VERSITA

Central European Journal of Medicine
DOI: 10.2478/s11536-007-0037-z
Research article
CEJMed 2(4) 2007 430–446

Effects of cholinesterase inhibitor metrifonate on naive rats and rats with a model of hypoxia-induced impaired memory

Darinka S. Dimitrova[1], Damianka P. Getova[1*], Vesselin T. Belovezdov[2]

[1] *Department of Pharmacology, Clinical Pharmacology and Drug Toxicology,*

[2] *Department of Pathology, Medical University, 15A V. Aprilov str, 4002 Plovdiv, Bulgaria*

Received 15 February 2007; accepted 11 July 2007

Abstract: Cholinesterase inhibitors are currently used in the therapy of different kind of dementia to improve brain memory functions. The acetylcholinesterase inhibitor metrifonate was studied in naive rats and in rats with a model of sodium nitrite-induced hypoxia. One active avoidance test and in two passive avoidance tests were used. In the active avoidance test metrifonate increased the number of avoidances during the learning session only. In both passive avoidance tests, metrifonate prolonged latency differently during the learning session and in short-term or in long-term memory retention. Hypoxic rats showed lower numbers of avoidances in learning and memory retention sessions. Metrifonate increased the number of avoidances during the learning session for hypoxic rats. In the step-through passive avoidance test, metrifonate increased the latency of reactions in the learning session and in long-term memory retention tests. In the step-down passive avoidance test, the groups with hypoxia and metrifonate did not change the latency of reaction in the learning and long-term memory retention sessions, but increased the latency of reactions in the short-term memory retention test. Morphological data showed a significant impaired neuronal structure in a CA1 zone of the hippocampus in hypoxic rats and a tendency to preserving in rats treated with metrifonate. Our results suggest that metrifonate improves cognitive functions in naive and in hypoxic rats.

Keywords: Sodium nitrite-induced hypoxia, cholinesterase inhibitor, metrifonate

1 Introduction

Dementia is a common symptom of many degenerative brain diseases [1, 2]. Reduced levels of acetylcholine are often seen during different kind of dementia [3], and the central

* E-mail: dgetova@yahoo.com

cholinergic deficit is one of the prominent features of the disease [4]. The cognitive decline is accompanied by the deficiency of cholinergic transmission [5].

Metrifonate is a cholinesterase inhibitor effective in the treatment of cognitive symptoms of dementia, with a smooth onset of action and stable long-lasting high levels of acetylcholinesterase inhibition [3]. Metrifonate has been used as an antihelminthic in tropical countries for more than 30 years [6], and even recently has been suggested as a treatment for dementias. Ringman JM and Cummings JL [7] indicate that metrifonate is an inactive prodrug. Its active metabolite is 2,2-dimethyldichlorovinyl phosphate (DDVP), which irreversibly inhibits the enzyme acethylcholinesterase. In 1998 Jann MW [8] observed that metrifonate and DDVP improved performance in young rats and cognitive functions in aged rats. Chronic treatment of aging rats with metrifonate induces a long-lasting increase of acetylcholine levels [9].

Chronic application of sodium nitrite induced long-lasting effects such as hypoxia, neuron damage and impaired behavior [10].

The aim was to study the effects of metrifonate on learning and memory in naive rats and in rats with hypoxia, induced by sodium nitrite (a model of impaired memory function).

2 Statistical methods and Experimental Procedures

2.1 Drug

Metrifonate (Bayer) is a dimethyl ester of (2, 2, 2-trichloro-1-hydroxyethyl)-phosphonic acid.

2.2 Animals

Male Wistar rats weighing 180-220g kept under standard laboratory conditions (08.00-20.00 light, food and water at libitum) were used. The animals were divided into the following experimental groups (n = 9): A: saline, s.c. + saline, p.o. (0.1 ml/100 g body weight); B: saline, s.c. + metrifonate, 30 mg/kg p.o.; C: saline, s.c. + metrifonate, 50 mg/kg p.o.; D: saline, s.c. + metrifonate, 80 mg/kg p.o.; E: sodium nitrite, 50mg/kg, s.c. + saline, p.o. F: sodium nitrite, 50mg/kg, s.c. + metrifonate, 30 mg/kg, p.o.; G: sodium nitrite, 50mg/kg, s.c. + metrifonate, 50 mg/kg, p.o.; H: sodium nitrite, 50mg/kg, s.c. + metrifonate, 80 mg/kg, p.o. Immediately after subcutaneous injection, the rats received per gavage the second application and the tests were performed 60 minutes later.

2.3 Behavior tests

An automatic reflex conditioner for active avoidance “shuttle box” (Ugo Basile, Commerio-Varese, Italy) was used. A learning session of 5 consecutive days was performed. Each day consisted of 30 trials with the following parameters: 6s light and buzzer (670 Hz and

70 dB), 3s 0.4 mA foot shock, and 12s pause. A memory retention session with the same parameters without foot shock was performed 7 days later (12th day). The parameters automatically counted were as follows: 1 - number of conditioned responses (avoidances); 2 - number of unconditioned stimuli (escapes from foot shock); 3 - number of intertrial crossings.

Two passive avoidance tests were used:

An automatic set-up for passive avoidance "step-through"" (Ugo Basile) was used. The test parameters were as follows: 6s door delay, open door for 12s, and 0.4 mA foot shocks 9s later. A learning session of 2 consecutive days was performed. A short-term memory retention session and a long-term one were done on the 3^{rd} day (24 hours later) and 10th day respectively. Each session consisted of 3 trials, and a 30-min pause between each trial. The learning criterion was a latency of reactions of 180 $\pm$ 2 seconds in the light chamber.

An automatic set-up for passive avoidance "step-down" wire cage with plastic platform (Ugo Basile) was used. The learning session consisted of 2 trials (electrical stimulation duration of 10s at intensity 0.4 mA) with a 60-min interval between trials. A learning session of 2 consecutive days was done. A short-term memory retention session and a long-term one were performed on the 3^{rd} day (24 hours later) and 10th day. The memory retention test with the same parameters without foot shock was done. The latency of reactions (the rat remaining on the platform for more than 60s) was accepted as the criterion for learning and retention.

2.4 Morphological methods

The rats from 2 groups, the sodium nitrite group (E) and the sodium nitrite + metrifonate 50 mg/kg (G) group, were used for morphological study.

Intracardial perfusion with 2,5% glutaraldehyde under ether narcosis was done. The separation of brain and subsequent 4% glutaraldehyde fixation was made. After fixation, the two hemispheres were sagitally cut.

2.5 Light microscopy

The left-brain hemispheres were cut 1 mm lateral from and parallel to the sagital line, corresponding to the anatomical localization of the hypocampus, dehydrated in ascending alcohol and stained with eosin-hematoxylin. The 4 μm brain slides were fixed on glass holders and observed at 200x magnification.

