DOI 10.1007/s11062-019-09758-z
Neurophysiology, Vol. 50, No. 5, October, 2018

Activities of the Dopaminergic System and Glutathione Antioxidant System in the Hippocampus of Stressed rats

N. Popović,[1] S. B. Pajović,[1] V. Stojiljković,[1] A. Todorović,[1] S. Pejić,[1] I. Pavlović,[1] and L. Gavrilović[1]

Received August 8, 2017

The effects of chronic restraint stress (CRS, 2 h during 14 days) on gene expression of tyrosine hydroxylase (TH), catechol-O-methyltransferase (COMT), and glutathione peroxidase (GPx) were studied in the rat hippocampus. Changes in the dopamine (DA) concentration and activities of monoamine oxidases (MAO A and MAO B) and GPx in this cerebral structure of chronically stressed rats were also examined. The investigated parameters were quantified using real-time RT-PCR, Western blot analyses, and assay of enzymatic activity. We found that CRS decreased the TH protein level and DA concentration, which probably confirms the statement that *de novo* synthesis of DA is suppressed under stress conditions. The increased activities of MAO B, as well as the increased level of COMT protein, are believed to be related to intensified DA catabolism conditions. Also, a decreased activity of GPx in the hippocampus of chronically stressed animals was found. The increased enzymatic activity of MAO B negatively correlated with the reduced activity of GPx under the above-mentioned stress conditions. These events in the hippocampus of chronically stressed animals could synergistically cause oxidative damage to the mitochondria.

Keywords: dopamine, glutathione peroxidase, chronic restraint stress, hippocampus, gene expression.

INTRODUCTION

The hippocampus is an area of the brain particularly susceptible to the action of chronic stress [1]. As was found, chronic stress causes hippocampal atrophy and functional impairment [2].

Dopaminergic signaling is regarded as one of the key mechanisms in the modulation of brain functions. Following stress-inducing stimulation, a variety of changes in gene expression [3] and dopamine (DA) metabolism [4] occurs in the hippocampus. Tyrosine hydroxylase (TH) is the "rate-limiting" enzyme in the DA biosynthesis. Monoamine oxidase (MAO) is a mitochondria-bound enzyme, which catalyzes oxidative deamination of monoamine neurotransmitters. The effects of a major DA metabolite, dihydroxyphenylacetate (DOPAC), lead to mitochondrial dysfunction and cell death, which may be an important factor in Parkinson's disease [5]. In addition, catechol-O-methyltransferase (COMT) catalyzes transformation of DOPAC into homovanillic acid (HVA). It is known that the by-products of oxidative deamination of monoamine neurotransmitters include a number of potentially neurotoxic agents, such as hydrogen peroxide and ammonia. It was found that hydrogen peroxide can trigger intensification of production of reactive oxygen species (ROSs) and induce mitochondrial damage and neuronal apoptosis. One of the crucial endogenous antioxidant enzymes, which repair or prevent ROS damage of macromolecular cellular structures, is glutathione peroxidase (GPx).

Because of the significant role of DA in the regulation of a number of brain functions, elucidation of correlations between the activity of enzymes catalyzing oxidation of monoamines and activity of the antioxidant enzyme in the hippocampus under stress conditions is important for estimation of their possible synergistic impact on oxidative damage to the mitochondria. Therefore, it is important to examine gene expression of key

[1] Institute of Nuclear Sciences "Vinča", Laboratory of Molecular Biology and Endocrinology, University of Belgrade, Belgrade, Serbia. Correspondence should be addressed to L. Gavrilović (e-mail: gljubica@vin.bg.ac.rs).

enzymes involved in monoamine metabolism, gene expression of the antioxidant enzymes, and their degree of correlation in the hippocampus under stress conditions.

