in liquid nitrogen. The mitochondrial fraction obtained was resuspended in 0.2 M phosphate buffer (pH 7.6 at 4°C), divided into portions and frozen in the same manner. In biochemical experiments, the above mentioned membrane fractions were obtained from brain of individual animals separately and each measurement was made in triplicate.

Measurement of Na/K-ATPase activity. Na/K-ATPase activity was determined in the synaptosomal fraction as the ouabain-sensitive portion of ATP hydrolysis under optimal conditions (Klodos et al. 1975). Total ATPase activity was measured in the mixture containing 130 mM NaCl, 20 mM KCl, 3 mM $MgCl_2$, 3 mM ATP, 30 mM piperazine-NN′-bis[2-ethanesulfonic acid] (PIPES) (pH 7.4 at 37°C), and the ouabain insensitive component was measured in the same suspension containing 1 mM ouabain. Na/K-ATPase activity was calculated as the difference between these two values. The results are presented as a mean from 3 independent measurements.

Measurement of glutamate binding to NMDA-receptors. Glutamate binding to N-methyl-D-aspartate (NMDA)-receptors of rat brain synaptosomes was measured by the radio-ligand method (Kyle et al. 1992). Membrane fractions were thawed, washed in 10 volumes of 50 mM Tris-acetate buffer (pH 7.4 at 4°C) and centrifuged for 20 minutes at 20 000 g. The pellet was resuspended in 10 volumes

of the buffer containing 1 mM ethylene glycol-bis(β-aminoethyl ester)-N,N,N′, N′-tetraacetic acid (EGTA). In order to determine the total glutamate binding, 60–120 μg protein samples were incubated for 40 minutes at 4°C in 1 ml medium containing 50 mM Tris-acetate buffer (pH 7.4), 1 μM glycine, 200 mM glutamate and 10 nM [^{3}H]-glutamate. Non-specific glutamate binding was measured in the samples of the same composition, containing additionally 1 mM NMDA. The reaction was terminated by vacuum filtration, using GF/C filters (Sigma Chemicals Co., St. Louis, MO, USA), 30 minutes pretreated with BSA (1 mg/ml) to decrease non-specific sorption. The filters were washed by 12 ml of cold buffer, dried and counted at "12 M RACBETTA LKB" Counter. The results were presented as maximal glutamate binding by NMDA-receptors (B_{max}) in pmoles/mg synaptosomal protein.

Measurement of MAO B activity. Monoamine oxidase B activity was measured in mitochondrial fraction at 37°C, using benzyl amine as substrate (McEven and Cohen 1963). Mitochondrial protein of 0.25 mg and 0.2 M Na/K-phosphate buffer (pH 7.4) were placed into the corked tubes in a 0.9 ml volume. The reaction was initiated by addition of 0.1 ml of 10 mM benzyl amine and stopped after an appropriate time by 0.25 ml of 30% cold trichloroacetic acid. Into the control tube benzyl amine was added after the reaction had been stopped. Three ml of heptane was added into each sample and the benzaldehyde (BA) accumulated was extracted into organic phase. BA was determined spectrophotometrically (with the molar extinction coefficient being equal to 13 080 $M^{-1} \cdot cm^{-1}$).

Measurement of protein content. Protein was determined in the presence of 0.1% sodium deoxycholate as was described earlier (Klodos et al. 1975).

Reagents: All reagents used were purchased from Sigma Chemicals Co.

Statistics. Behavioral characteristics and mortality data were analyzed using non-parametric statistic method (chi-square and Mann-Whitney U-test) (Glantz 1994). The biochemical measurements were made at least in triplicates and Student's *t*-test was used to estimate the statistically significant difference (Sigma Chemicals Co.).

Results and Discussion

Results of training of animals in T-Maze and Open Field tests are presented in Tables 1 and 2. As one can see, the common feature for both tests used is a reduction of numerical values of all the parameters with days of training (from 1 to 10) which reflects the learning process. Carnosine treated rats were characterized by a higher rate of learning between 3–6 days of the experiment while to the end of experiment the parameters for animals of both groups were equalized.