2.6 Ultrastructure

The right-brain hemispheres were cut 1 mm lateral from and parallel to the sagital line and fragments from CA1 and CA2 regions of the hypocampus measuring 1,5 mm^3 were chosen. This was followed by fixation in 4% glutaraldehyde for 24 hours. Post-fixation

in osmium tetraoxide was performed, and then they were buffered in cacodilatic buffer solution and dehydrated in ascending alcohol, before the embedding in durcopan. The 0,03 μm cut sections were obtained, fixated in copper girds and examined by transmission electron microscopy Philips CM 12/STEM (The Netherlands).

2.7 Statistical analysis

The data were analyzed with one-way ANOVA using the INSTAT computer program. The mean ± S.E.M. for each group was calculated. Two-way ANOVA for repeated measurements was used to compare the experimental groups with the corresponding control groups.

3 Results

3.1 Effects of metrifonate on naive rats

3.1.1 In the shuttle-box active avoidance test

The control naive rats showed a statistically significant increase in the number of conditioned stimuli responses (avoidances) on the 4^{th} ($P < 0.05$) and 5^{th} ($P < 0.01$) days of learning session compared to the 1^{st} day, and maintained the increase in the memory retention session on the 12th day ($P < 0.01$) (Fig. 1).

The group treated with 30 mg/kg doses of metrifonate significantly increased the number of avoidances on the 1^{st}, 2^{nd}, 3^{rd} and 4^{th} days of the learning session ($P < 0.05$) compared to the same days control group (Fig. 1), as did the group treated with 50 mg/kg doses of metrifonate. The rats with higher dose metrifonate 80 mg/kg increased the number of avoidances on the 2^{nd} and 3^{rd} days of the learning session compared to the same control (Fig. 1). In the memory retention test, the groups with metrifonate did not change the number of avoidances compared to the control on the 12th day (Fig. 1).

The three different doses of metrifonate used did not change the number of unconditioned stimuli responses (escapes) and intertrial crossings during the learning session and memory retention test (Table 1 and 2).

3.1.2 In the step-through passive avoidance test

The control naive rats increased the latency of reaction ($P < 0.05$) on the 2^{nd} day of the learning session and in the short-term memory retention test (3^{rd} day), compared to the 1^{st} day control (Fig. 2).

The groups with metrifonate (at all doses used) did not change the latency of reactions in the learning session and in the short-term memory retention test, but significantly prolonged the latency of reaction in the long-term memory retention on the 10th day ($P < 0.05$), compared to the same day saline group (Fig. 2).

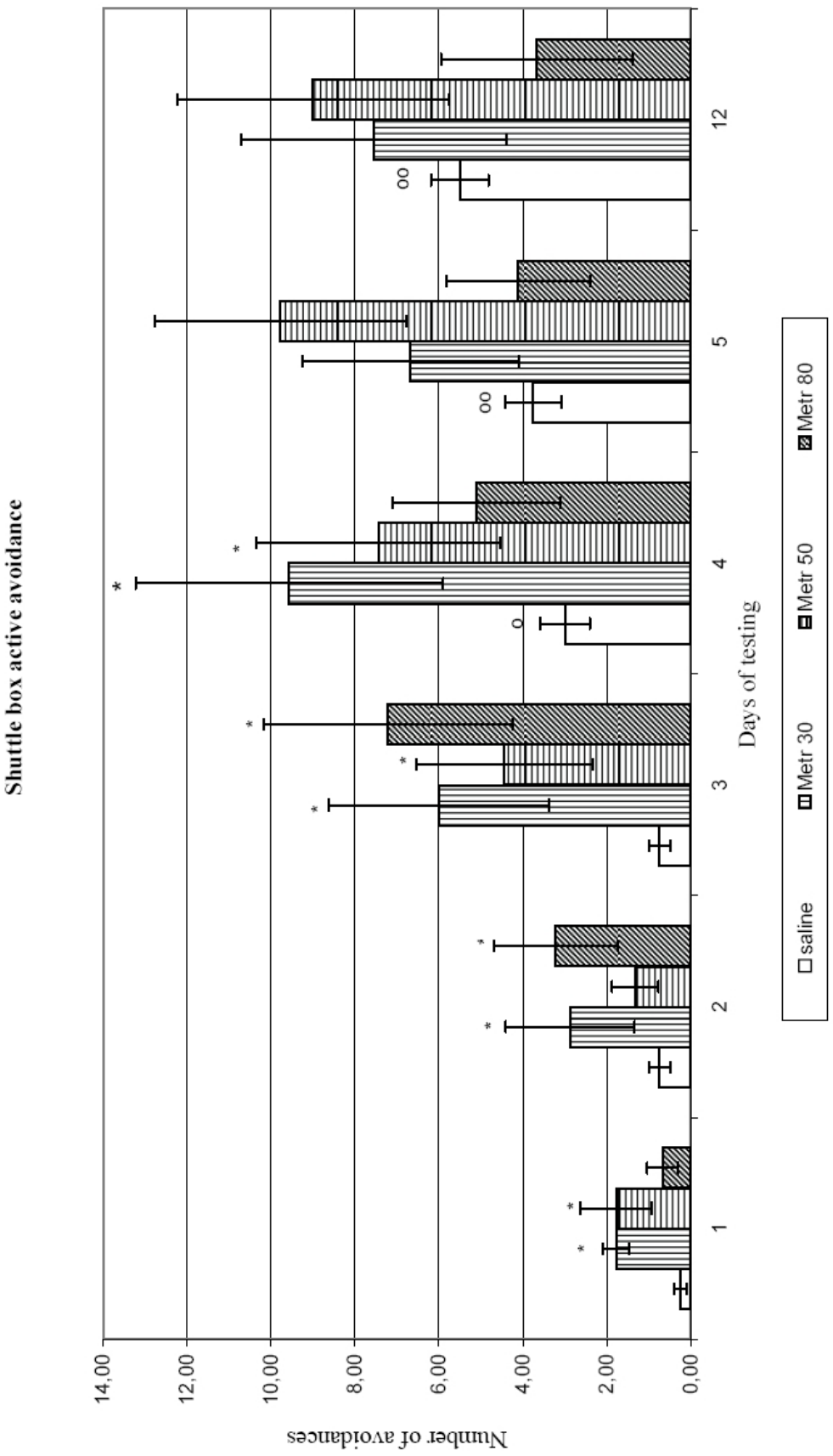

Fig. 1 Effects of metrifonate (Metr) on the conditioned stimuli responses (avoidances) in the active avoidance test. On the abscissa - days of testing; on the ordinate - number of avoidances. $^{o}P < 0.05$ and $^{oo}P < 0.01$ compared to the 1^{st} day control; $^{*}P < 0.05$ compared to the same day control.