We investigated how chronic restraint stress (CRS, 2 h during 14 days) of experimental rats affects gene expression of TH, GPx, and COMT, as well as concentrations of DA and activity of MAO A and MAO B in the hippocampus. Taking into account that the rise in catecholamine catabolism results in increased ROS production, we also measured gene expression and activity of the antioxidant enzyme (GPx) in this brain structure. We have decided to measure gene expression and activity of GPx because information on the correlation between activities of the stress-activated dopaminergic system and glutathione antioxidant defense system in the hippocampus is rather limited. In these experiments, we presumed a high degree of correlation between the activity of enzymes catalyzing oxidation of monoamines and also the activity of GPx.

METHODS

Animals and a Stress Model. Wistar 11-week-old male rats were kept under standard vivarium conditions with water and food *ad libitum*, three to four per cage [6]. In accordance with our previous protocol [7], the animals were divided into two groups, control one (n = 10) that was not exposed to any treatment, and group CRS (n = 10), in which the animals were exposed to chronic restraint stress (CRS). The latter was performed by placing each animal in a 25 × 7 cm plastic bottle, as described previously by Gamaro et al. [8]. Animals in the CRS group were exposed to stressing every day during 14 days within random times during the light period of the light/dark cycle, to avoid habituation [9]. To reduce the variability of the physiological parameters due to circadian rhythms, the remaining animals were sacrificed at the same time point in the circadian cycle, between 9:00 and 11:00 am, i.e., one day after the last treatment. The rats were sacrificed with minimization of stress conditions (by rapid decapitation) [10]. The hippocampi were rapidly dissected, frozen in liquid nitrogen, and stored at –70°C until analyzed.

Dopamine Measurements. Hippocampal tissues were homogenized in 0.01 N HCl in the presence of EDTA and sodium metabisulfite. The DA concentration in hippocampal fractions was measured using 3-CAT Research ELISA kits (Labor Diagnostica Nord, Germany) according to the manufacturer's protocol. The concentrations were normalized to 1 g of tissues in the homogenate. The respective values were expressed in nanograms of DA per 1.0 g of tissue, which is in accordance with our previous protocol [7].

RNA Isolation and cDNA Synthesis. Methods of RNA isolation and cDNA synthesis were described previously by Gavrilović et al. [10]. Total RNAs were isolated from hippocampal tissue using the TRIZOL reagent (Invitrogen, USA). Reverse transcription was performed using Ready-To-Go You-Prime First-Strand Bead (Amersham Biosciences, Great Britain) and a pd(N)6 Random Hexamer (Amersham Biosciences, Great Britain) primer according to the manufacturer's protocol. Twelve milliliters of the sample, which contained 1500 ng mRNA, were incubated for 10 min at 65°C. Then, 21 µl of the solution of reverse transcriptase with the pd(N)6 primer (final, 0.2 µg) were added per sample and incubated 1 h at 37°C, which is in accordance with the above-mentioned protocol [10].

Real-Time RT-PCR. The TH and GPx mRNA levels were quantified using the quantitative real-time RT-PCR, as described previously by Gavrilović et al. [10]. TaqMan PCR assays were carried out using Assay-on-Demand Gene Expression Products (Applied Biosystems, USA) for TH (Rn00562500_m1) and GPx (Rn00577994_g1). The reference gene (endogenous control) was included in each analysis to correct the differences in the inter-assay amplification efficiency, and all transcripts were normalized with respect to cyclophyline A (Rn00690933_m1) expression. The relative expression of the target gene was normalized to cyclophyline A and expressed in relation to the calibrator, i.e., to the control sample, as previously described [10].

Hippocampal Tissue Homogenization and Measurement of the Protein Concentration. The hippocampus was homogenized in 0.05 M sodium phosphate buffer (pH 6.65). Subsequently, the protein concentration was determined using the BCA method (Thermo Scientific Pierce, USA) [11].