After surgical procedure inducing ischemic attack 1 day off period was used then the trials were re-started again. Mainly the parameters used were found to be un-changed by ischemia with exception of Parameters I and II in T-Maze test

TABLE 1. *Parameters of animals training in the T-Maze test*

Days of experiment	Parameter I			Parameter II			Parameter III			Parameter IV		
	Group A	Group B		Group A	Group B		Group A	Group B		Group A	Group B	
1	13.0±12.9	13.3±13.3	$p=0.98$	82.0±50.5	53.4±28.7	$p=0.070$	3.8±2.1	3.4±2.1	$p=0.65$	5.1±3.2	4.6±2.4	$p=0.93$
2	39.7±60.3	18.4±40.1	$p=0.027$	108.3±65.2	75.5±67.3	$p=0.12$	2.5±1.6	2.1±1.7	$p=0.34$	5.1±2.4	3.8±2.1	$p=0.059$
3	48.5±64.5	15.8±13.5	$p=0.047$	96.0±70.3	82.8±67.6	$p=0.59$	3.1±2.2	1.7±1.6	$p=0.024$	4.1±2.6	2.5±2.6	$p=0.046$
4	31.0±45.2	9.7±7.2	$p=0.048$	65.6±57.2	31.3±24.8	$p=0.023$	2.9±2.7	1.2±1.5	$p=0.010$	3.8±2.8	2.2±2.4	$p=0.050$
5	19.6±34.1	6.2±5.1	$p=0.033$	39.0±44.6	18.6±12.3	$p=0.046$	2.2±1.8	1.2±1.4	$p=0.042$	2.2±2.5	1.0±1.6	$p=0.050$
6	11.1±15.7	6.9±6.5	$p=0.28$	25.2±25.7	13.2±10.7	$p=0.061$	1.1±1.2	0.6±1.2	$p=0.064$	1.4±2.3	0.6±1.5	$p=0.37$
7	9.4±15.6	7.7±8.2	$p=0.60$	18.7±35.6	15.6±12.7	$p=0.37$	0.5±0.8	0.6±1.0	$p=1.0$	1.1±2.4	0.7±1.7	$p=0.80$
8	7.3±12.9	7.7±7.4	$p=0.84$	19.2±30.5	16.4±15.4	$p=0.51$	1.5±2.5	0.8±1.0	$p=0.60$	1.3±2.0	1.2±1.7	$p=0.91$
9	11.2±14.1	7.4±5.3	$p=0.26$	22.3±26.1	15.7±14.6	$p=0.60$	1.3±1.4	1.5±1.3	$p=0.55$	2.0±2.4	2.5±1.6	$p=0.21$
10	9.9±9.4	9.3±7.8	$p=0.80$	19.2±15.6	19.7±14.6	$p=0.60$	1.4±1.8	1.7±1.4	$p=0.21$	1.3±2.4	1.3±2.2	$p=0.74$
12	41.4±59.4	72.5±83.5	$p=0.45$	61.9±61.0	161.7±44.9	$p=0.01$	1.3±0.7	0.8±0.5	$p=0.17$	0	0.5±0.8	$p=0.21$
13	39.3±59.9	78.3±72.3	$p=0.31$	48.3±59.9	111.3±72.6	$p=0.11$	1.5±0.8	0.7±0.4	$p=0.049$	0.4±1.1	0.7±1.0	$p=0.62$
14	35.8±53.6	75.4±75.7	$p=0.35$	70.9±75.7	102.7±84.8	$p=0.49$	1.3±0.7	0.35±0.2	$p=0.010$	0.7±1.9	0.5±1.2	$p=0.83$
15	30.8±54.9	75.0±75.8	$p=0.24$	53.0±35.8	109.7±79.1	$p=0.11$	1.0±0.5	0.35±0.2	$p=0.012$	0.1±0.4	0.3±0.5	$p=0.44$
16	39.3±40.3	70.0±70.8	$p=0.35$	56.6±52.7	108.0±81.7	$p=0.20$	1.2±0.5	0.5±0.3	$p=0.012$	0.4±1.1	0.2±0.4	$p=0.66$

1-10 days, before and 12-16 days after brain ischemia. The difference between parameters (see text, Materials and Methods) obtained for groups A (control animals) and B (carnosine-treated animals) is taken as statistically significant when $p \leq 0.05$. Statistical analysis was made using Mann-Whitney U-test. An amount of animals at the beginning of experiment in both groups, $n=30$.