Table 1 Effects of metrifonate on active avoidance test (shuttle box): number of unconditioned stimuli responses (escapes).

Days	Saline 0.1 ml/100 g	Saline 0.1 ml/100 g + metrifonate 30 mg/kg	Saline 0.1 ml/100 g + metrifonate 50 mg/kg	Saline 0.1 ml/100 g + metrifonate 80 mg/kg
1	14.75 ± 1.95	13.67 ± 2.34	16.00 ± 2.69	13.67 ± 3.15
2	15.63 ± 2.41	13.13 ± 2.83	19.56 ± 2.25	16.78 ± 2.53
3	16.13 ± 2.52	14.22 ± 2.16	20.44 ± 1.78	12.78 ± 2.84
4	11.75 ± 2.23	10.89 ± 2.92	17.67 ± 2.53	17.89 ± 1.68
5	12.25 ± 1.90	14.67 ± 3.13	14.11 ± 2.20	10.11 ± 3.28
12	15.50 ± 3.17	13.89 ± 3.28	12.78 ± 2.75	17.11 ± 2.94

Table 2 Effects of metrifonate on active avoidance test (shuttle box): number of intertrial crossings.

Days	Saline 0.1 ml/100 g	Saline 0.1 ml/100 g + metrifonate 30 mg/kg	Saline 0.1 ml/100 g + metrifonate 50 mg/kg	Saline 0.1 ml/100 g + metrifonate 80 mg/kg
1	11.00 ± 0.91	13.00 ± 2.12	$19.33 \pm 4.13*$	12.11 ± 3.03
2	11.00 ± 3.06	14.78 ± 2.75	16.33 ± 3.16	15.11 ± 2.45
3	14.63 ± 3.39	13.33 ± 2.48	20.89 ± 3.12	15.56 ± 2.93
4	15.88 ± 3.73	15.78 ± 3.48	18.67 ± 2.35	23.44 ± 3.75
5	19.63 ± 4.12	19.33 ± 5.32	16.78 ± 2.61	6.67 ± 2.65
12	11.50 ± 1.60	12.78 ± 3.85	13.22 ± 2.59	16.67 ± 4.38

3.1.3 In the step-down passive avoidance test

The naive rats increased the latency of reactions in the short-term (3^{rd} day) and long-term (7^{th} day) memory retention tests ($P < 0.05$) compared to the 1^{st} day of the learning session. The groups treated with 30, 50 and 80 mg/kg metrifonate doses significantly increased ($P < 0.05$) the latency of reactions in both days of the learning session and in the short-term memory retention test, compared to the controls. In the long-term memory retention test, animals with the 30 mg/kg metrifonate doses significantly increased the latency of reactions ($P < 0.05$) compared to the same day saline group (Fig. 3).

3.2 Effects of metrifonate on rats with hypoxia-induced impaired memory

3.2.1 In the shuttle box active avoidance test

The rats treated with sodium nitrite only significantly increased the number of avoidances ($P < 0.05$) on the 4^{th} and 5^{th} days of the learning session and maintained the increase in the memory retention session, compared to the 1^{st} day (Fig. 4).

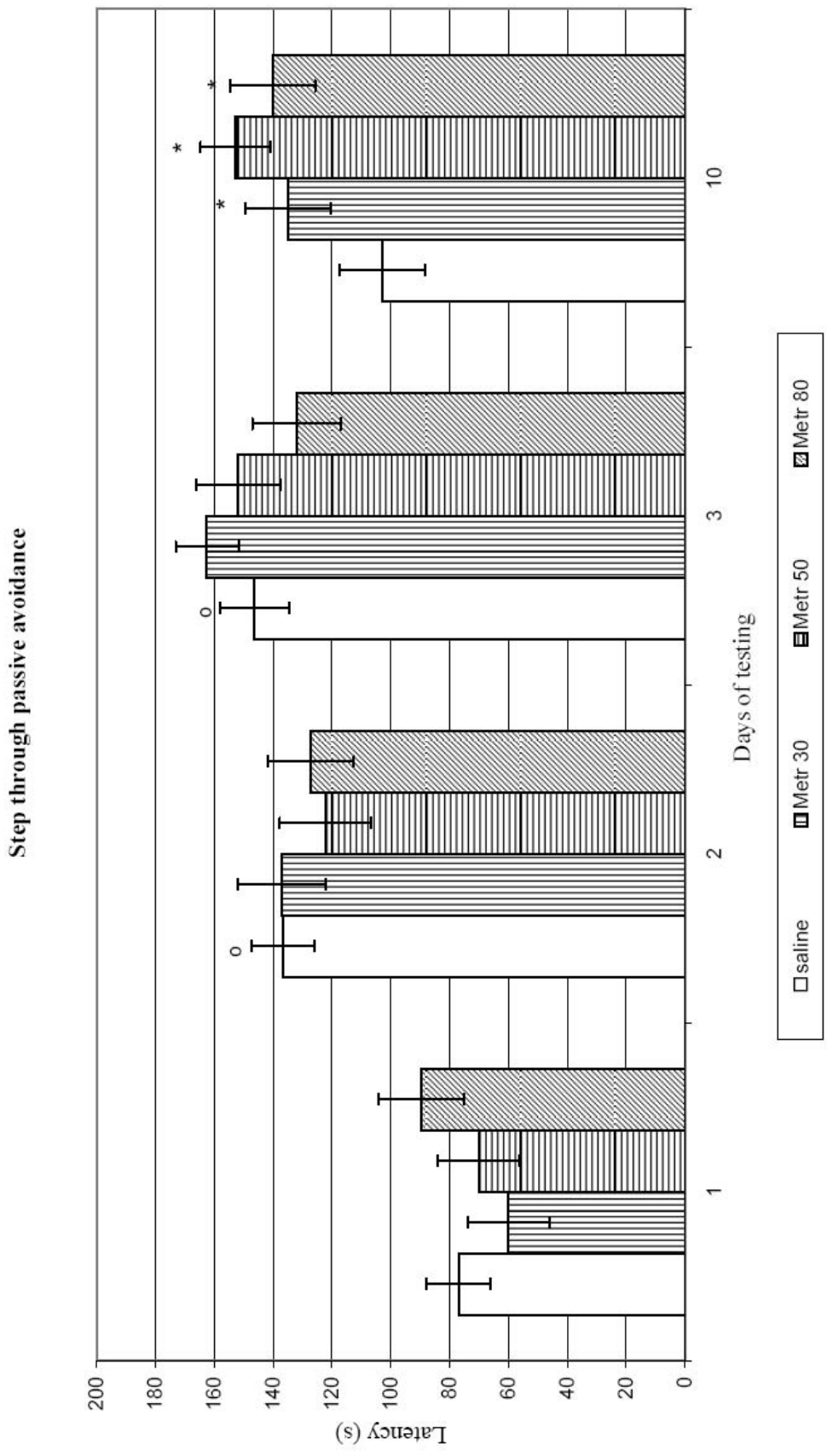

Fig. 2 Effects of metrifonate (Metr) on latency in the step-through passive avoidance test. On the abscissa - days of testing; on the ordinate - latency of time spent on the light chamber in seconds. $^{o}P < 0.05$ compared to the 1st day control; $^{*}P < 0.05$ compared to the same day control.