Western Blot Analysis. TH, COMT, and GPx proteins were assayed by Western blot analysis, as described previously [10]. The samples were boiled in a denaturing buffer, according to Laemmli [12], for 5 min at 95°C. A protein extract from the hippocampus (15 µg) was separated

by 10% SDS-polyacrylamide gel electrophoresis and then transferred to a supported nitrocellulose membrane (Hybond™ C Extra, Amersham Biosciences, Great Britain). The membrane was blocked in 5% non-fat dry milk in Tris-buffered saline-Tween (TBST). All following washes and antibody incubations were also carried out in TBS-T at room temperature on a shaker. Antibodies used for quantification of specific proteins were as follows: for TH, ab51191 (Abcam, USA); for COMT, ab208647; for GPx, sc-30147 (Santa Cruz Biotechnology, USA), and for β-actin, ab8227 (Abcam, USA). After washing, the membranes were incubated with secondary anti-mouse and anti-rabbit antibodies (dilution 1:5000; Amersham ECL™ Western Blotting Analysis System, Great Britain) conjugated to horseradish peroxidase. A secondary antibody was then visualized by a Western blotting enhanced chemiluminiscent detection system (ECL, Amersham Biosciences, Great Britain). The membranes were exposed to an ECL film (Amersham Biosciences, Great Britain). The densitometry of protein bands on the ECL film was performed using Image J analysis PC software. The results were expressed in arbitrary units normalized with respect to β-actin, which is in accordance with the protocol of Gavrilović et al. [10].

Monoamine Oxidase Enzyme Activities. Determination of MAO A and MAO B activity was performed using the Amplex Red Monoamine Oxidase Assay (A12214, Molecular Probes, USA) [13]. This assay is based on the detection of H_2O_2 in a horseradish peroxidase-coupled reaction using N-acetyl-3,7-dihydroxyphenoxazine (Amplex Red), a highly sensitive and stable probe for H_2O_2. The fluorescence intensity was measured with a fluoremeter using excitation at 560 ± 10 nm and fluorescence detection at 590 ± 10 nm. The monoamine oxidase activity was expressed in units per milligram of protein (U/mg protein), which is in accordance with our previous protocol [7].

Assay of GPx Activity. The GPx activity was determined using previously described methods of Stojiljković et al. [14]. This activity was assessed using the Oxis Bioxytech GPx-340 Assay (Oxis International, USA). The final result for the enzyme activity was expressed in U/mg protein.

Data Analysis. The numerical data are presented as means ± s.e.m. Differences between values of gene expressions (mRNA and protein levels) of TH, COMT, and GPx, concentrations of DA, as well as activity of the enzymes (MAO A, MAO B, and GPx), in the hippocampi of control and CRS animals were analyzed using the *t*-test. The statistical significance in intergroup comparisons was accepted at $P < 0.05$.

Correlations of MAOs and GPx enzyme activities were analyzed by the Pearson test, using the Sigma Plot v10.0 (with SigmaStat integration).

RESULTS

Differences of the DA Concentration in the Hippocampus. We found that the concentration of DA in the hippocampus of CRS animals was significantly (by 12%; $P < 0.05$, *t*-test, Fig. 1) lower than that in control animals.

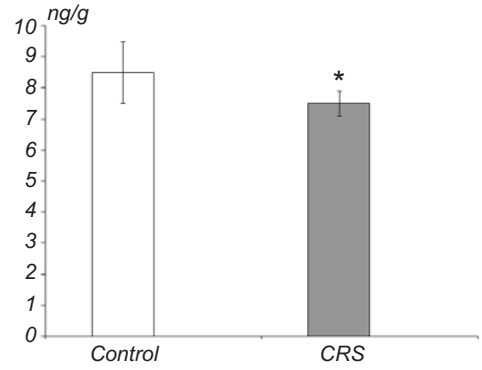
Changes of the TH Gene Expression in the Hippocampus. The animals exposed to CRS showed a practically unchanged level TH of mRNA (Fig. 2A) and a lower level of TH protein (by 32%; $P < 0.05$, *t*-test, Fig. 2B) in the hippocampus, compared to those in control animals.