TABLE 2. *Parameters of animals training in the Open Field test*

Days of experiment	Parameter I		Parameter II		Parameter III		Parameter IV		Parameter V	
	Group A	Group B	Group A	Group B	Group A	Group B	Group A	Group B	Group A	Group B
1	42.0±19.0	46.6±13.0	27.1±13.3	31.2±8.0	15.6±7.3	18.7±7.5	21.5±28.3	13.7±12.2	5.5±2.7	5.6±3.1
	$p=0.15$		$p=0.058$		$p=0.14$		$p=0.35$		$p=0.91$	
2	35.0±18.7	30.5±14.9	25.8±12.1	21.5±9.4	12.0±7.0	9.8±6.5	45.9±43.9	25.6±24.7	4.1±3.6	4.6±3.4
	$p=0.43$		$p=0.17$		$p=0.26$		$p=0.047$		$p=0.55$	
3	38.9±19.9	32.4±11.1	28.7±11.8	23.3±8.3	16.8±8.5	12.4±6.5	35.4±32.5	22.9±20.3	2.6±3.4	3.1±3.3
	$p=0.53$		$p=0.093$		$p=0.10$		$p=0.084$		$p=0.55$	
4	42.3±19.1	32.5±16.7	28.5±11.5	21.7±0.1	15.9±8.1	11.5±6.3	32.8±16.7	16.7±11.2	2.8±3.3	1.2±1.8
	$p=0.047$		$p=0.048$		$p=0.048$		$p=0.050$		$p=0.045$	
5	39.7±15.0	31.3±10.6	27.9±11.3	21.9±8.2	15.7±8.1	10.9±4.5	50.2±50.3	23.2±16.2	2.7±2.7	1.2±1.7
	$p=0.050$		$p=0.043$		$p=0.045$		$p=0.047$		$p=0.026$	
6	31.5±14.8	23.3±12.4	22.2±8.7	16.7±8.1	10.2±6.3	6.6±5.5	41.6±35.9	21.9±17.0	3.0±3.3	1.2±1.9
	$p=0.067$		$p=0.038$		$p=0.028$		$p=0.047$		$p=0.046$	
7	29.0±11.2	27.0±12.7	22.9±7.7	18.5±7.7	10.5±6.1	8.9±6.0	52.1±43.7	32.7±31.5	2.0±3.1	1.6±2.6
	$p=0.46$		$p=0.049$		$p=0.46$		$p=0.048$		$p=0.78$	
8	31.9±12.1	23.7±16.1	22.2±8.9	15.7±9.7	9.1±5.0	7.8±5.7	42.5±34.7	26.1±23.8	2.1±3.1	1.5±2.8
	$p=0.059$		$p=0.025$		$p=0.43$		$p=0.056$		$p=0.44$	
9	28.7±15.9	29.2±13.8	22.3±9.8	18.8±7.9	10.8±6.4	10.6±5.7	49.9±54.4	48.6±76.9	1.7±2.7	1.3±2.1
	$p=0.65$		$p=0.18$		$p=0.96$		$p=0.061$		$p=0.72$	
10	30.6±14.8	29.9±16.1	22.0±9.8	19.2±9.7	12.8±7.7	11.8±8.1	37.0±27.9	23.8±15.9	1.6±2.7	1.2±2.1
	$p=0.59$		$p=0.33$		$p=0.76$		$p=0.068$		$p=0.92$	
12	13.3±3.6	10.0±3.8	10.3±3.4	7.2±3.1	5.3±3.9	2.0±1.3	52.7±33.8	17.7±19.7	0.3±0.7	0
	$p=0.14$		$p=0.12$		$p=0.075$		$p=0.044$		$p=0.34$	
13	16.6±5.9	14.9±4.6	13.4±4.6	11.2±3.7	4.7±3.7	3.7±2.7	18.3±24.2	16.2±9.0	0.7±2.2	0
	$p=0.58$		$p=0.37$		$p=0.57$		$p=0.84$		$p=0.086$	
14	16.6±5.3	10.2±5.4	12.0±4.7	6.7±3.1	5.7±2.2	2.2±1.7	36.6±40.4	33.2±28.2	0.6±1.5	0.5±1.2
	$p=0.054$		$p=0.038$		$p=0.048$		$p=0.87$		$p=0.99$	
15	21.4±7.5	13.0±7.5	18.3±6.2	11.2±4.7	6.9±1.5	3.7±3.1	36.1±58.5	44.1±37.2	0	0.7±1.2
	$p=0.065$		$p=0.043$		$p=0.037$		$p=0.78$		$p=0.17$	
16	14.9±5.4	13.0±5.3	12.9±5.0	11.1±4.1	6.4±2.6	2.9±2.9	27.7±21.9	55.3±45.3	0	0.7±1.2
	$p=0.61$		$p=0.48$		$p=0.040$		$p=0.83$		$p=0.17$	

