



The anxiolytic effects of atorvastatin and simvastatin on dietary-induced increase in homocysteine levels in rats

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Abstract

The aim of this study was to evaluate the effects of atorvastatin and simvastatin on behavioral manifestations that followed hyperhomocysteinemia induced by special dietary protocols enriched in methionine and deficient in B vitamins (B6, B9, B12) by means of alterations in anxiety levels in rats. Simultaneously, we investigated the alterations of oxidative stress markers in rat hippocampus induced by applied dietary protocols. Furthermore, considering the well-known antioxidant properties of statins, we attempted to assess their impact on major markers of oxidative stress and their possible beneficial role on anxiety-like behavior effect in rats. The 4-week-old male Wistar albino rats were divided (eight per group) according to basic dietary protocols: standard chow, methionine-enriched, and methionine-enriched vitamins B (B6, B9, B12) deficient. Each dietary protocol (30 days) included groups with atorvastatin (3 mg/kg/day i.p.) and simvastatin (5 mg/kg/day i.p.). The behavioral testing was performed in the open field and elevated plus maze tests. Parameters of oxidative stress (index of lipid peroxidation, superoxide dismutase, catalase activity, glutathione) were determined in hippocampal tissue samples following decapitation after anesthesia. Methionine-load dietary protocols induced increased oxidative stress in rat hippocampus, which was accompanied by anxiogenic behavioral manifestations. The methionine-enriched diet with restricted vitamins B intake induced more pronounced anxiogenic effect, as well as increased oxidative stress compared to the methionine-load diet with normal vitamins B content. Simultaneous administration of statins showed beneficial effects by means of both decreased parameters of oxidative stress and attenuation of anxiety. The results obtained with simvastatin were more convincing compared to atorvastatin.

Keywords Homocysteine · Atorvastatin · Simvastatin · Anxiety · Oxidative stress · Rats

Abbreviations

Met	Methionine	SH	Sulfhydryl
SAM	<i>S</i> -adenosylmethionine	SOD	Superoxide dismutase
SAH	<i>S</i> -adenosylhomocysteine	CAT	Catalase
Hcy	Homocysteine	OF	Open field
MS	Methionine synthase	EPM	Elevated plus maze
GSH	Glutathione	TBARS	Thiobarbituric acid reactive substance
		MDA	Malondialdehyde

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Introduction

Methionine (Met) is essential, sulfur-containing amino-acid that has an important role in one-carbon metabolism. Methionine is converted to its active form, *S*-adenosylmethionine (SAM), the major donor of methyl-group in the numerous intracellular transmethylation reactions. SAM is converted to *S*-adenosylhomocysteine (SAH), which is intermediate of all transmethylation reactions. SAH is hydrolyzed

by a reversible enzyme SAH-hydrolase to homocysteine (Hcy) and adenosine [1]. Homocysteine is sulphurated, non-proteinogenic amino acid exclusively derived from ingested methionine in the reaction of demethylation. Homocysteine–methionine cycle plays a crucial role in maintaining the biochemical balance by methylation reactions within the central nervous system [2]. Hcy is normally metabolized via two biochemical pathways—remethylation, which converts Hcy back to methionine and transsulfuration that result in the conversion of Hcy to cysteine and taurine, with a notable role of B complex vitamins (B6, B9, B12). Under normal conditions, Hcy levels are maintained in a narrow range (5–15 μM) as a result of a balance between remethylation and transsulfuration processes [3].

However, Hcy metabolism in the brain is significantly different compared to other organs and tissues, resembling the folate/B12 remethylation pathway as the exclusive mechanism for maintaining normal levels of Hcy in the brain. Therefore, the lack of important enzymes involved in Hcy metabolism may explain the higher vulnerability of CNS to the increased Hcy levels [4]. In addition, the methylation reactions that are required for the proper synthesis of serotonin, other monoamine neurotransmitters and catecholamines [5], which play an important role in normal brain functioning and mood regulation, are largely relying on B vitamins as necessary cofactors in these reactions [6].

Dysregulation of Hcy metabolism is implicated in a number of adverse clinical outcomes. Numerous epidemiological studies have confirmed that hyperhomocysteinemia represents a risk factor for various disorders affecting CNS, such as neurological cognitive deficit [7], mental retardation [8], demyelination [9], Alzheimer's disease [10], Parkinson's disease [11], and stroke [12]. There is a growing interest in the causative relationship between impaired Hcy metabolism and psychiatric disorders [13, 14]. Several studies confirmed that elevated Hcy levels have been present in patients with major depression [15, 16]. Hyperhomocysteinemia, vitamin B12 deficiency, and folate deficiency are significantly related to depressive disorders [17]. Besides the role in depressive disorders, the effect of impaired one-carbon metabolism on anxiety disorders has been established in several studies [18], especially in obsessive–compulsive disorder [19, 20]. Animal studies have also confirmed the role of Hcy and folate metabolism in the supply of methyl groups and regulation of the biochemical pathways for methylation processes [21, 22].

The toxicity of Hcy on neuronal cells has been the focus of many investigations in past years [23, 24]. The underlying mechanisms of Hcy toxicity on brain tissue have not yet been completely clarified. One of the suggested mechanisms of neurotoxicity caused by hyperhomocysteinemia is increased oxidative damage [25, 26]. The brain tissue is more vulnerable to oxidative stress due to its modest antioxidative defense

compared to other organs and high oxygen utilization, that is necessary for normal brain functioning and, therefore, represents a source of free radical by-products [27]. In addition, the metabolism of catecholamines, neurotransmitters that are released in anxiety disorders, also represents an important source of free radicals in the brain [28]. Lipid-rich brain constitution also favors lipid peroxidation in the presence of oxidative imbalance [29]. As a result, oxidative stress may alter neurotransmission, neuronal function, and overall brain activity [30].

The role of oxidative stress as a possible pathogenic mechanism underlying psychiatric disorders, including anxiety, has been intensively studied in past years. Oxidative pathophysiology in psychiatric disorders is strongly supported by numerous human studies. The results of these studies confirmed alterations in antioxidant enzyme activities [31, 32] and decreased levels of non-enzymatic antioxidants such as vitamins C and E, glutathione (GSH), and specific antioxidant components such as free sulfhydryl (SH) groups, uric acid, and bilirubin [33, 34]. Still, it has been reported that antioxidant status was not diminished in patients with Parkinson's disease and obsessive–compulsive disorder [35, 36]. Increased lipid peroxidation (the most studied marker of oxidative stress in psychiatric disorders) has been consistently reported in numerous clinical trials [37, 38]. It has been shown that methionine-enriched diet leads to the development of hyperhomocysteinemia. The alterations in oxidative status observed in those studies included modifications in the lipid peroxidation, superoxide dismutase (SOD), and catalase (CAT) activity, as well as GSH content. Both of these studies confirmed that hyperhomocysteinemia induced by methionine nutritional overload increased anxiety-related behavior in rats [39, 40].

The proposed causal role of oxidative stress in anxiety disorders implicated the potential benefits of antioxidative therapy in the treatment of anxiety. Numerous studies have confirmed the efficacy of the conventional antioxidants, such as vitamins C and E, and selenium, in reducing anxiety symptoms [41]. The sulfur-containing amino acids, such as methionine and *N*-acetylcysteine, have been also reported to have an important role in the reduction of neurotoxicity [42]. Strong evidence exist for the use of herbal supplements containing extracts of passionflower or kava and combinations of L-lysine and L-arginine, magnesium-containing supplements, inositol [43], and some polyphenols [44].

Statins, 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors, are widely used in the treatment of dyslipidemia and in the primary and secondary prevention of cardiovascular diseases and stroke. Statins also exert pleiotropic effects independent of their hypolipidemic action. They improve blood-flow, reduce coagulation, modulate the immune system, and reduce oxidative damage [45]. Their strong antioxidant effects have been the focus of many

studies [46]. Statins' beneficial effects on CNS disorders have received increasing attention in recent years. However, the underlying mechanisms of their neuroprotective effects are not completely understood [47]. Studies on animal models concerning the effects of statins on anxiety-like and depressive-like behavior are very scarce. Under various pathological conditions, such as hypertension [48], diabetes [49], chronic mild stress [50], increased intake of high-fat diet [51], absence epilepsy [52], and depression [53], the administration of statins showed beneficial effects. Since oxidative stress has been implicated as a possible underlying pathogenic mechanism in all of these conditions, these beneficial effects can be, at least in a part, attributed to their antioxidant properties.

In order to estimate the potential benefits of statins in treatment of dietary-induced behavioral alterations, we evaluated the effects of hyperhomocysteinemia induced by special dietary protocols enriched in methionine and deficient in B vitamins (B6, B9, B12) on anxiety levels in rats by means of behavioral testing (open field—OF and elevated plus maze—EPM tests). Simultaneously, we investigated the alterations of oxidative stress markers in rat hippocampus induced by applied dietary protocols. Furthermore, considering the well-known antioxidant properties of statins, we attempted to assess their impact on major markers of oxidative stress and their possible beneficial role on anxiety-like behavior effect in rats.