Changes of the MAO A and MAO B Activity in the Hippocampus. The CRS treatment dramatically increased the enzymatic activity of MAO B (by 220%; $P < 0.001$, *t*-test, Fig. 3B), compared with that in control animals. However, the animals exposed to CRS showed nearly unchanged enzymatic activity of MAO A (Fig. 3A), compared with that in the control group.

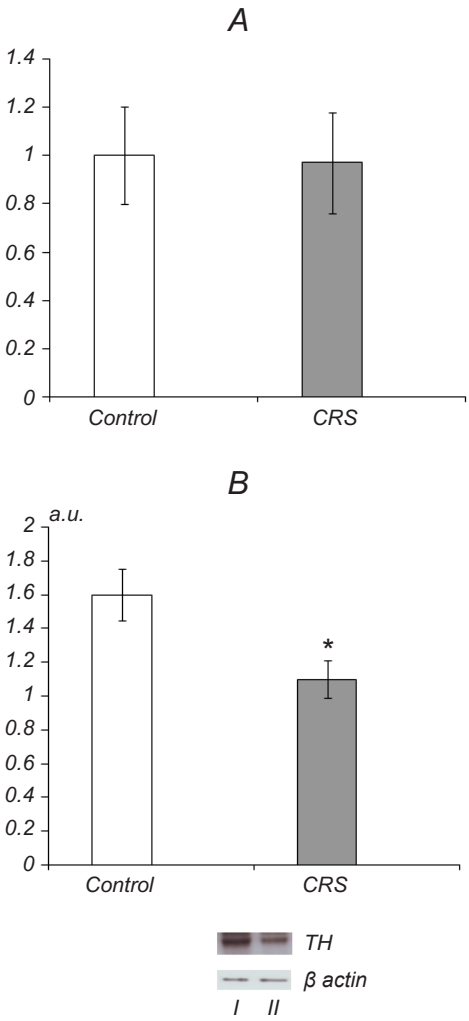
Changes of the COMT Protein Levels in the Hippocampus. CRS resulted in dramatically increased protein level of COMT (by 133%; $P < 0.001$, *t*-test, Fig. 4), compared with the control.

Changes of the GPx Gene Expression and Enzyme Activities in the Hippocampus. The animals exposed to CRS showed practically unchanged levels of mRNA and protein of GPx (Fig. 5A, B) compared with the control. However, CRS treatment significantly decreased the enzyme activity of GPx (by 28%, on average, $P < 0.05$, *t*-test, Fig. 5C).

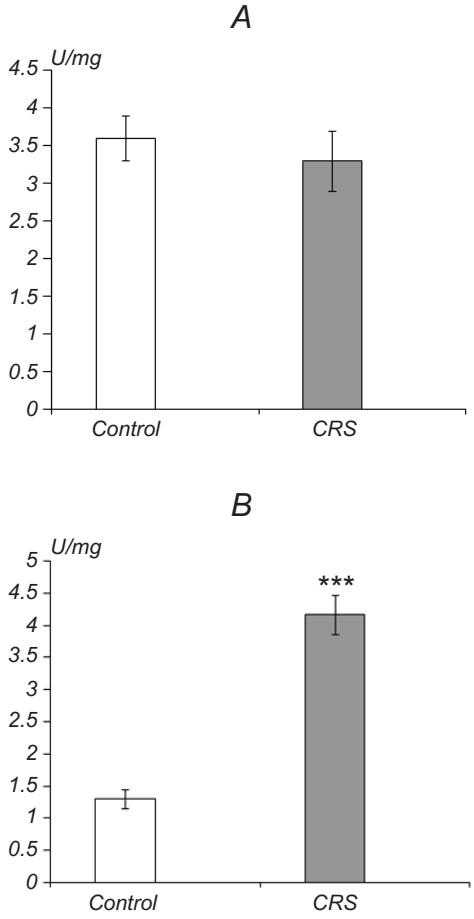
A significant negative correlation was found between the enzymatic activity of MAO B and GPx in the hippocampus (Pearson's $r = -0.679$; $P < 0.05$) of animals exposed to CRS.