1-10 days, before and 12-16 days after brain ischemia. The difference between parameters (see text, Materials and Methods) obtained for groups A (control animals) and B (carnosine-treated animals) is taken as statistically significant when $p \leqq 0.05$. Statistical analysis was made using Mann-Whitney U-test. An amount of animals at the beginning of experiment in both groups, $n=30$.

which were sufficiently increased and not changed with subsequent days of training. Carnosine treated rats were characterized by significantly better learning results in relation to parameter III (T-Maze), as well as parameters II and III (Open Field). In all cases (12–16 days) the difference measured was found to be significant ($p<0.05$). One example demonstrating the learning tendency before and after ischemia for the control and carnosine treated rats is presented in Fig. 1.

Common characterization of the learning capacity of group A animals in T-Maze studies resulted in the following distribution between the above mentioned sub-groups: non-satisfactory animals—17%, satisfactory animals—34% and successful animals—49% (Fig. 2-A). Within the group B rats, treated with carnosine, the distribution between the sub-groups was found to be distinct from the control, namely the amount of animals with satisfactory learning abilities significantly increased (from 34 to 52%), while that with non-satisfactory learning abilities decreased from 17 to 5%—Fig. 2-B. At the same time, the number of animals with the successful learning was slightly (not significantly) diminished (from 49 to 43%).

Behavior of animals in the Open Field showed the following distribution of control animals (Fig. 2-C): non-satisfactory animals—25%, satisfactory animals—64%, and successful animals—11%. Among the animals kept on carnosine containing diet, percentage of rats with satisfactory result was not changed, while

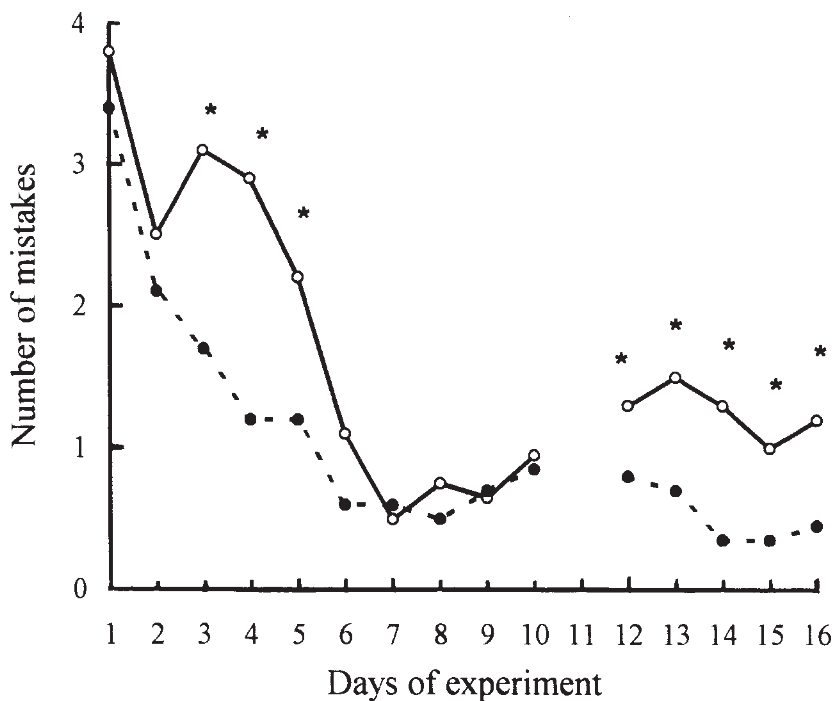

Fig. 1. Change in Parameter III (number of mistakes) during training rats in T-Maze test (1–10 days, before ischemia; 12–16 days, after experimental ischemia). Open circles, continuous line-control (Group A), derk circles, dotted line-carnosine treated animals (Group B). The difference between values obtained for 3–6 days and 12–14 days ($p<0.05$ using Mann-Whitney U-test) is statistically significant (marked by *).