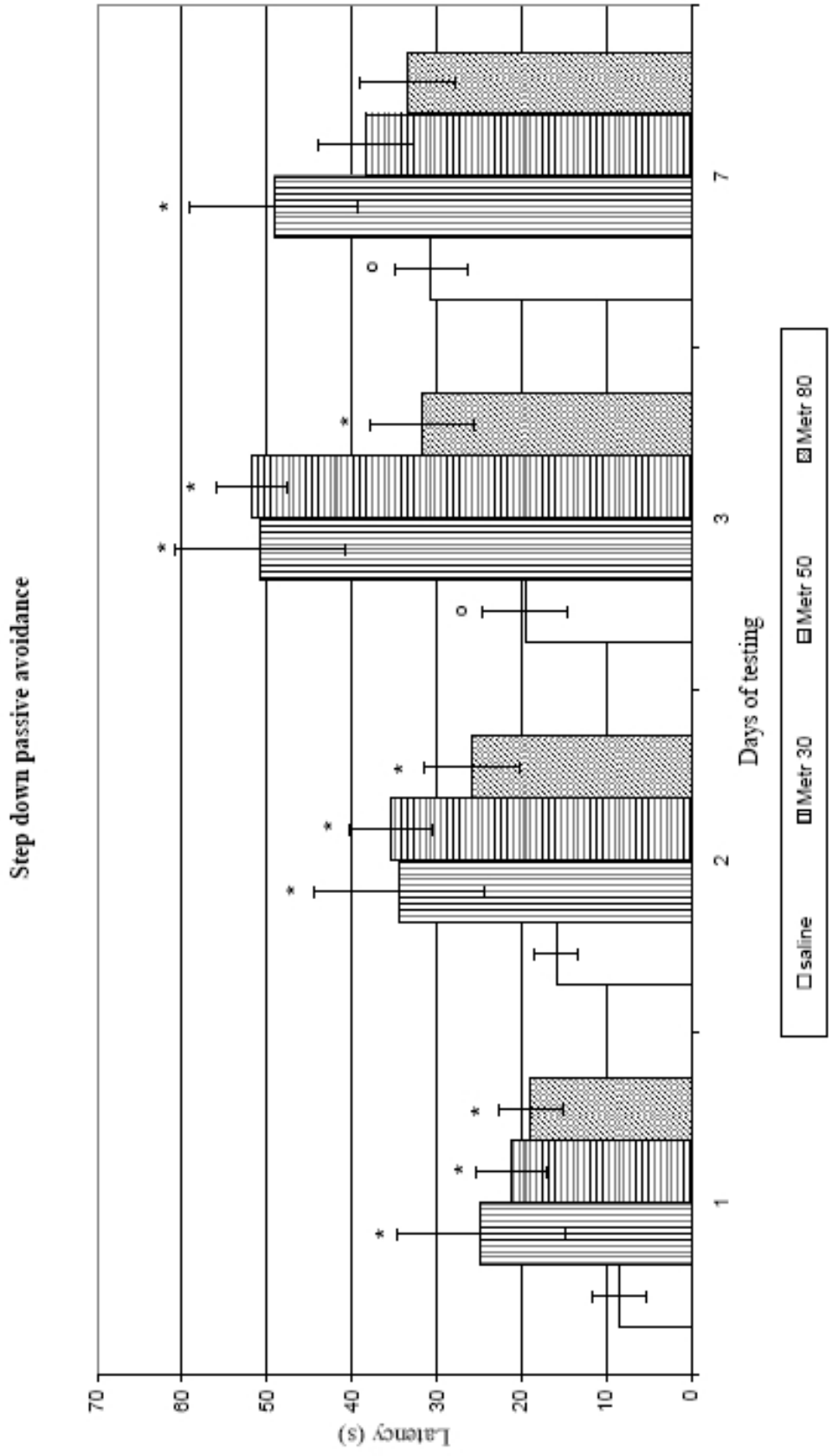

Fig. 3 Effects of metrifonate (Metr) on latency in the step-down passive avoidance test. On the abscissa - days of testing; on the ordinate - latency of time spent on the platform in seconds. $^{o}P < 0.05$ compared to the 1^{st} day control; $^{*}P < 0.05$ compared to the same day control.