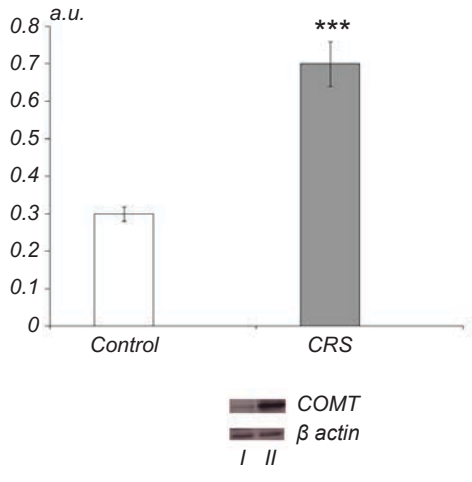
F i g. 1. Effects of chronic restraint stress (CRS) on the concentration of dopamine (DA) in the hippocampus. Data are shown as means ± s.e.m. for 10 rats in each group. *$P < 0.05$ in comparison of the group exposed to CRS (dashed column) compared to the control (open column; *t*-test).



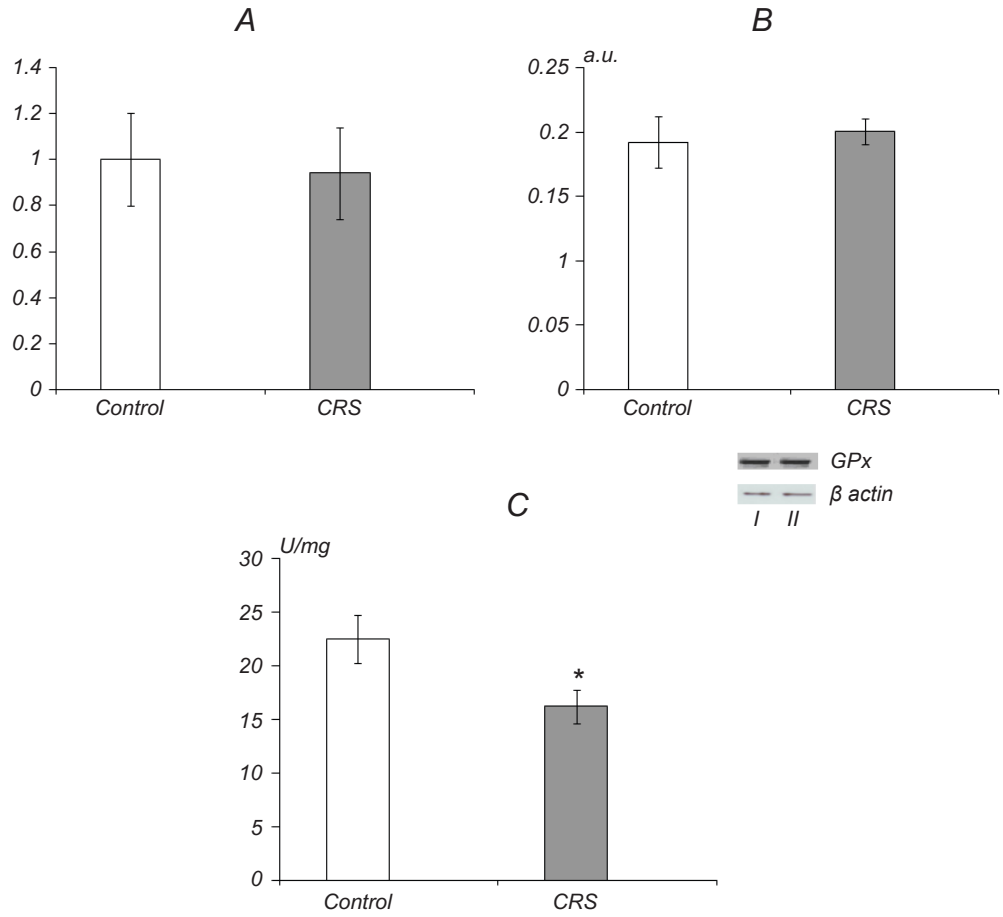
F i g. 2. Effects of chronic restraint stress (CRS) on the tyrosine hydroxylase (TH) mRNA (A) and TH protein levels (B) in the hippocampus. Designations are similar to those in Fig. 1.