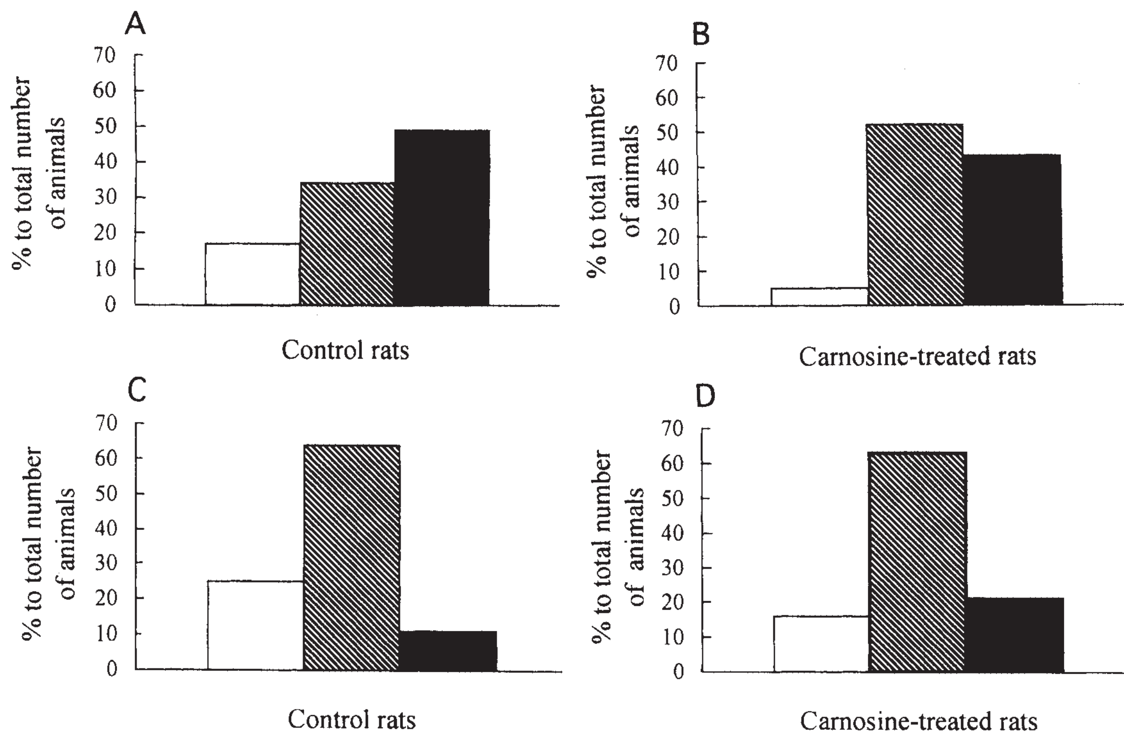

Fig. 2. Effect of carnosine on distribution of rats between different learning groups. Results of first 10 days of animals learning in the T-Maze (A and B) or in the Open Field (C and D) are expressed as % to the total number of animals relating to the efficiency of learning (see Materials and Methods). A and C-control rats (Group A), B and D-carnosine treated animals (Group B). White bars, non-satisfactory animals; striped bars, satisfactory animals; black bars, successful animals.

that with non-satisfactory learning decreased, and that with successful learning increased (Fig. 2-D). Thus, according to both tests used, the positive effect of carnosine on the learning process was demonstrated, namely carnosine treated animals were characterized by more adequate orientational behavior.

Analysis of the viability of rats during long-term ischemia showed that animals were dying mostly during the first 24 hours after ischemia, and percentage of died animals in both groups studied was significantly different (Fig. 3). The viability in the control group (A) was 45%, and in the group, treated with carnosine (B)—83%. At the same time, the neurological symptomatics of the surviving animals did not exceed 1.5 grades of the modified McGrow scale (Boldyrev et al. 1996), and differences in symptoms between control and carnosine treated groups of the animals surviving to the 6th day of ischemia was not statistically significant.