Materials and methods

Ethical statement

All research procedures were carried out in accordance with European Directive for the welfare of laboratory animals No. 86/609/EEC and principles of Good Laboratory Practice (GLP), approved by Ethical Committee of the Faculty of Medical Sciences, University of Kragujevac, Serbia.

Animals and treatments

The dietary protocols were performed on male Wistar albino rats ($n = 72$; 4 weeks old; 100 ± 15 g body weight). Animals were housed in standard environmental conditions (temperature 23 ± 1 °C, 12/12 light/dark cycle) in polycarbonate cages (four animals per cage). Rats had free access to water and standard or special laboratory food. Special dietary protocols were applied for inducing hyperhomocysteinemia [40]. Commercial methionine-enriched chow contained a double amount of methionine compared to standard rodent chow (7.7 vs. 3.85 g methionine/kg). Based on the previous studies that confirmed the relationship between levels of B-vitamins (pyridoxine—B6, folic acid—B9, and

cobalamine—B12) with anxiety disorders [54], methionine nutritional overload was combined with variable vitamin B content (Mucedola SRL., Milan, Italy). Animals received methionine-enriched chow with regular vitamin B content (folate, pyridoxine and cobalamine; 2.0, 70.0, and 0.03 mg/kg, respectively) or methionine-enriched B vitamin complex deficient chow (folate, pyridoxine and cobalamine; 0.08, 0.01 and 0.01 mg/kg, respectively). The content of other compounds was identical in both diets. Application of statins along with chronic dietary protocols was defined on the basis of previous studies that have shown that treatment with statins has the beneficial effect on oxidative stress [55, 56]. Animals were exposed to treatment with atorvastatin at a dose of 3 mg/kg/day i.p. or simvastatin at a dose of 5 mg/kg/day i.p. at the same time every day (between 8 and 9 a.m.). Applied dietary protocols lasted for 30 days.

Animals were randomly divided into nine different groups (8 animals per group) as follows:

1. Control group—animals fed with standard rodent chow (C)
2. Animals fed with standard rodent chow and with the administration of atorvastatin (St + A)
3. Animals fed with standard rodent chow and with the application of simvastatin (St + S)
4. Animals fed with the methionine-enriched diet with regular vitamin B content (M^+)
5. Animals fed with a diet rich in methionine with atorvastatin application ($M^+ + A$)
6. Animals fed with a diet rich in methionine with the application of simvastatin ($M^+ + S$)
7. Animals fed with the methionine-enriched diet with the deficiency in vitamin B complex—B6, B9, B12 (M^+B^-)
8. Animals fed with a diet rich in methionine with the deficiency in B vitamins with the administration of atorvastatin ($M^+B^- + A$)
9. Animals fed with a diet rich in methionine with the deficiency in B vitamins with simvastatin administration ($M^+B^- + S$)

To ensure that all experimental groups have similar experiences and consistent treatment before behavioral testing, control group, M^+ group, M^+B^- group received at the same time approximately the same amount of saline in the same manner (by means of volume and route of administration) as other six groups received therapy (i.p. injections of statins).

Behavioral testing was performed 24 h following the completion of dietary pretreatment. Rats were transported in their home cages to the testing room (approximately at 9 a.m.), and allowed to accommodate for 1 h before behavioral testing. The same-housed animals were tested on the same day. Open field test and elevated plus maze test were performed under proper conditions of silence and illumination

for this kind of behavioral testing. Both tests were performed one by one (for all experimental groups) in the following order: the open field test and afterward elevated plus maze test. In order to avoid (minimize) the cumulative effects of the repeated anxiety-provoking testing, inter-trial interval of approximately 15 min between these tests was allowed. During the behavioral testing, mazes were cleaned with water and ethanol (70%) following the trial for each animal to remove possible interfering scents.

Open field test

The open field test is one of the commonly used tests for evaluation of anxiety-like behavior. The apparatus consisted of a square arena (60×60×30) made of black wood. At the beginning of the test, each rat was placed in the center of the arena and spontaneous exploration activity was recorded. During a trial, the experimenter was not present in the testing room. The movements of the rats were recorded for 5 min by a digital video camera mounted 150 cm above the open field and then analyzed. The following parameters were obtained during OF test: cumulative duration in the centre zone—CDCZ (s), frequency to the centre zone—FCZ, total distance moved—TDM (cm), velocity (cm/s), the percentage of time moving—%TM, the number of rearings. The moving pattern in arena provides information about the anxious-like state. The total time spent in the centre zone was determined as the major index for anxiety and more ambulations toward centre zone of the open field reflect less anxiety [57].

Elevated plus maze test

The elevated plus maze (EPM) test is widely used behavioral assay for rodents and is considered as a standard for measuring the anxiety responses. EPM consisted of two opposite open (50×20 cm²) and two opposite enclosed arms (50×20×30 cm³) and an open roof. The entire maze was elevated 100 cm from the floor. Each rat was placed in the center of the elevated plus maze facing the open arm and was allowed 5 min for free exploration. This test enables determining the emotional reactivity of animals by means of a conflict between secure parts of the maze (2 enclosed arms) and aversive parts of the maze (open arms). The following parameters were estimated by this test: cumulative duration (the total time spent) in the open arms—CDOA (s), frequency (the number of entries) to open arms—FOA, total distance moved—TDM (cm), velocity (cm/s), percentage of time moving—%TM, the number of rearings, the number of head dippings, and the number of total exploratory activity (TEA) episodes. These parameters are considered as indicators of anxiogenic effect [58, 59]. In order to estimate the overall exploratory activity in EPM test, we used a recently proposed parameter—the total exploratory activity [60] that

includes patterns of exploratory activity observed in different zones of EPM (closed and open arms). TEA is calculated as the sum of the numbers of rearings and head dippings during 5 min of testing in EPM. The activity of the rats was recorded by a digital video camera mounted centrally 250 cm above the elevated plus maze.

Video recording system and analysis

OF and EPM tests were recorded by the digital video camera mounted above mazes at the appropriate height. Video files were analyzed using Ethovision software, an integrating video tracking system for automatic recording of activity movement and interactions of animals [Noldus Information Technology, the Netherlands].

After the completion of behavioral tests, animals were anaesthetized by short-term narcosis, induced by intraperitoneal application of ketamine (10 mg/kg) and xylazine (5 mg/kg), and then sacrificed by decapitation. Brains were carefully removed from the skull, hippocampal tissue was dissected according to Li [61], and tissue samples were homogenized in PBS (Phosphate-buffered saline, 0.01 M, pH 7.4) with a manual homogenizer and stored in a freezer (−80 °C) until analysis, as previously described [62].

Parameters of oxidative stress quantification

Hippocampus tissue homogenates were centrifuged at 4000 rpm for 15 min at 4 °C. Supernatants obtained by this procedure were utilized for the evaluation of oxidative stress parameters including thiobarbituric acid reactive substance (TBARS) level, catalase (CAT), and superoxide dismutase (SOD) activities and reduced glutathione (GSH) level, by spectrophotometric assays. TBARS was measured as malondialdehyde (MDA) level in hippocampal tissue according to the method of Okawa and coworkers [63] and calculated using the standard curve of 1,1,3,3-tetraethoxypropane. Results were expressed as nanomoles of MDA per milligram of protein (nmol/mg protein). The enzymatic activity of SOD was determined by following the inhibition of adrenochrome formation from adrenalin at 480 nm [64]. The activity of CAT in hippocampal tissue homogenate was determined spectrophotometrically by monitoring the decomposition rate of hydrogen peroxide at 240 nm following the procedures described by Beers and Sizer [65]. Both SOD and CAT activities were expressed as enzymatic units per milligram of protein (U/mg protein). The concentration of GSH was determined spectrophotometrically according to the method of Ellman [66] on the basis of the reaction with 5,5-dithio-bis-(2-nitrobenzoic acid). Results were expressed as milligrams of GSH per g of protein (mg/g protein). Protein concentrations were determined according to

the method of Lowry et al. [67], using bovine serum albumin as the standard.

Statistical analysis

The results were expressed as the means \pm SEM. Parameters obtained in OF test and EPM test and oxidative stress markers were initially submitted to Levene's test for homogeneity of variance and to Shapiro–Wilk test of normality. Comparisons between groups were performed using One-way ANOVA, followed by Bonferroni test. A value of $p < 0.05$ was considered to be significant. Statistical analysis was performed with SPSS version 20.0 statistical package (IBM SPSS Statistics 20).