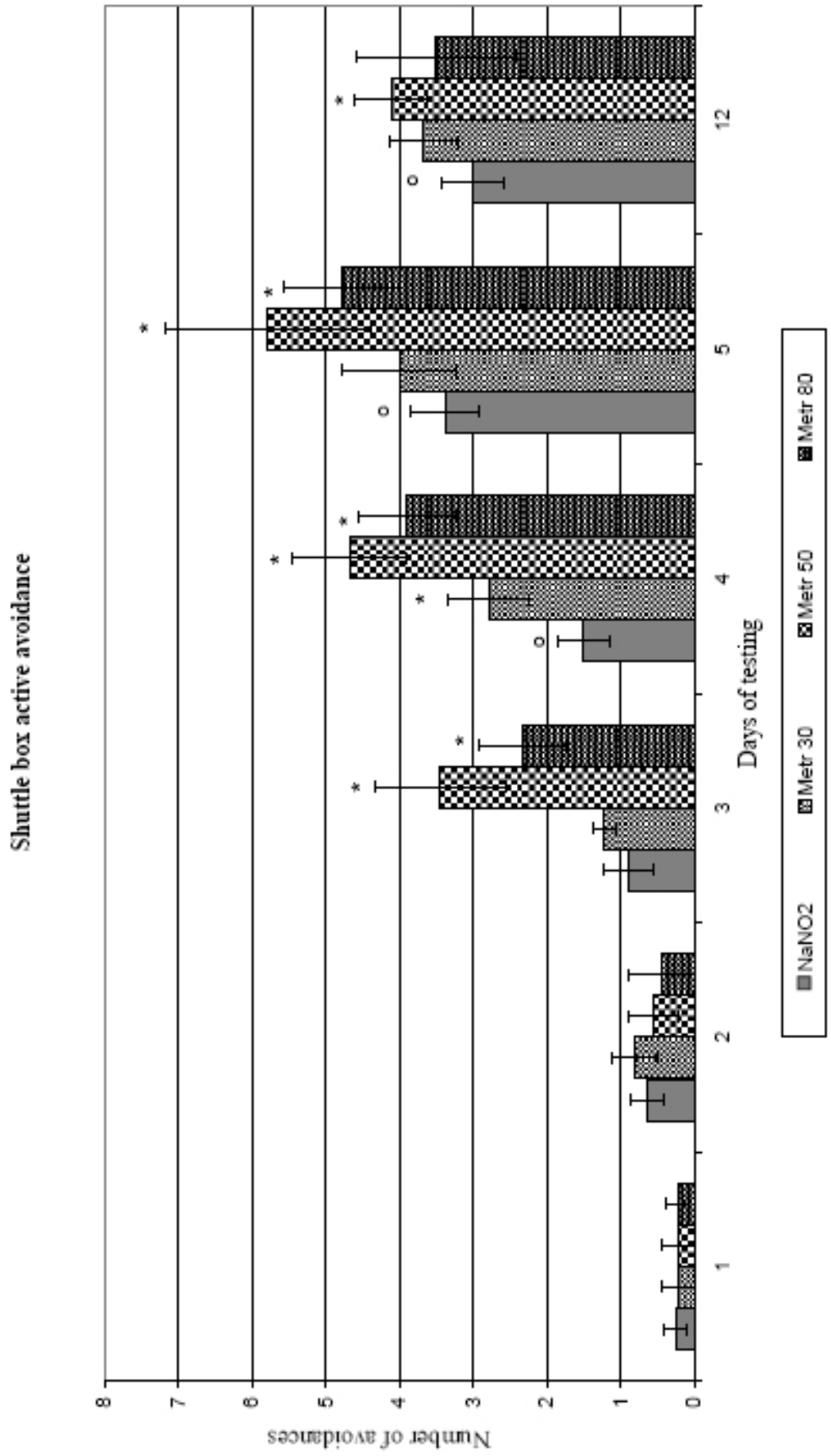

Fig. 4 Effects of metrifonate (Metr) on the conditioned stimuli responses (avoidances) in the active avoidance test. On the abscissa - days of testing; on the ordinate - number of avoidances. $^{o}P < 0.05$, compared to the 1^{st} day rats treated with sodium nitrite only; $^{*}P < 0.05$ compared to the same day rats treated with sodium nitrite only.

The animals with hypoxia that received bigger doses of metrifonate 50 mg/kg and 80 mg/kg had significant increased number of avoidances on the 3^{rd}, 4^{th} and 5^{th} days ($P < 0.05$) of the learning session compared to the group with sodium nitrite only. In the memory retention test only rats treated with metrifonate 50 mg/kg significantly increased ($P < 0.05$) the number of avoidances compared to the same day sodium nitrite group (Fig. 4).

The groups with the model of hypoxia and metrifonate (all used doses) did not significantly change the number of unconditioned stimuli responses (escapes) and the number of intertial crossings during the learning and memory retention sessions (Table 3 and 4).

Table 3 Effects of metrifonate on active avoidance test (shuttle box) in a model of hypoxia: number of unconditioned stimuli responses (escapes).

Days	$NaNO_2$ 50 mg/kg + Saline 0.1 ml/100g	$NaNO_2$ 50 mg/kg + metrifonate 30 mg/kg	$NaNO_2$ 50 mg/kg + metrifonate 50 mg/kg	$NaNO_2$ 50 mg/kg + metrifonate 80 mg/kg
1	6.88 ± 2.11	4.89 ± 0.87	6.67 ± 1.70	9.22 ± 2.31
2	5.75 ± 2.34	7.00 ± 2.09	8.11 ± 1.99	7.00 ± 2.15
3	8.63 ± 2.35	6.78 ± 2.44	7.89 ± 1.93	6.78 ± 1.49
4	6.88 ± 2.92	5.44 ± 1.98	7.67 ± 2.40	8.22 ± 1.90
5	8.00 ± 3.32	7.44 ± 2.80	5.67 ± 1.76	8.33 ± 2.27
12	8.25 ± 3.30	6.22 ± 2.22	5.89 ± 2.85	7.38 ± 2.88

Table 4 Effects of metrifonate on active avoidance test (shuttle box) in a model of hypoxia: number of intertrial crossings.

Days	$NaNO_2$ 50 mg/kg + Saline 0.1 ml/100g	$NaNO_2$ 50 mg/kg + metrifonate 30 mg/kg	$NaNO_2$ 50 mg/kg + metrifonate 50 mg/kg	$NaNO_2$ 50 mg/kg + metrifonate 80 mg/kg
1	7.25 ± 2.68	4.00 ± 0.65	6.33 ± 2.10	12.67 ± 5.46
2	8.75 ± 3.86	7.00 ± 2.12	7.11 ± 4.18	5.22 ± 1.53
3	14.63 ± 4.43	9.89 ± 3.96	7.00 ± 2.89	5.11 ± 1.74
4	14.38 ± 5.46	7.00 ± 2.89	6.33 ± 2.25	7.00 ± 2.88
5	17.13 ± 5.65	9.44 ± 4.40	5.67 ± 2.12	7.89 ± 2.41
12	12.38 ± 4.31	6.89 ± 2.98	8.45 ± 3.49	5.13 ± 1.90

3.2.2 In the step-through passive avoidance test

The rats with sodium nitrite only significantly increased the latency of reactions ($P < 0.05$) during the short-term (3^{rd} day) and long-term (10th day) memory retention tests, compared to the 1^{st} day (Fig. 5).