It was interesting to elucidate the biochemical causes of protective effect of carnosine during experimental brain ischemia. More than 70% of food carnosine was demonstrated to penetrate through the alimentary canal of rodents into the blood; a low level of serum carnosinase allows it to circulate in the blood for a relatively long time (several hours), penetrating into the liver and brain (Tamaki

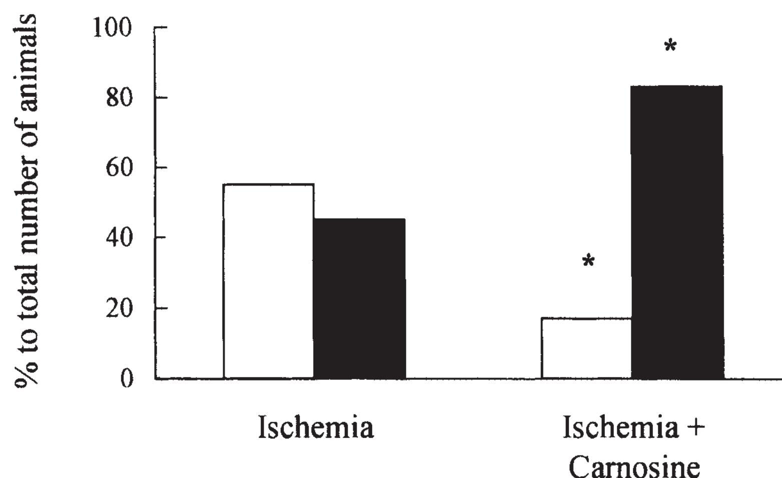

Fig. 3. Mortality of rats after experimental ischemia in control (left) and carnosine treated (right) groups of animals. White bar, viability; black bar, mortality; $p<0.05$ (Chi-square test was used for comparison between control and carnosine treated groups, and (*) marks the date significantly differed [$p<0.05$] from control.).

and Ikeda 1985; Boldyrev 1999). It was found recently (Matthews and Webb 1995) that tissues of carnosine fed animals are characterized by higher level of this compound; moreover, these tissues were more stable against oxidative damage (Chan and Decker 1994; Yuneva et al. 1999). Increased level of tissue carnosine was observed only for a short time after injection due to its participation in cellular metabolism (Boldyrev 1999), but the consequences of carnosine administration affects metabolic reactions for one day or longer (Boldyrev et al. 1997; Stvolinsky et al. 1997). These facts let us make the conclusion that carnosine possesses a long-term effect on cellular metabolism.

The carnosine effect can be both a direct one and also be caused by the appearance of the products of its hydrolysis, β-alanine and histidine. The second one is more biologically active being an essential amino acid for rodent, taking part in protein synthesis as well as in histamine accumulation. In agreement with this fact, histidine affects the behavior of animals, and lack of this amino acid in a diet can be compensated by carnosine (Tamaki et al. 1984; Orthen-Gambill 1988). It cannot be excluded that in our experiments not only direct effect of carnosine but also the action of its metabolites (mainly histidine) was observed. However, according to the literature data injection of histidine into rats does not intensify but even depresses the moving and investigating activity, and this could be a result of increased histamine production (Dutra-Filho et al. 1989). Moreover, addition of the mixture of (histidine+β-alanine) to the mice diet in the same molecular proportion as in carnosine molecule did not increase resistance of the animals against oxidative stress as carnosine did (Gallant et al. 2000). Therefore, the suggestion that protective effect of carnosine on the brain of ischemic animals is caused by its own action is more probable.

In order to characterize the biochemical parameters of the ischemic brain we

chose the proteins, which activity is directly related to excitatory processes and their regulation-Na/K-ATPase and NMDA-receptors in synaptosomes, and MAO B in mitochondria (Bulygina et al. 1999). Na/K-ATPase activity regulates the asymmetric distribution of the monovalent cations on the neuronal membrane and thus the excitability of the neurons (Beauge et al. 1997; Mintorovitch et al. 1994). NMDA-receptors take part in behavioral reactions and long-term potentiation processes closely connected with memory (Mattson 1998). MAO B also takes part in brain excitation, influencing tissue level of biogenic amines (Gorkin 1983).

For intact animals, the parameters studied were as follows: Na/K-ATPase activity -34.7 ± 4.9 μmoles Pi/mg protein per hour, glutamate binding to NMDA receptors -3.5 ± 1.7 pmoles/mg of protein and MAO B -109.8 ± 18.4 nmoles BA/mg of protein per hour. These data are in a good agreement with the literature (Gorkin 1983; Mintorovitch et al. 1994; Beauge et al. 1997). Na/K-ATPase and MAO B activity in the group B (animals treated with carnosine) did not change practically, whereas glutamate binding was two times higher (Table 3). These results correlate well with an improvement in learning ability, demonstrated by these animals.