Results

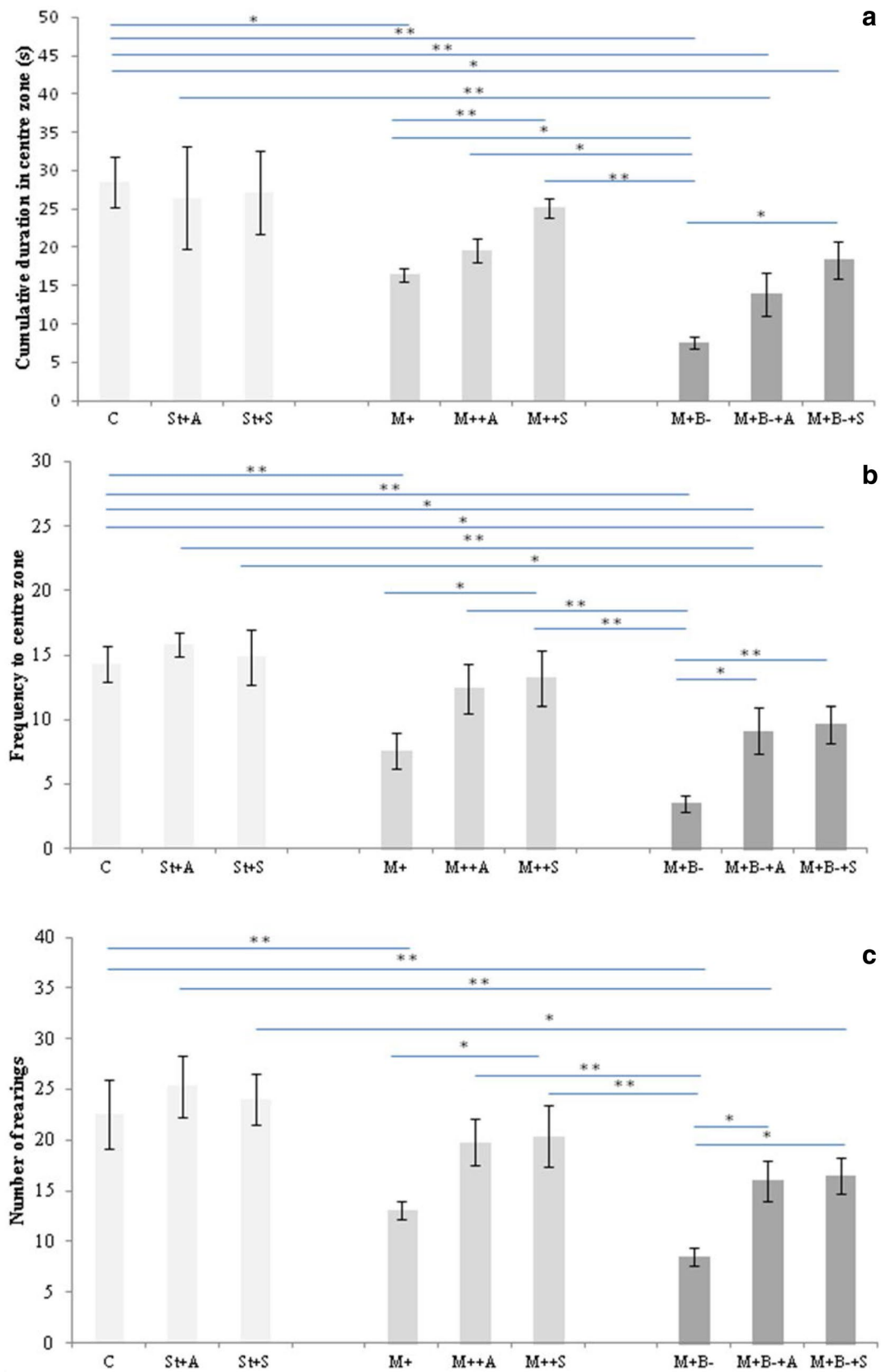
Both the methionine-enriched diet and methionine-enriched folate, vitamin B₆ and B₁₂ deficient diet lowered cumulative duration in the centre zone (Fig. 1a) and frequency to the centre zone (Fig. 1b) compared to the group fed with standard rodent chow ($df=8$, $F=5.368$ and 6.672 , respectively, $p < 0.01$). Diet rich in methionine and deficient in B vitamins also lowered cumulative duration in the centre zone compared to the group fed with methionine-enriched diet ($p < 0.05$). Administration of simvastatin along with methionine-enriched diet significantly increased cumulative duration and frequency to the centre zone ($p < 0.01$ and $p < 0.05$, respectively) compared to the group that was fed with the methionine-enriched diet without statins supplementation. The treatment with simvastatin along with methionine-enriched diet with B vitamin complex deficiency also increased cumulative duration and frequency to the centre zone ($p < 0.05$ and $p < 0.01$, respectively) compared to the group that was fed with methionine-enriched vitamin B deficiency diet. Atorvastatin application along with methionine-enriched and B complex deficiency diet increased only frequency to the centre zone compared to the group fed with methionine-enriched and B complex deficiency diet ($p < 0.05$). The treatment with atorvastatin along with both dietary protocols, methionine-enriched, and methionine-enriched vitamin B deficiency protocol, showed no significant alterations in total time spent in the centre zone and no alterations in frequency to the centre zone when applied with methionine-enriched diet.

As shown in the Fig. 1c, increased methionine intake and increased methionine intake with folate and vitamin B6 and B12 deficiency induced significant decrease in the vertical exploratory activity in OF test, expressed as the number of rearings, compared to the control group ($F=5.516$, $p < 0.01$). Administration of atorvastatin along with methionine-enriched folate and vitamin B6 and B12

deficient diet significantly increased the number of rearings ($p < 0.05$) compared to the group with methionine-enriched folate and vitamin B6 and B12 deficient diet. Simvastatin application along with methionine-enriched diet and methionine-enriched folate and vitamin B6 and B12 deficient diet also significantly increased the number of rearings ($p < 0.05$) compared to both dietary protocols without simvastatin applied.

The parameters of locomotor activity—total distance moved (Fig. 1d), velocity (Fig. 1e) and percentage of time moving (Fig. 1f) were significantly reduced in the methionine-enriched diet and methionine-enriched diet with deficiency in B vitamins compared to the control group ($df=8$, $F=8.073$, 8.073 and 12.395 , respectively, $p < 0.01$). Diet rich in methionine with vitamin B complex deficiency resulted in even more pronounced decline in locomotor activity by means of all three parameters compared to the group on diet rich in methionine ($p < 0.01$). Administration of atorvastatin along with methionine-enriched diet and methionine-enriched vitamin B complex deficient diet resulted in significant increase in all three parameters of locomotor activity compared to the groups that were fed with same dietary protocols without atorvastatin applied ($p < 0.05$ and $p < 0.01$, respectively). Simvastatin application along with the diet rich in methionine increased total distance moved and velocity compared to the group fed with food rich in methionine ($p < 0.05$), but with no effect on the percentage of time moving. Administration of simvastatin along with the diet rich in methionine and deficient in B vitamin complex also significantly increased parameters of locomotor activity compared to the group on diet rich in methionine and deficient in B vitamin complex ($p < 0.01$ for TDM and velocity, and $p < 0.05$ for %TM).

Both dietary protocols, methionine-enriched diet with no deficiency in B vitamins (folic acid, B6, and B12) and methionine-enriched diet deficient in B vitamins, significantly reduced the total time spent in open arms (Fig. 2a) and frequency to open arms (Fig. 2b) compared to the group fed with standard rodent chow ($df=8$, $F=6040$, and 6612 , respectively, $p < 0.01$). The methionine-enriched diet deficient in B vitamins additionally reduced total time spent in open arms and the number of entries to open arms when compared to the group on the methionine-enriched diet ($p < 0.05$). Cumulative duration in open arms and the number of entries to open arms were increased in the groups that were exposed to chronic simvastatin treatment along with methionine-enriched diet and the methionine-enriched diet deficient in B vitamins ($p < 0.05$) compared to the groups on the same dietary regimens without statins. Simultaneous treatment with atorvastatin along with methionine-enriched vitamin B deficient diet also increased total time spent in open arms compared to the group on methionine-enriched vitamin B deficient diet



◀ **Fig. 1** Parameters calculated from the open field test: **a** CDCZ, **b** FCZ, **c** number of rearings, **d** TDM, **e** velocity, **f** %TM. *C* control group, *St+A* standard chew + atorvastatin group, *St+S* standard chew + simvastatin, *M⁺* methionine rich, *M⁺+A* methionine rich + atorvastatin group, *M⁺+S* methionine rich + simvastatin group, *M⁺B⁻* methionine rich, vitamin B (B6, B9, B12) deficient group, *M⁺B⁻+A* methionine rich, vitamin B (B6, B9, B12) deficient + atorvastatin group, *M⁺B⁻+S* methionine rich, vitamin B (B6, B9, B12) deficient + simvastatin group, (mean ± SEM, *significant difference $p < 0.05$, **significant difference $p < 0.01$)

($p < 0.05$), with no significant effect on frequency to open arms.

Locomotor activity observed in EPM was significantly reduced by both chronic dietary protocols when compared to the control group by means of reduction in total distance moved (Fig. 2c), velocity (Fig. 2d), and percentage of time moving (Fig. 2e) ($F = 11.067$, 11.067 and 9.189 , respectively, $p < 0.01$). The reduction of locomotor activity was even more pronounced in the group with the methionine-enriched diet deficient in B vitamins compared to the methionine-enriched dietary regime ($p < 0.05$). Atorvastatin administration along with methionine-enriched diet and methionine-enriched vitamin B deficient diet increased locomotor activity by means of TDM and velocity compared to the groups on methionine-enriched diet and methionine-enriched vitamin B deficient diet ($p < 0.05$). After application of atorvastatin, the percentage of time moving was significantly increased in the group with methionine-enriched vitamin B deficient group compared to the methionine-enriched vitamin B deficient group ($p < 0.01$), with no significant alteration compared to the methionine-enriched group. Chronic simvastatin administration resulted in significant increase in all three parameters of locomotor activity compared to both methionine-enriched and methionine-enriched vitamin B deficient group ($p < 0.05$).