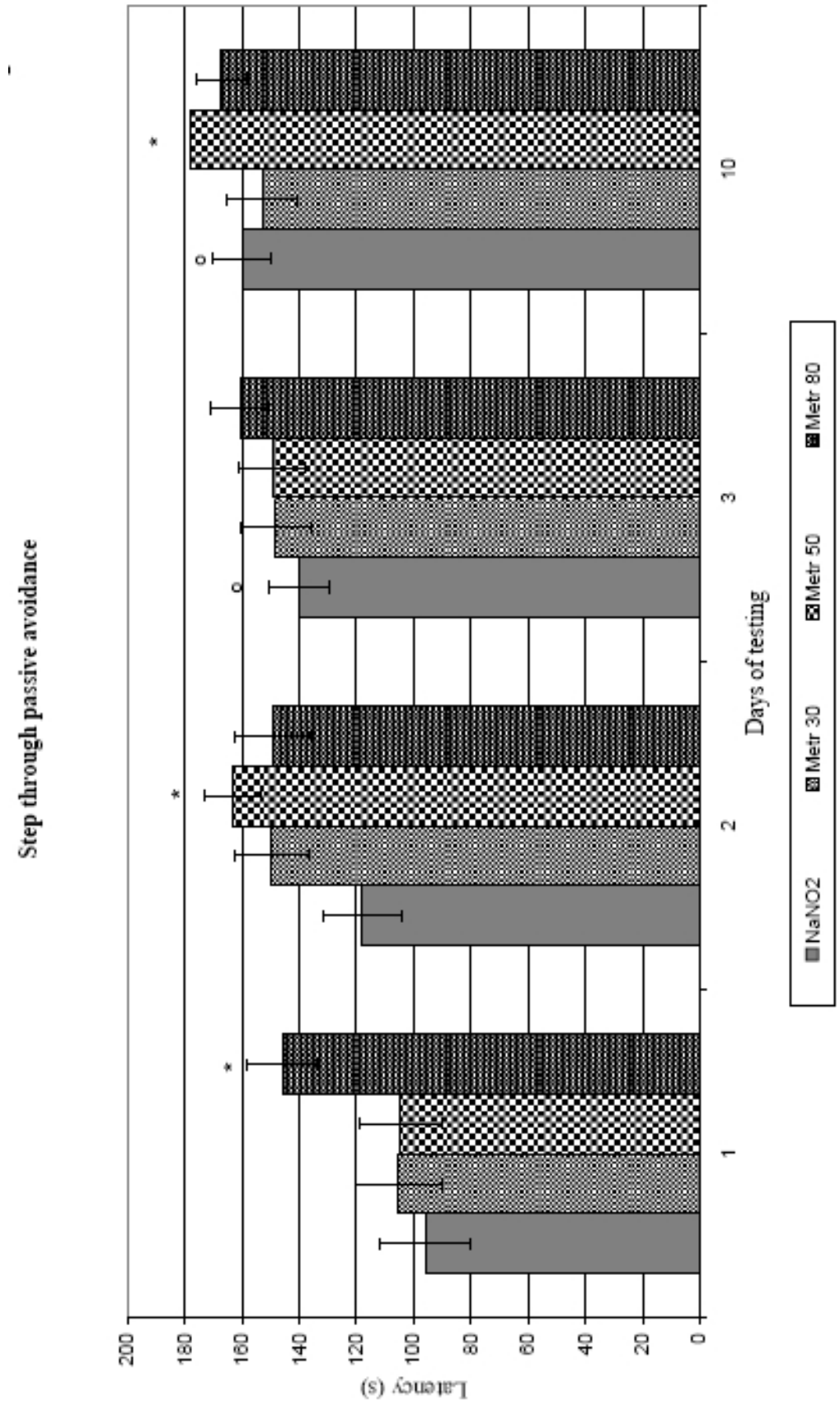

Fig. 5 Effects of metrifonate (Metr) on latency in the step-through passive avoidance test. On the abscissa - days of testing; on the ordinate - latency of time spent on the light chamber in seconds. $^{o}P < 0.05$ compared to the 1st day rats treated with sodium nitrite only; $^{*}P < 0.05$ compared to the same day rats treated with sodium nitrite only.

The animals subjected to hypoxia that received the 30 mg/kg doses of metrifonate expressed no change in the latency of reactions during the learning session, and short-term and long-term memory retention tests compared to the sodium nitrite group. The rats with the model of hypoxia and 50 mg/kg doses of metrifonate significantly increased the latency of reactions ($P < 0.05$) on the 2^{nd} day of the learning session and in the long-term memory retention test compared to the group with hypoxia only (Fig. 5). The hypoxic group treated with 80 mg/kg doses of metrifonate increased the latency of reactions ($P < 0.05$) on the 1^{st} day of the learning session compared to same day rats treated with sodium nitrite only. No significant differences were observed in the short-term memory retention test on rats treated with metrifonate, compared to the group with the model only (Fig. 5).

3.2.3 In the step-down passive avoidance test

The rats injected with sodium nitrite only did not change in the latency of reaction during the learning session, and the short-term and long-term memory sessions, compared to the controls (Table 5).

Table 5 Effects of metrifonate on passive avoidance test (step-down) in a model of hypoxia: latency of reactions.

Days	$NaNO_2$ 50 mg/kg + Saline 0.1 ml/100g	$NaNO_2$ 50 mg/kg + metrifonate 30 mg/kg	$NaNO_2$ 50 mg/kg + metrifonate 50 mg/kg	$NaNO_2$ 50 mg/kg + metrifonate 80 mg/kg
1	29.80 ± 6.13	27.41 ± 5.71	30.17 ± 6.25	37.32 ± 6.53
2	37.73 ± 6.15	44.54 ± 4.99	45.66 ± 5.67	45.02 ± 6.12
3	31.76 ± 5.80	41.34 ± 5.80	$47.31 \pm 5.07*$	44.84 ± 5.90
7	43.43 ± 5.58	48.89 ± 4.86	50.64 ± 4.89	43.16 ± 6.02

The groups with the model of hypoxia and metrifonate treatment at doses of 30, 50 and 80 mg/kg did not significantly alter the latency of reactions in the two-day learning session and long-term memory retention test. In the short-term memory retention test, the animals subjected to hypoxia that received 50 mg/kg doses of metrifonate significantly increased ($P < 0.05$) the latency of reactions compared to the same day sodium nitrite rats (Table 5).

3.2.4 Morphological data

The light microscopy slices from regions CA1 and CA2 of the hippocampus in animals treated with sodium nitrite only (group E) reveal a significant number of neurons with picnotic nuclei and condensed eosinophillic cytoplasm (Fig. 6). The nuclei ultrastructurally display increased amounts of functionally inactive chromatin, i.e. heterochromatin. In some of the neurons, the heterochromatin is placed at the periphery of the nuclear membrane (Fig. 6). Profiles of the rough endoplasmic reticulum are dilated and

the mitochondria are filled with electron-dense granules. The number of glial cells is increased.