According to the literature data, Na/K-ATPase activity in rat brain after short-term experimental ischemia become 25–30% less (Mintorovitch et al. 1994; Kurella et al. 1997) while glutamate binding by NMDA receptors significantly increases (Sauer and Fagg 1992). MAO B activity decreases after ischemic attack by 20–30% (Gorkin 1983), and this decrease is in a good correlation with neurological symptomatics-the more neurological deficit is expressed the more MAO B activity decreased (our unpublished observation). Stabilization of parameters studied is usually observed at the steps of recovery following ischemic injury.

In our experiments brain Na/K-ATPase of the animals surviving after long-term ischemia (6 days) was slightly lower than in the control (the difference being statistically insignificant), MAO B activity still significantly (by 29%) decreased, and glutamate binding was nearly twice of that of the control (Table 3). These data point out that suppression of MAO B activity and activation of NMDA-receptors are both the long-term effects contrary to Na/K-ATPase activity which is characterized by faster recovery rates.

It was interesting to compare these parameters of brain with those obtained under protecting effect of carnosine (Group A and B, intact rats treated and untreated with carnosine). Table 3 demonstrates that glutamate binding by synaptosomal membranes isolated from brain of carnosine treated ischemic rats decreased approaching the control data. At the same time, Na/K-ATPase activity is at the level close to that of ischemic rats, not treated with carnosine. MAO B activity is nearly totally recovered. This fact proved the importance of the parameters measured for survival of animals during ischemia. Moreover, the data obtained demonstrate that carnosine is able to protect brain cells from oxidative stress not only in vitro (Boldyrev et al. 1999a), but in vivo as well. The fact that

TABLE 3. *Na/K-ATPase, MAO B, and glutamate binding to NMDA-receptors in brain membrane fractions of rats after 120 hours experimental ischemia*

Groups of the animal studied	Na/K-ATPase, μmoles Pi/mg per hour	MAO B, nmoles BA/mg per hour	Glutamate binding to NMDA-receptors, (B_{max}, pmoles/mg)
Group A			
Intact rats ($n=8$)	34.7±4.9	109±15	3.5±1.8
Ischemic rats ($n=7$)	31.2±7.2	77±9 $p<0.05$ †	6.4±0.5 $p<0.05$ †
Group B			
Intact rats ($n=8$)	37.0±8.7	104±11	7.0±0.8 $p<0.01$ †
Ischemic rats ($n=8$)	32.5±5.9	88±11	3.7±1.2 $p<0.05$ ††

A, control animals; B, carnosine-treated animals. Significance of the differences was estimated in relation to data obtained for intact (†) or to ischemic rats from Group A (††). Student's t-test was used.

carnosine decreased the activity of NMDA-receptors during ischemia correlates with its ability to avoid the cell damaging component of excitotoxic glutamate effect on the neurons (Boldyrev et al. 1999b).

The possible mechanism of the described effect of carnosine may consist in prevention of ROS generation within the cells, particularly, to lower the ROS level, to protect MAO B and, consequently, to recover normal metabolism of biogenic amines, and to suppress NMDA-receptors, which are known to provide excitotoxic mechanisms of neuronal damage during ischemia (Sauer and Fagg 1992; Viola and Rodriguez-de Lores Arnaiz 1994; Boldyrev 1999). This multiple protection is suggested to be the immediate cause for prevention of rat mortality.

Generally, carnosine demonstrates dual effect on NMDA-receptors activity-during long-term carnosine treatment it increases NMDA binding in the glutamatergic brain system, probably due to the expression of subsequent proteins (Sauer and Fagg 1992; Ikeda et al. 1999) and this action causes a more effective training process. On the other hand, activation of NMDA-receptors as a result of oxidative stress, caused by a direct effect of ROS on receptor's protein, is suppressed by carnosine, which is in agreement with its antioxidative action (Boldyrev 1999). Thus, carnosine, as real biological modulator demonstrates two distinct abilities, using various mechanisms to provide adequate response of brain to different environmental factors. At the same time, possible effect of products of its metabolism on the parameters studied requires additional analysis.

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