Exploratory activity in EPM, expressed by means of the number of rearings (Fig. 2f), the number of head dippings (Fig. 2g) and the number of TEA episodes (Fig. 2h) was significantly decreased by both dietary protocols, diet rich in methionine and diet rich in methionine and deficient in B vitamins, compared to the control ($F = 8.018$, 3.989 and 10.751 , respectively, $p < 0.01$). The methionine-enriched diet deficient in B vitamins resulted in an additional decline in the number of rearings and the number of TEA episodes when compared to the group on the methionine-enriched diet ($p < 0.05$, $p < 0.01$, respectively), with no significant decrease in the number of head dippings compared to methionine-enriched diet group. Atorvastatin administration along with methionine-enriched diet resulted in significant increase in the number of rearings and the number of TEA episodes ($p < 0.05$ and $p < 0.01$, respectively) compared to the group on the methionine-enriched diet. The same effect on these two parameters of exploratory activity

was observed after the simvastatin application along with methionine-enriched diet compared to the group on the methionine-enriched diet ($p < 0.01$). The application of both statins along with methionine-enriched diet had no significant effect on the number of head dippings compared to the group on the methionine-enriched diet. Results obtained in the groups with simultaneous administration of atorvastatin and simvastatin along with methionine-enriched vitamin B deficient diet showed a significant increase in all three parameters for evaluation of exploratory activity in EPM test compared to the group on the methionine-enriched vitamin B deficient diet ($p < 0.01$).

The applied diet protocols also significantly altered the oxidative stress markers in hippocampal tissue. As shown in the Fig. 3a, methionine-enriched diet and methionine-enriched vitamin B deficient diet induced a significant increase in TBARS when compared to the control group ($df = 8$, $F = 4.162$, $p < 0.05$ and $p < 0.01$, respectively). On the other hand, application of simvastatin along with methionine-enriched vitamin B deficient diet protocol resulted in a significant decrease in the index of lipid peroxidation (expressed in nmol of MDA per mg of tissue proteins) compared to the methionine-enriched vitamin B deficient diet group ($p < 0.05$). Simvastatin induced more pronounced decline in lipid peroxidation compared to atorvastatin group ($p < 0.01$) following the methionine-enriched vitamin B complex deficient diet. Chronic supplementation with atorvastatin had no effect on TBARS values compared to control, neither to the group with methionine-enriched diet nor to the methionine-enriched vitamin B complex deficient diet group.

Both methionine-enriched and methionine-enriched vitamin B (B6, B9, B12) deficient diet (Fig. 3b) significantly altered enzymatic activity of SOD ($df = 8$, $F = 2.739$) resulting in a significant decline in SOD activity compared to control group ($p < 0.05$, $p < 0.01$, respectively). Although with no effect following standard chow and methionine-enriched diet, atorvastatin and simvastatin supplementation significantly increased SOD activity following the methionine-enriched vitamin B deficient diet ($p < 0.05$, $p < 0.01$, respectively). As shown in Fig. 3c, increased methionine intake, with or without vitamin B complex deficiency, decreased hippocampal tissue CAT levels ($df = 8$, $F = 3.262$, $p < 0.01$). While atorvastatin supplementation failed to increase CAT levels, chronic administration of simvastatin significantly increased CAT levels in hippocampus, following both methionine-enriched and methionine-enriched vitamin B complex deficient dietary protocols ($p < 0.01$ and $p < 0.05$, respectively). The increase in hippocampal CAT values following simvastatin administration was significant even compared to atorvastatin supplemented group in methionine-enriched chow diet group ($p < 0.05$). As shown in Fig. 3d,

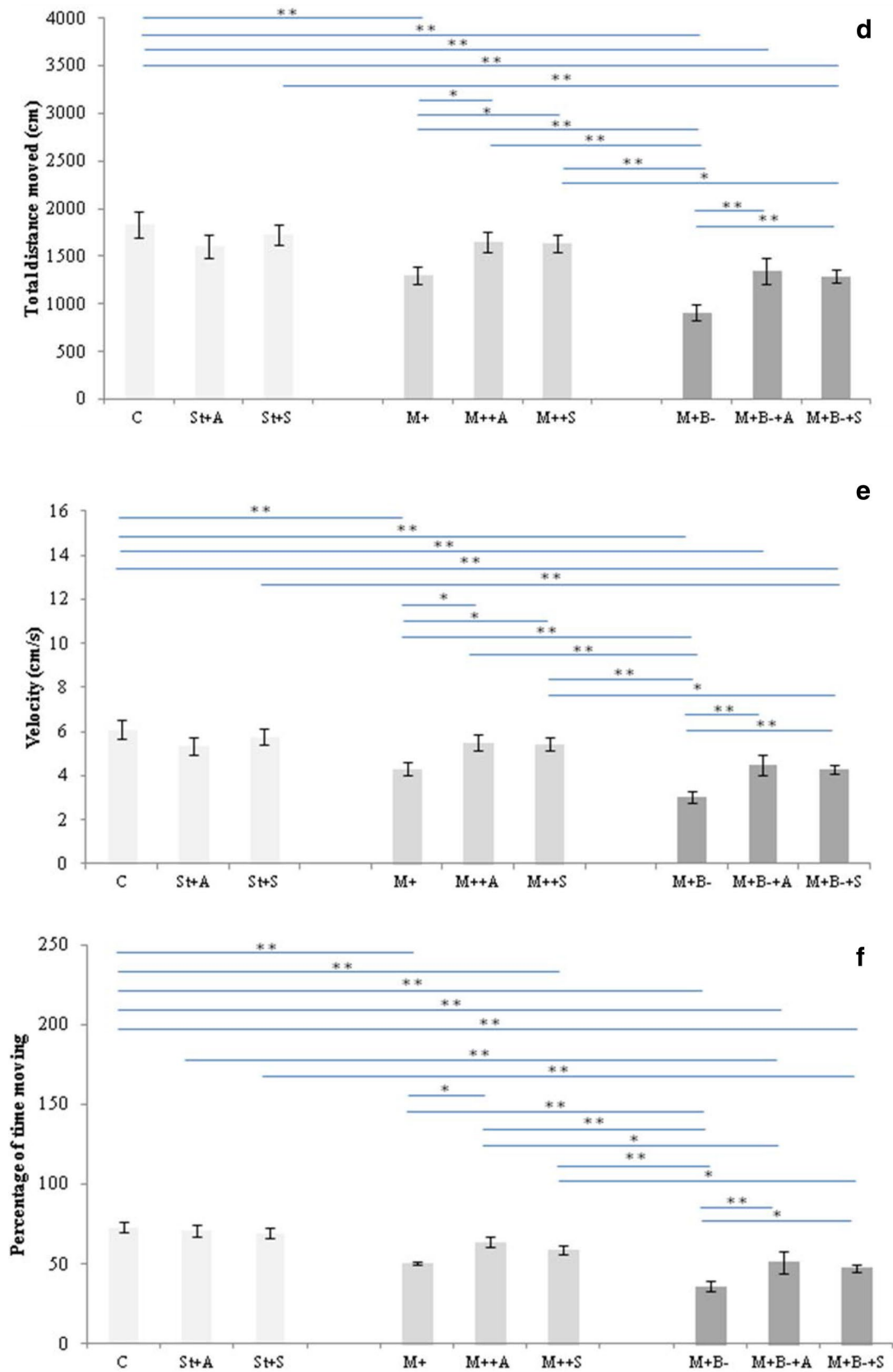


Fig. 1 (continued)

none of the applied dietary protocols resulted in significant alteration of total GSH levels in hippocampal tissue samples.

Simple regression analysis revealed that both CDCZ (Fig. 4a) and CDOA (Fig. 5a) significantly (negatively) correlated with TBARS in hippocampal tissue samples (Pearson's $r=0.70$ and $r=0.64$, $p=2.07^{-10}$ and $p=1.75^{-8}$, respectively). The regression analysis also confirmed that CDCZ (Fig. 4b, c) and CDOA (Fig. 5b, c) significantly (positively) correlated with SOD and CAT activity in rat hippocampal tissue (Pearson's $r=0.61$ and $r=0.78$, $p=1.31^{-07}$ and $p=6.89^{-14}$, for CDCZ, and Pearson's $r=0.69$ and $r=0.82$, $p=5.57^{-10}$ and $p=3.74^{-16}$, for CDOA).

Discussion

There is growing evidence for the impact of hyperhomocysteinemia on various dysfunctions of the central nervous system. Therefore, it seems very reasonable to investigate the possible interventions considering therapeutic regulation of methionine metabolism, especially in the brain. Since the imbalance in methionine levels in the brain has been accompanied with increased oxidative damage [68], it is legitimate to search for potential improvement of oxidative status altered by hyperhomocysteinemia, in order to prevent amelioration of various behavioral patterns, including the mood disorders.