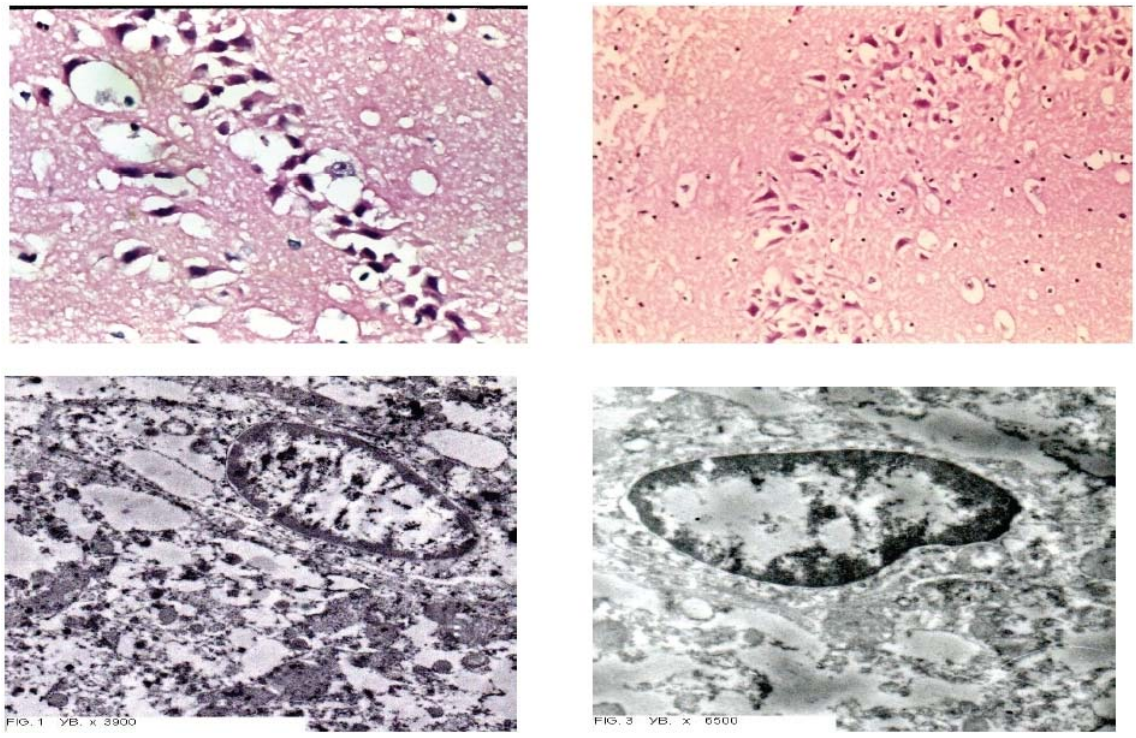

Fig. 6 Morphological changes in the CA1 zone of the hippocampus in rats with the model of hypoxia (left panels) and rats with hypoxia treated with metrifonate (right panels). Upper panels are data observed under light microscopy; lower panels are data observed under electronic microscopy.

In group G the number of neurons is decreased in both zones CA1 and CA2 of the hippocampus. The decrease in picnotic nuclei in neurons was observed to be less expressed and the increase of glial cells was only moderate compared with group E (Fig. 6). The degenerative changes are less expressed; there were zones with low electronic density, necrosis and apoptosis in some neurons. The glial cells proliferation was less expressed as well.

4 Discussion

In the active avoidance test, the naive control rats showed a well-expressed learning ability that was preserved during the memory retention test, suggesting formation of memory traces. Metrifonate, applied alone, improves learning ability in the shuttle-box test during the 5-day learning session.

In the step-through passive avoidance test, control rats learned the task and maintained it for the short-term memory retention test only, but in the step-down passive avoidance test control rats not only learned the task but also kept it in both memory retention tests. In the step-down passive avoidance test, the animals treated with all doses

of metrifonate learned the task very well and maintained it for the short-term memory retention test. In the step-through passive avoidance test, the same groups of rats had increased latency of reactions only in the long-term memory test. However, it is possible to suggest, that metrifonate in the three doses used helped the formation of short-term and long-term memory traces.

The effects of metrifonate on one-trial learning in intact healthy adult rats have been studied in passive avoidance tasks (Schmidt and De Jonge [11]). In this study metrifonate was administered orally 30 minutes before the acquisition trial. It is likely that the compound affected the acquisition processes, because under this condition, the compound had a beneficial effect on the retention performance 24 hours after the acquisition trial.

Jos Prickaerts *et al.* [12] observed in healthy adult rats the memory-enhancing effects of metrifonate in the object recognition task, and concluded that metrifonate (30 mg/kg) given before the first trial showed an improved memory performance in rats. According to their findings, the administration of a drug before the learning trial should have an effect on the acquisition of information. Our results are in favor of such suggestion.

In our experiments using the shuttle-box active avoidance test, the animals with the model of hypoxia only showed lower learning ability. Metrifonate at doses of 50 mg/kg had improving effects on learning and memory performance. In the passive avoidance tests the rats with the model of hypoxia did not learned the task well and had increased latency of reactions in the short-term and long-term memory retention tests (step-through), probably due to decreased motor activity induced by sodium nitrite. The chronic application of sodium nitrite induced long-term consequences, mainly impaired behavior [13], suggesting that this increased latency is probably not a real memory improvement. In our experiments metrifonate did not improved learning and memory retention in the step-down passive avoidance tests in rats subjected to hypoxia.

There are data that haemic hypoxia caused by the methemoglobin-inducing agent sodium nitrite evokes disturbed habituation in the open field and impaired learning and memory in the passive avoidance paradigm [14]. Others [15] have studied the short-term effects and long-term consequences of sodium nitrite hypoxia on the spontaneous behavior of rats. They found delayed structural alterations in the CNS due to exposure to sodium nitrite for several days. Our results are in accordance with such suggestions.

Our data showed that the cholinesterase inhibitor metrifonate probably has some effect on brain functions, improving the active avoidance learning in the model of hypoxia. Metrifonate antagonized the damaging effect of sodium nitrite and improved learning ability in the last three days of the learning period and maintained it during the memory retention test taken seven days later. The rats treated with metrifonate showed less stimulating motor activity (changes in the number of intertrial crossings) in the learning period.

Giovannini M. *et al.* [16] published that in aged rats ($24 - 26$ months), a subchronic treatment with metrifonate results in a long-lasting cholinesterase inhibition and a persistent increase in acetylcholine extracellular levels, which compensate for the age-associated cholinergic hypofunction. They conclude that metrifonate is therefore a potentially use-

ful agent for the treatment of memory disturbances accompanying Alzheimer's disease and other types of dementia. They suggest that the long-lasting increase of extracellular acetylcholine levels after metrifonate treatment reflects the persistent inhibition of cholinesterase activity, caused by its active metabolite DDVP. This mechanism of action of metrifonate can explain its long-acting cholinesterase inhibition and the ensuing persistent rise in extracellular acetylcholine levels.

Furthermore, the large effect of metrifonate on the cortical cholinergic system did not lead to changes in choline acetylesterase activity, or in the number or affinity of muscarinic or nicotinic receptors, as demonstrated by Schmidt *et al.* [17]. It has been also shown [18] that cognitive improvement due to metrifonate becomes long lasting in subchronically treated rats. The subchronic oral administration of metrifonate (80 mg/kg) in aged rats results in a profound, long-lasting cholinesterase inhibition paralleled by an increase in extracellular acetylcholine levels, both in the cortex and hippocampus. The increase in acethylcholine levels in the cortex was much larger than in the hippocampus [16]. Our study rats, with hypoxia and metrifonate at the same dose for the similar experimental period, showed changes in the hippocampus, probably due to higher acetylcholine levels that allow the improvement of the cognitive functions.