F i g. 3. Effects of chronic restraint stress (CRS) on enzyme activity of monoamine oxidase A (MAO A) (A) and monoamine oxidase B (MAO B) (B) in the hippocampus. ***$P < 0.001$ in the intergroup comparison. Other designations are similar to those in Figs. 1 and 2.



F i g. 4. Effects of chronic restraint stress (CRS) on the catechol-O-methyltransferase (COMT) protein level in the hippocampus. Designations are similar to those in Figs. 1-3.



F i g. 5. Effects of chronic restraint stress (CRS) on the glutathione peroxidase (GPx) mRNA level (A), GPx protein level (B), and GPx enzyme activity (C) in the hippocampus. Designations are similar to those in Figs. 1-4.

DISCUSSION

In the above-described study, we have found a significantly lower mean DA concentration in the hippocampus of chronically stressed animals. Also, we observed that CRS reduced the protein levels of TH, which coincides with the reduced concentration of DA in the hippocampus. This finding probably confirms suppression of *de novo* synthesis of DA in the hippocampus of chronically stressed animals. It is possible that excessive stimulation by chronic stress could provoke desensitization of the cells of this monoamine system, which results in decreased DA synthesis in the hippocampus. In addition, the CRS treatment significantly increased the enzyme activity of MAO B and levels of COMT protein. Stress-induced activation of MAO may increase conversion of DA into its metabolites (DOPAC and HVA) [4], which may cause oxidative damage to the mitochondria [15]. It is known that inhibition of MAO and/or increases in the monoamine neurotransmitter levels are considered important therapeutic strategies for a few neuropsychiatric disorders. For example, an increase in the DA level is caused by MAO B inhibitors [16, 17]. Also, the literature data confirm that inhibitors of COMT are commonly used in patients suffering from Parkinson's disease [18].

An important result in our study is that CRS induces a significant decrease in GPx enzyme activity in the rat hippocampus. Our result is in accordance with the reports of Nilakantan et al. [19], Hsu et al. [20], and Nunes et al. [5]. These authors have found that nitric oxide (NO) or NO-derived products inhibit the GPx activity. In addition, we have found that increased enzyme activity of MAO B negatively correlated with reduced enzyme activity of GPx in the hippocampus of chronically

stressed animals. Nunes et al. [5] demonstrated that, under the combined action of DOPAC and NO, an early significant decrease in the intracellular glutathione content was detected in the cells. Several studies have independently implicated an excessive/inappropriate formation of NO (and its derivatives) [21–25] and an abnormal DA metabolism, involving the potential formation of reactive derivatives, such as free radicals [26–29].

Our results support the idea that the increased catabolism of DA and reduced enzyme activity of GPx in the hippocampus of chronically stressed animals could synergistically cause strong oxidative damage to the mitochondria. A significant negative correlation between the increased enzyme activity of MAO B and reduced enzyme activity of GPx is indicative of such a statement.

Acknowledgment. This work was supported by the Ministry of Education, Science, and Technological Development of the Republic of Serbia; Contract No.III 41027, OI 173041 and III41022.

Care was taken to minimize pain and discomfort of the animals according to the recommendations of the Ethical Committee of the Vinča Institute of Nuclear Sciences, Belgrade, Serbia, which follows the guidelines of the Serbian Society for the Use of Animals in Research and Education.

The authors, N. Popović, S. B. Pajović, V. Stojiljković, A. Todorović, S. Pejić, I. Pavlović, and L. Gavrilović, declare the absence of any conflict in commercial or financial relations, relationships with organizations or persons that in any way could be related to the study, and also in interrelations of the co-authors.