Protocols applied in this study resulted in significant alterations of both behavioral (Figs. 1, 2), evaluated by means of estimation of anxiety levels, and parameters of oxidative stress (Fig. 3). An average methionine intake in this study was 160 ± 10 mg/kg/daily. Literature data confirm that this methionine load was sufficient to increase homocysteine levels. Our previous study with the same dietary protocols induced increase serum Hcy levels up to the ~ 22 $\mu\text{M/L}$ with methionine-enriched diet and ~ 62 $\mu\text{M/L}$ following methionine-enriched vitamins B deficient diet, compared to control levels at 8 $\mu\text{M/L}$ [69]. Depending on the duration of dietary protocols, increased methionine intake induced the elevation of serum Hcy levels by four (30 days protocol) [40] and seven times (8 weeks protocol) [39]. The vulnerability of Hcy metabolism in the brain was previously confirmed following dietary protocols with increase methionine intake and decreased the content of B vitamins (B6, B9 and B12), such as performed in this study, that resulted in increased serum Hcy levels by 5–6-fold in mice [70].

The evaluation of statins influence on Hcy levels, except for one report (with lovastatin) [71], leads to the conclusion that statins do not influence homocysteine concentrations significantly in healthy subjects by means of results observed in numerous clinical trials [72]. Still, the beneficial effects of statins (simvastatin, 20 mg/day, for 8 weeks) on lowering serum Hcy levels were reported in patients with

primary hyperlipidemia and this effect was dependent on the initial levels of serum Hcy [73]. The doses of statins applied in this study (atorvastatin 3 mg/kg/day, simvastatin 5 mg/kg/day) were chosen according to previously reported effects that cover both behavioral alterations [56] and changes in oxidative stress [55]. The applied doses of both statins were within the therapeutic range determined for cardioprotection and treatment of various dyslipidemia patterns in numerous studies [74], and significantly below the reported toxic doses [75].

The results of behavioral tests performed in this study clearly demonstrate that described dietary interventions, considering (increased) methionine load in the chow, resulted in anxiogenic effect. Methionine-enriched diet induced a significant increase of anxiety indicators in both OF (Fig. 1) and EPM test (Fig. 2). The anxiogenic effect of two-fold increased methionine load was unequivocal by means of alterations in "direct indicators" of anxiety in OF test, such as CDCZ and FCZ (Fig. 1a, b), and in EPM test - CDOA and FOA (Fig. 2a, b). Additionally, observed anxiogenic effect was confirmed by means of significantly decreased parameters of locomotor activity in both mazes (Figs. 1d, e, f, 2c–e), indicating previously described influence of anxiety levels on locomotor performance [76]. Furthermore, the parameters of exploratory activity in OF and EPM tests (Figs. 1c, 2f–h), alternative indicators of increased anxiety state [59], also revealed the anxiogenic effect of methionine-enriched diet. Moreover, restriction in vitamins B (B6, B9, and B12) intake, performed simultaneously with increased methionine load, resulted in even more pronounced anxiogenic effect compared to behavioral manifestations of the methionine-enriched diet itself (Figs. 1, 2). The overall enhancement of anxiogenic behavioral patterns following 30 days of simultaneously increased methionine load and deficient vitamin B complex intake was obvious by means of decreased time and frequency in the centre zone of the OF (Fig. 1a, b) and decreased cumulative duration and frequency to open arms in EPM test (Fig. 2a, b), as well as by means of significant additional decline in both exploratory and locomotor activity estimated in behavioral tests (Figs. 1, 2). The results obtained in this study are in accordance with previously reported anxiogenic effects following both acute [77] and chronic [39] increased methionine intake, with subsequent elevation of homocysteine levels, although the simultaneous increase of methionine load and restriction in vitamins essential for cysteine metabolism in brain (vitamin B6, B9, and B12) on behavioral alterations, including anxiogenic effects, has not been evaluated yet.

Administration of statins, atorvastatin and simvastatin, in the doses with confirmed beneficial therapeutic effects of both agents [55, 56], still far from defined toxic doses, did not influence anxiety level parameters when applied with standard chow. Since there is no similar experimental

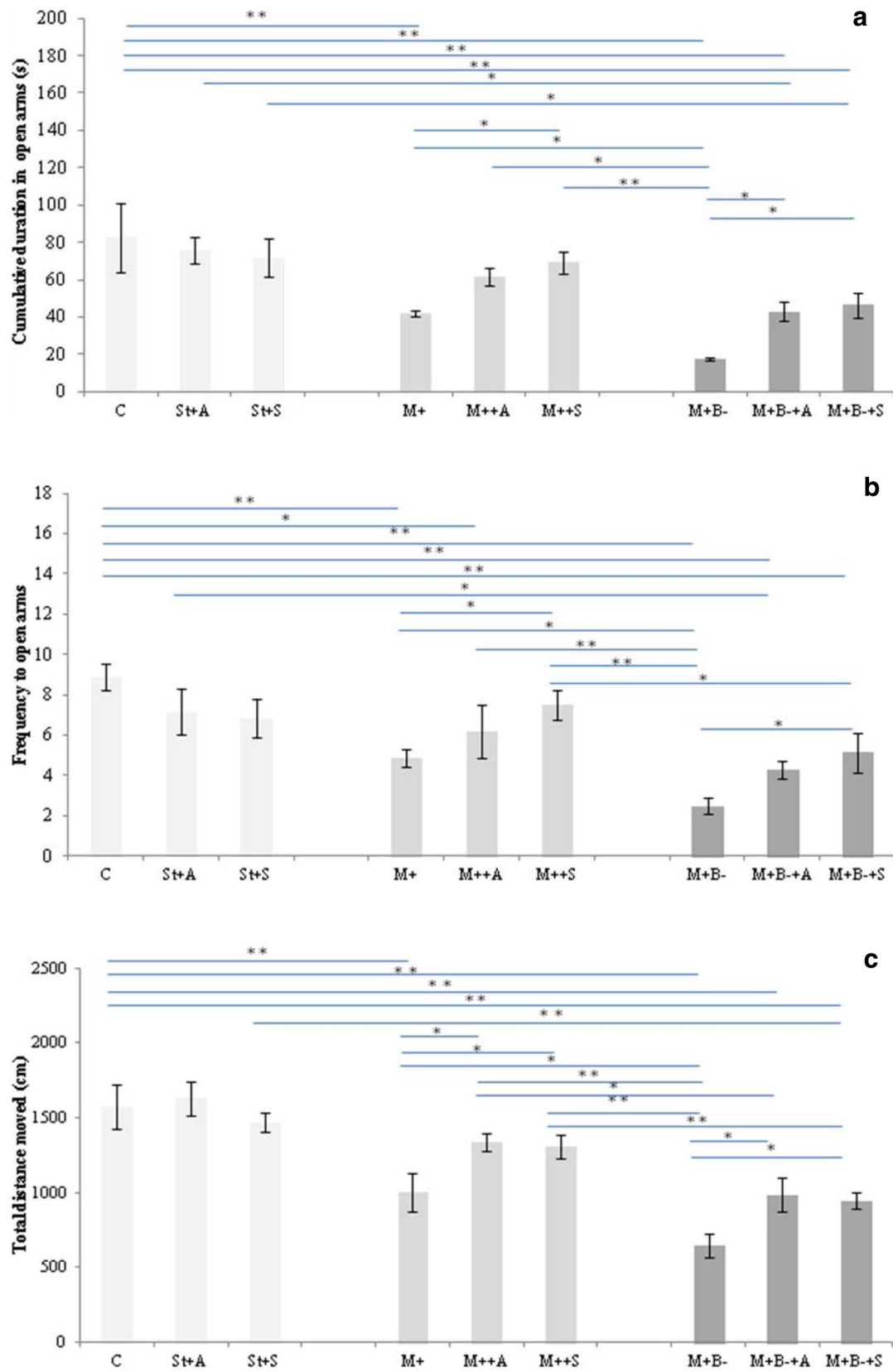


Fig. 2 Parameters calculated from the elevated plus maze test: **a** CDOA, **b** FOA, **c** TDM, **d** velocity, **e** %TM, **f** number of rearings, **g** number of head dippings, **h** number of TEA episodes. *C* control group, *St*+*A* standard chew+atorvastatin group, *St*+*S* standard chew+simvastatin, *M*⁺ methionine rich, *M*⁺+*A* methionine rich+atorvastatin group, *M*⁺+*S* methionine rich+simvastatin group, *M*⁺*B*⁻ methionine rich, vitamin B (B6, B9, B12) deficient group, *M*⁺*B*⁻+*A* methionine rich, vitamin B (B6, B9, B12) deficient+atorvastatin group, *M*⁺*B*⁻+*S* methionine rich, vitamin B (B6, B9, B12) deficient+simvastatin group, (mean±SEM, *significant difference $p < 0.05$, **significant difference $p < 0.01$)

design described in literature (significant differences in the applied doses, durations of protocols, various statins, etc.), it is hard to compare the results considering the effects of statins on anxiety levels with literature data, and we can only compare our results with previously reported data based on different experimental protocols. Unlike in our study, acute administration of simvastatin induced the anxiolytic effect in healthy rats [78]. That effect was observed following application of 2–10-fold higher doses than applied in this study. The similar (anxiolytic) effect was observed after subchronic (14 days) treatment of both atorvastatin and simvastatin [79]. The only study considering the effects of statins on anxiety state levels in animal experimental models showed that chronic administration of both statins applied in our study resulted in anxiolytic effect, although with the doses that were 2–3-fold above the doses applied in this study [52].