The histochemical study suggests that the development of cholinergic innervation after long-term prenatal hypoxia (induced by chronic exposure to sodium nitrite) was impaired. The animals had a transient delay in the cholinergic innervation of parietal neocortex and dental gyrus and retardation of the cholinergic fiber development and outgrowth in the cortical target area [19]. In our experiments the morphological changes are corresponding to initial and reversible neuronal damages with subsequent compensatory regeneration presented with the increased presence of glial cells. The reduced learning ability and impaired memory in our experiments were probably due to sodium nitrite-induced hypoxia leading to decreased oxygen content in the brain, as suggested by Koziar *et al.* [14].

Our results suggest that metrifonate improve learning and memory processes in naive rats and rats with hypoxia-induced impaired memory, and are in favor of the hypothesis [11] of its role in preventing the fast alteration and malfunction of neurons. Experiments demonstrate that metrifonate increases neuronal excitability in CA1 pyramidal neurons and also enhances the learning rate of hippocampus-dependent tasks, especially in aged animals [20]. It is also pointed out that the amelioration of hippocampal impairment with the cholinergic compound metrifonate may in part be due to the enhanced excitability of hippocampal pyramidal neurons [21]. Our morphological results are in favor of such suggestions.

Our data taken together with literature findings allow us to conclude that in the rat model of hypoxia, the anticholinesterase drug metrifonate have moderate improving effects on learning and memory processes.

Acknowledgments

This work was supported by the Grant U-L-05/2003 of the National Science Fund at the Bulgarian Ministry of Education and Science.

References

[1] A.J. Lerner and P.J. Whitehouse: "Primary dementias", In: *Clinical Neurology of Aging*, 2nd ed, M.L. Albert and J.E. Knoefel (Eds.), New York, Oxford Press, 1994.

[2] I. Gelinas and S. Auer: "Functional autonomy", In: *Clinical Diagnosis and Management of Alzheimer's disease* S. Gauthier (Ed.); Martin Dunitz Ltd, London, 1996.

[3] J.M. Lopez–Arrieta and L. Schneider: "Metrifonate for Alzheimer's disease", *Cochrane Database Syst. Rev.*, Vol. 19, (2006).

[4] P.J. Whitehouse, D.L. Price, A.W. Clark, J.T. Coyle AND M.R. DeLong: "Alzheimer's disease: Evidence for selective loss of cholinergic neurons in the nucleus basalis", *Ann. Neurol.*, Vol. 10, (1981) pp. 122–126.

[5] R.T. Bartus, R.L. III Dean, B. Beer and A.S. Lippa. "The cholinergic hypothesis of geriatric memory dysfunction", *Science*, Vol. 217, (1992), pp. 408–417.

[6] J. Cerf, A. Lebrun and J. Dierichx: "A new approach in helminthiasis control: The use of an organophosphors compound", *Am. J. Trop. Med. Hyg.*, Vol. 11, (1962), pp. 514–517.

[7] J.M. Ringman and J.L. Kummings: "Metrifonate: update on a new antidementia agent", *J. Clin. Psychiatry*, Vol. 60, (1999), pp. 776–782.

[8] M.W. Jann: "Preclinical pharmacology of metrifonate", *Pharmacotherapy*, Vol. 18, (1998), pp. 55–67, pp.79–82.

[9] C. Scali, F. Casamenti, A. Belluci, C. Costagli, B. Schmidt and G. Pepeu: "Effect of subchronic administration of metrifonate, rivastigmine and donepezil on brain acetylcholine in aged F344 rats", *J. Neural. Transm.*, Vol. 109, (2002), pp. 1067–1080.

[10] Z. Hlinak and I. Krejci: "Long-term behavior consequences of sodium nitrite hypoxia: an animal model", *Acta Nerv. Super.*, Vol. 32, (1990), pp. 48–53.

[11] B.H. Schmidt and M. De Jonge: "Behavioral and biochemical studies on metrifonate: a novel putative Alzheimer therapeutic", *Biol. Psychiatry*, Vol. 29, (1991), p. 486S.

[12] J. Prickaerts, A. Sik, F.J. van der Staay, J. de Vente and A. Blokland: "Dissociable effects of acethylcholinesterase inhibitors and phosphodiesterase type 5 inhibitors on object recognition memory: acquisition versus consolidation", *Psychopharmacology*, Vol. 177, (2005), pp. 381–390.

[13] C.L. Liu, B.K. Siesjo and B.R. Hu: "Pathogenesis of hippocampal neuronal death after hypoxia-ischemia changes during brain development", *Neuroscience*, Vol. 127, (2004), pp. 113–123.

[14] V.S. Koziar, S.S. Trofimov, R.U. Ostrovskaia, A.K. Sariev and V.P. Zherdev. "Prenatal exposure to sodium oxybutirate prevents a disorder of general behaviour, learning and memory in the progeny of rats subjected to chronic haemic hypoxia", *Eksp. Klin. Farmakol.*, Vol. 57, (1994), pp. 8–11.

[15] Z. Hlinak, I. Krejci, .J. Hondlik and A.Yamamoto: "Behavioral consequences of sodium nitrite in male rats: amelioration with alaptide treatment", *Methods. Find. Exp. Clin. Pharmacol.*, Vol. 12, (1990), pp. 385–393.

[16] G.M. Govannini, C. Scali, L. Bartolini, B. Schmidt and G. Pepeu. "Effect of subchronic treatment with metrifonate and tacrine on brain cholinergic function in aged F344 rats", *Eur. J. Pharmacol.*, Vol. 354, (1998), pp. 17-24.

[17] B.H. Schmidt, V.C. Hinz and F.J. van der Staay: "The preclinical pharmacology of metrifonate, a long-acting and well tolerated cholinesterase inhibitor for Alzheimer therapy", In: *Progress in Alzheimer and Parkinson's Disease*, A. Fisher, M. Yoshida, I. Hanin (Eds.), Plenum, New York, 1997.

[18] M. Riekkinen, B.H. Schmidt and P.J. Riekkinen: "Subchronic treatment increases the duration of the cognitive enhancement induced by metrifonate", *Eur. J. Pharmacol.*, Vol. 338, (1997), pp. 105–110.

[19] C. Nyakas, B. Buwalda, R.J. Kramers, J. Traber and P.G. Luiten: "Postnatal development of hyppocampal and neocortical cholinergic and serotonergic innervation in rat: effects of nitrite-induced prenatal hypoxia and nimodipine treatment", *Neuroscience*, Vol. 59, (1994), pp. 541–559.

[20] J.F. Disterhoft and M.M. Oh: "Pharmacological and molecular enhancement of learning in aging and Alzheimer's disease", *J. Physiol. Paris*, Vol. 99, (2006), pp. 180–192.

[21] J.F. Disterhoft and M.M. Oh: "Modulation of cholinergic transmission enhances excitability of hippocampal pyramidal neurons and ameliorates learning impairments in aging animals", *Neurobiol. Learn. Mem.*, Vol. 80, (2003), pp. 223–233.