However, simultaneous administration of simvastatin diminished the anxiogenic effect that appeared following the methionine-enriched diet for 30 days, by means of a significant increase of anxiety indicators in both OF, CDCZ and FCZ (Fig. 1a, b) and EPM test, CDOA and FOA (Fig. 2a, b). The beneficial effect of simvastatin, manifested as the significant attenuation of anxiogenic effect expressed by the alterations in the same parameters, was also observed after performing dietary protocol with methionine-enriched diet along with restriction in vitamins B (Figs. 1, 2). The enhancement of exploratory and locomotor activity observed in OF (Fig. 1) and EPM test (Fig. 2) confirmed that applied dose of simvastatin was sufficient to attenuate anxiogenic effects induced by methionine-enriched diet, and even under conditions with simultaneous restriction in B6, B9, and B12 vitamins. The administration of atorvastatin along with described dietary protocols showed similar, but less pronounced, effects to simvastatin. The application of atorvastatin failed to significantly reduce anxiogenic effects of methionine-enriched and methionine-enriched vitamins B deficient intake for 30 days (except for FCZ in OF test, Fig. 1b). Still, the dose of atorvastatin such as applied in this study significantly improved parameters of locomotion following a dietary-induced reduction in both tests (Figs. 1, 2). The similar beneficial effect of atorvastatin was manifested by means of increased exploratory activity under the dietary protocol

that included high methionine and restricted vitamins B intake (Figs. 1, 2). The behavioral manifestations, which include estimation of anxiety state levels, of prolonged statins' administration under the common pathological conditions that require indicated usage of statins in animal experimental models that parallel human pathology, have not yet been described in the literature. Since there is no follow-up study that analyzed the effects of chronic administration of statins on anxiety state levels under pathophysiological conditions in animal experimental models, we assume that the findings in this study are in line with the results obtained in our investigation, confirming that long-term administration of statins (in the therapeutic doses as applied in this study) may be beneficial in avoiding anxiogenic effects in chronic metabolic disorders sufficient to induce pathological alterations, such as coronary artery disease [80].

Both homocysteine load-increasing dietary protocols applied in this study resulted in enhanced lipid peroxidation. Methionine-enriched diet for 30 days significantly increased TBARS, while simultaneously applied dietary protocol with double fold methionine intake with restricted B vitamins (B6, B9, and B12) intake induced additional increase in lipid peroxidation, although not significant compared to methionine-enriched group (Fig. 3). Our results correspond with previously published data based on in vitro studies that also showed the potentiation of lipid peroxidation in rat hippocampus induced by increasing homocysteine levels in a dose-dependent manner [68]. At the same time, the postulated mechanism of Hcy-induced increase of lipid peroxidation was proposed by Jara-Prado and coworkers, and it was based on the Hcy-induced alterations in NMDA receptors function [29]. The results considering diminishing effects of Hcy-loading diets on antioxidant enzymes activity obtained in this study (Fig. 3) confirm that both dietary protocols resulted in decreased SOD and CAT activity. Decreased antioxidant capacity was more pronounced following restriction of vitamins B intake, although it was not significant compared to the effects of methionine-enriched diet only. Since there is no literature data considering the effects of increased methionine load on the activity of antioxidant enzymes in vivo, we can only confirm that our results are in line with reported total radical trapping antioxidant potential in rat hippocampus [68]. The total GSH levels remained unaffected by dietary protocols performed in this study, which corresponds to reported lack of influence of Hcy levels on hippocampal glutathione in rats [68].

Simultaneous administration of statins with Hcy overloading dietary protocols showed their beneficial effects by means of decreased oxidative stress. The antioxidant effect of atorvastatin was clearly observed only by increased CAT activity following methionine-enriched vitamin B restricted diet (Fig. 3c), while by far more pronounced effect of simvastatin was obvious by means of decreased

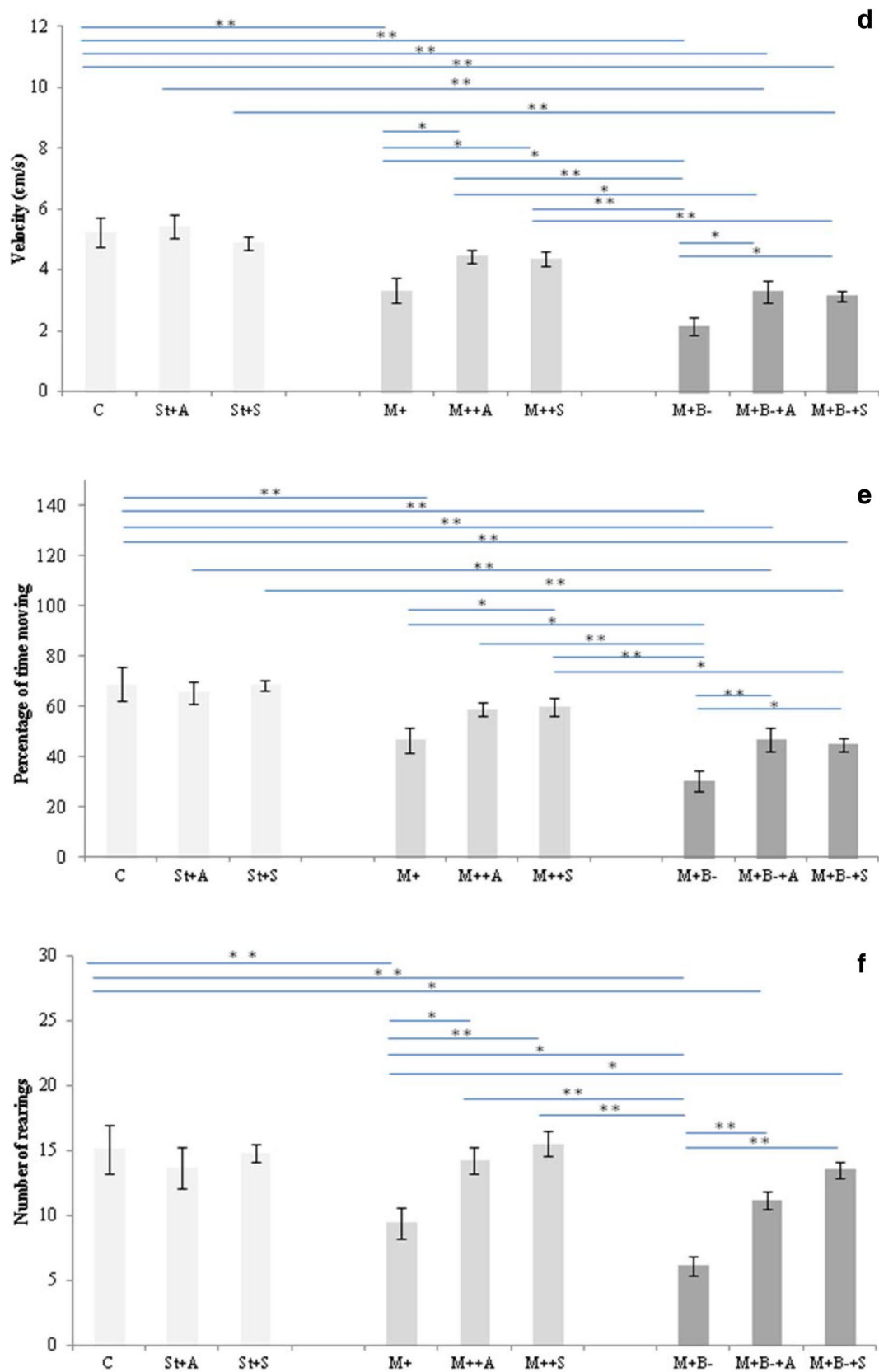


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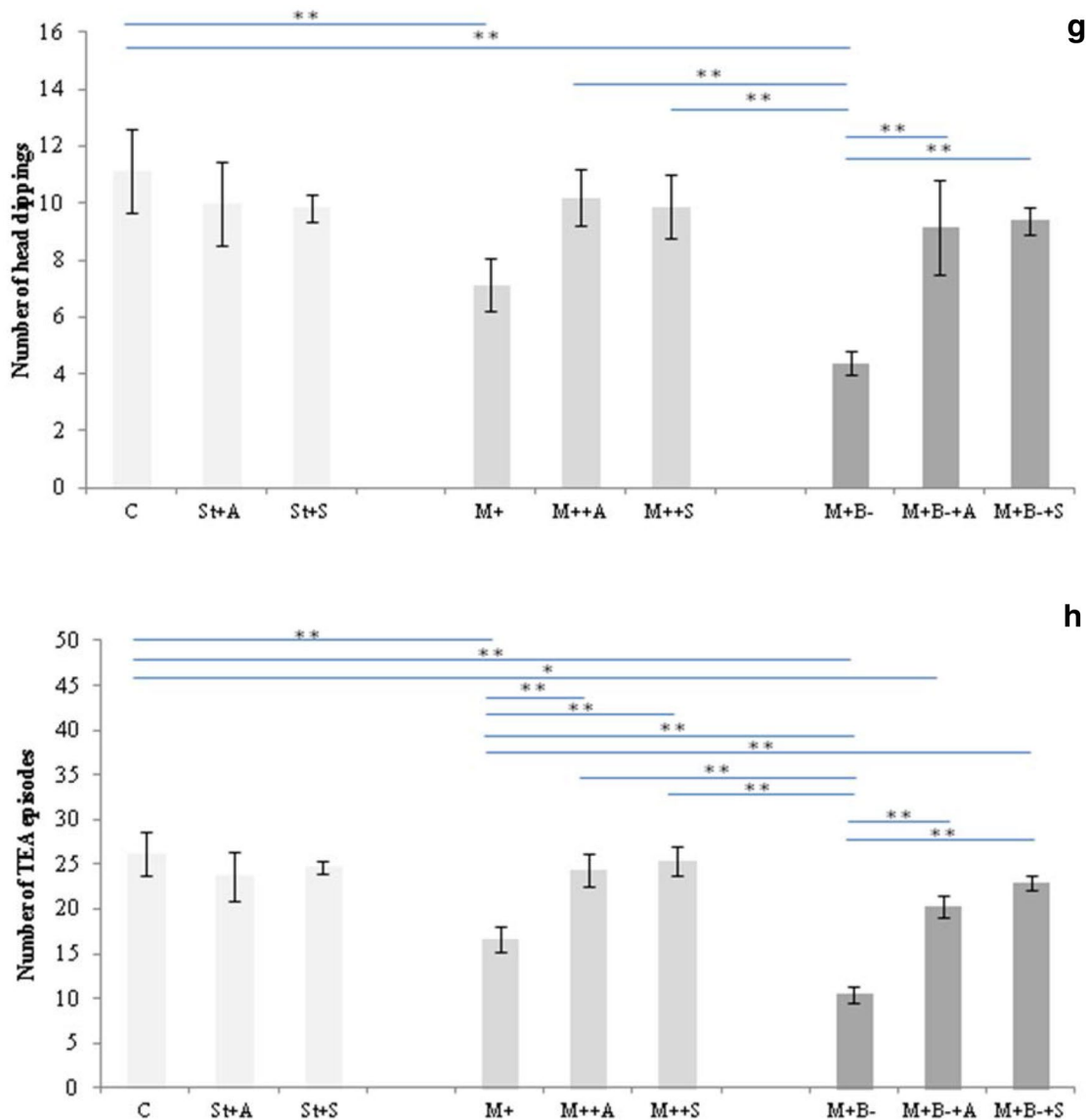


Fig. 2 (continued)

lipid peroxidation after methionine-enriched vitamin B restricted diet (Fig. 3a), as well as by increased activity of both estimated antioxidant enzymes (Fig. 3b, c). Our findings correspond to previously reported beneficial effects of atorvastatin expressed by a reduction of lipid peroxidation in rat brain tissue [81]. The results of our study are also in line with recently demonstrated findings that administration of simvastatin up-regulates SOD and CAT in oxidative stress processes in rat hippocampal cells [82].

The most sensitive indicators of anxiety state level in OF (Fig. 4) and EPM (Fig. 5) tests, the cumulative duration in centre zone and open arms, significantly correlated with oxidative stress markers. The beneficial (anxiolytic) effects

of statin supplementation on the increased anxiety state levels following methionine-enriched diet, and even more pronounced anxiogenic effect of methionine-enriched diet deficient in B vitamins, were negatively correlated to lipid peroxidation by means of parameters obtained in both OF (Fig. 4a) and EPM (Fig. 5a) tests. On the other hand, the anxiolytic effect of prolonged atorvastatin and simvastatin administration significantly positively correlated to both SOD and CAT activity (Figs. 4b, c, 5b, c). Our results strongly confirm that oxidative damage may contribute to the regulation of anxiety state levels. This is in line with previously reported findings based on the results obtained in in vitro study and clinical trial confirming that elevated

Fig. 3 Parameters of oxidative status in rat hippocampus: **a** TBARS, **b** SOD, **c** CAT, **d** GSH. *C* control group, *St+A* standard chew + atorvastatin group, *St+S* standard chew + simvastatin, *M⁺* methionine rich, *M⁺+A* methionine rich + atorvastatin group, *M⁺+S* methionine rich + simvastatin group, *M⁺B⁻* methionine rich, vitamin B (B6, B9, B12) deficient group, *M⁺B⁻+A* methionine rich, vitamin B (B6, B9, B12) deficient + atorvastatin group, *M⁺B⁻+S* methionine rich, vitamin B (B6, B9, B12) deficient + Simvastatin group, (Mean \pm SEM, *significant difference $p < 0.05$, **significant difference $p < 0.01$)

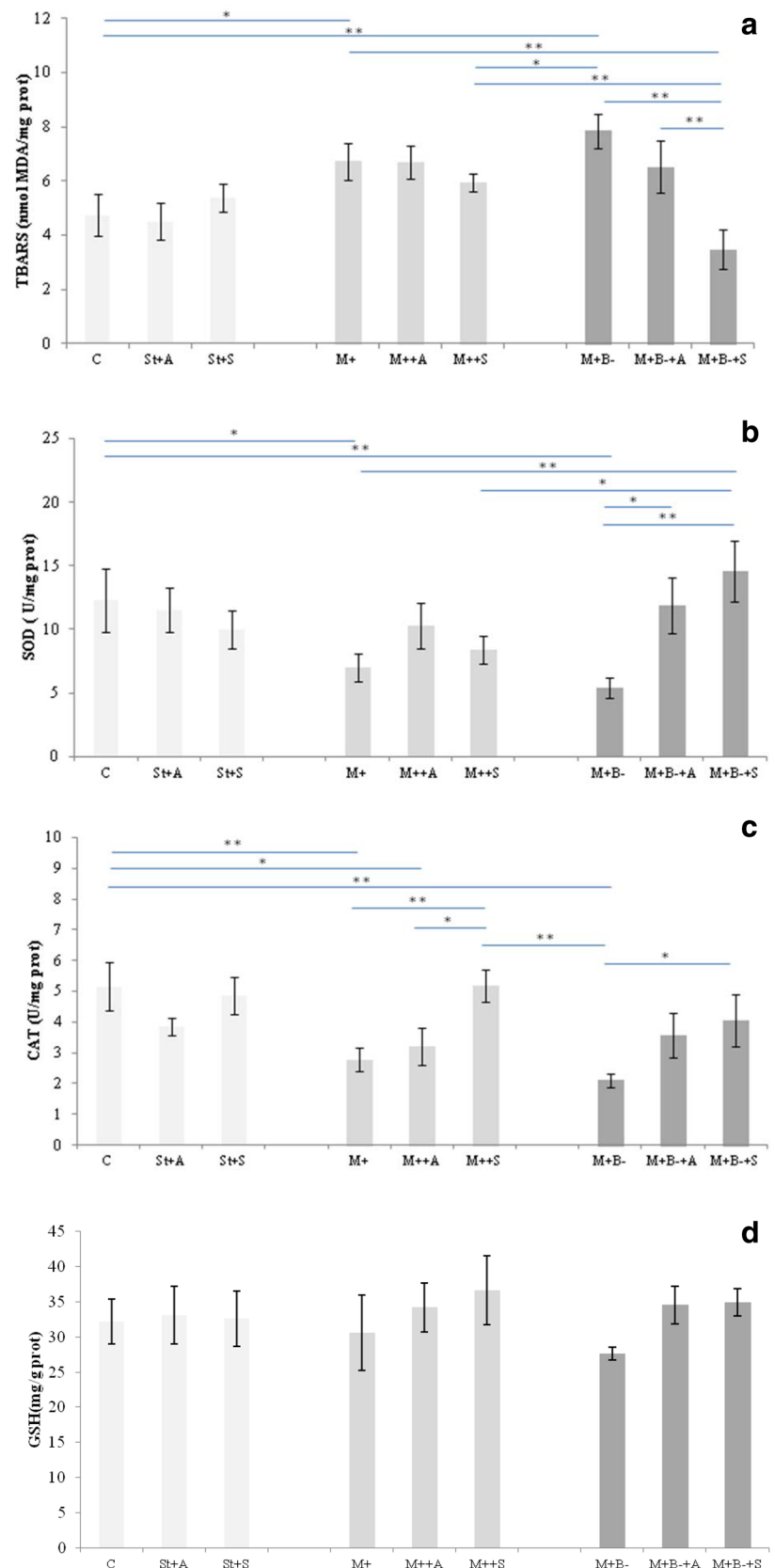


Fig. 4 Relationship between the cumulative duration in centre zone and **a** index of lipid peroxidation (expressed as TBARS), **b** SOD activity, **c** CAT activity in rat hippocampus for all investigated groups. Simple regression analysis ($n=72$) indicated that the cumulative duration in centre zone and TBARS were significantly and negatively correlated ($r=0.70$, $p=2.07^{-10}$), while significantly and positively correlated with SOD and CAT ($r=0.61$, $p=1.31^{-07}$ and $r=0.78$, $p=6.89^{-14}$, respectively)

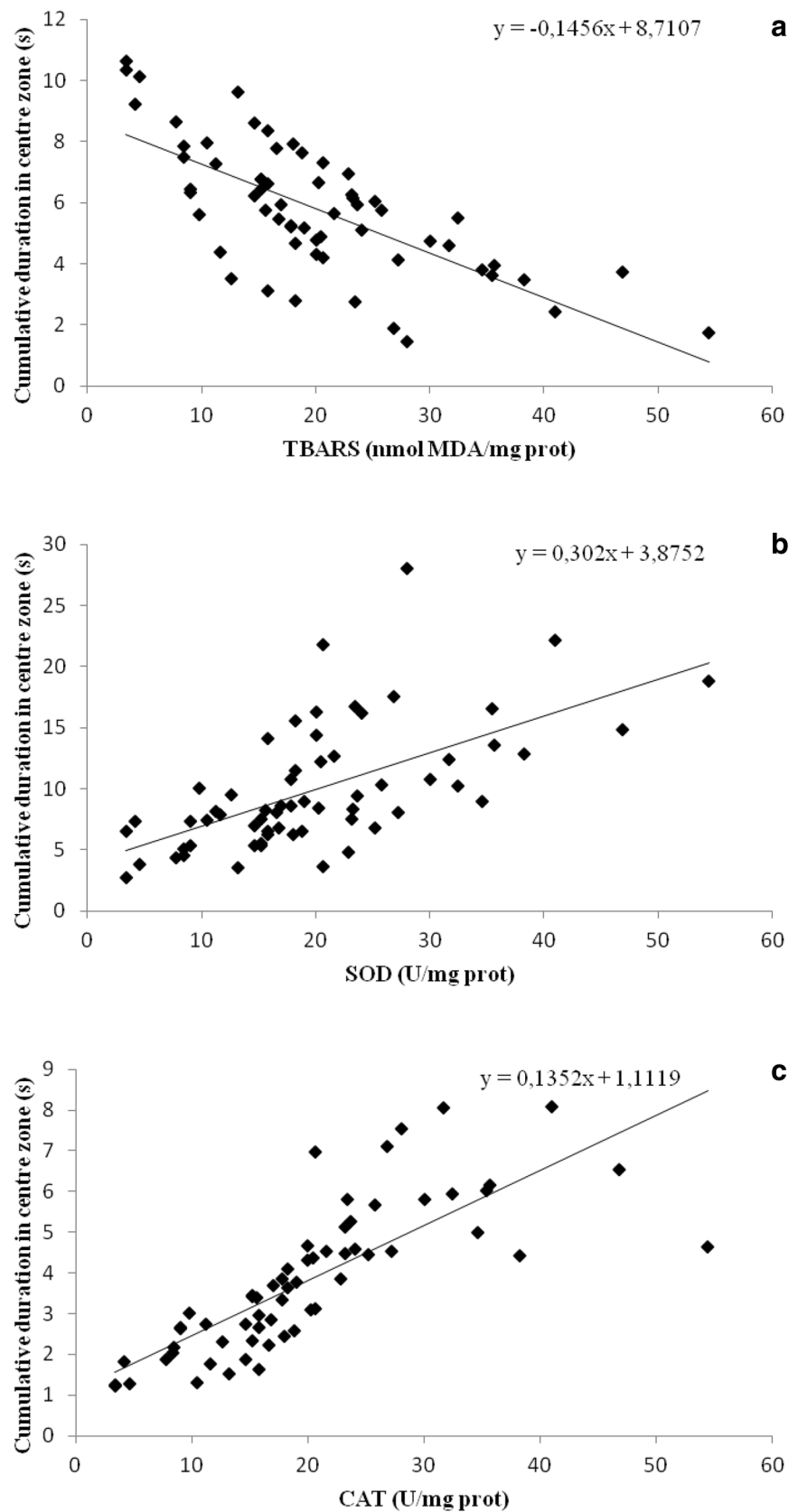
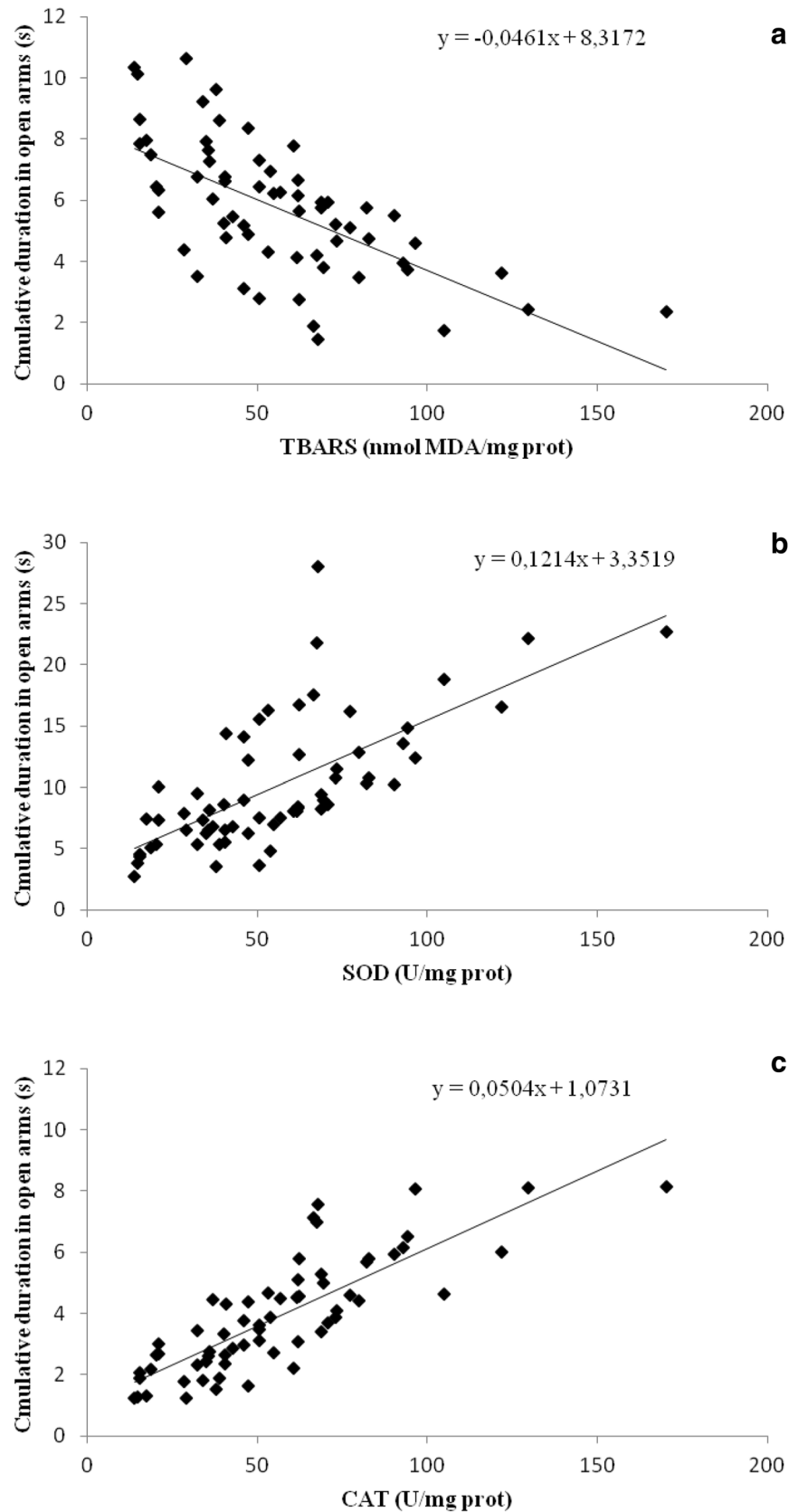


Fig. 5 Relationship between the cumulative duration in open arms and **a** index of lipid peroxidation (expressed as TBARS), **b** SOD activity, **c** CAT activity in rat hippocampus for all investigated groups. Simple regression analysis ($n = 72$) indicated that the cumulative duration in open arms and TBARS were significantly and negatively correlated ($r = 0.64$, $p = 1.75^{-8}$), while significantly and positively correlated with SOD and CAT ($r = 0.69$, $p = 5.57^{-10}$ and $r = 0.82$, $p = 3.74^{-16}$, respectively)



homocysteine levels increased oxidative stress [68] and also are accompanied to behavioral alterations including increased anxiety levels [18].

In conclusion, the results obtained in this study confirmed that methionine-load dietary protocols induced increased oxidative stress in rat hippocampus, which was accompanied by anxiogenic behavioral manifestations. Simultaneous administration of statins showed beneficial effects by means of both decreased parameters of oxidative stress and attenuation of anxiety. The results obtained with simvastatin were more convincing compared to atorvastatin. Altogether, our findings support that increasing number of hyperhomocysteinemia (of different origin) with various clinical manifestations (including behavioral) may be considered as an indication for (pre)medication with statins.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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