REFERENCES

1. Y. C. Tse, I. Montoya, A. S. Wong, et al., "A longitudinal study of stress-induced hippocampal volume changes in mice that are susceptible or resilient to chronic social defeat," *Hippocampus*, **24**, 1120-1128 (2014).
2. B. S. McEwen and A. M. Magarinos, "Stress effects on morphology and function of the hippocampus," *Ann. N.Y. Acad. Sci*., **821**, 271-284 (1997).
3. Xh Li, Jx Chen, Gx Yue, et al., "Gene expression profile of the hippocampus of rats subjected to chronic immobilization stress," *PLoS One*, **8**, No. 3, e57621 (2013).
4. A. Ahmad, N. Rasheed, K. Chand, et al., "Restraint stress-induced central monoaminergic & oxidative changes in rats & their prevention by novel *Ocimum sanctum* compounds," *Indian J. Med. Res*., **135**, No. 4, 548-554 (2012).
5. C. Nunes, R. M. Barbosa, L. Almeida, and J. Laranjinha, "Nitric oxide and DOPAC-induced cell death: from GSH depletion to mitochondrial energy crisis," *Mol. Cell Neurosci*., **48**, No. 1, 94-103 (2011).
6. L. Gavrilovic, N. Spasojevic, and S. Dronjak, "Subsequent stress increases gene expression of catecholamine synthetic enzymes in cardiac ventricles of chronic-stressed rats," *Endocrine*, **37**, 425-429 (2010).
7. N. Popović, S. B. Pajović, V. Stojiljković, et al., "Prefrontal catecholaminergic turnover and antioxidant defense system of chronically stressed rats," *Folia Biol.*, **65**, No. 1, 43-54 (2017).
8. G. D. Gamaro, M. B. Michalowski, D. H. Catelli, et al., "Effect of repeated restraint stress on memory in different tasks," *Braz. J. Med. Biol. Res.*, **32**, No. 3, 341-347 (1999).
9. K. S. Kim and P. L. Han, "Optimization of chronic stress paradigms using anxiety- and depression-like behavioral parameters," *J. Neurosci. Res*., **83**, No. 3, 497-507 (2006).
10. L. Gavrilović, V. Stojiljković, J. Kasapović, et al., "Treadmill exercise does not change gene expression of adrenal catecholamine biosynthetic enzymes in chronically stressed rats," *Ann. Acad. Bras. Cienc*., **85**, No. 3, 999-1012 (2013).
11. T. M. Stich, "Determination of protein covalently bound to agarose supports using bicinchoninic acid," *Ann. Biochem*., **191**, 343-346 (1990).
12. U. K. Laemmli, "Cleavage of structural proteins during the assembly of the head of bacteriophage T4," *Nature*, **227**, 680-685 (1970).
13. M. Zhou and N. Panchuk-Voloshina, "A one-step fluorometric method for the continuous measurement of monoamine oxidase activity," *Analyt. Biochem*., **253**, No. 2, 169-174 (1997).
14. V. Stojiljković, A. Todorović, S. Pejić, et al., "Antioxidant status and lipid peroxidation in small intestinal mucosa of children with celiac disease," *Clin. Biochem*., **42**, Nos. 13/14, 1431-1437 (2009).
15. M. Bortolato, K. Chen, and J. C. Shih, "Monoamine oxidase inactivation: from pathophysiology to therapeutics," *Adv. Drug. Deliv. Rev*., **60**, 1527-1533 (2008).
16. A. M. Cesura and A. Pletscher, "The new generation of monoamine oxidase inhibitors," *Prog. Drug. Res*., **38**, 171-297 (1992).
17. J. Knoll, "(-)Deprenyl (selegiline): past, present and future," *Neurobiology*, **8**, 179-199 (2000).
18. T. Müller, "Catechol-O-methyltransferase inhibitors in Parkinson's disease," *Drugs*, **75**, No. 2, 157-174 (2015).
19. V. Nilakantan, X. Zhou, G. Hilton, et al., "Hierarchical change in antioxidant enzyme gene expression and activity in acute cardiac rejection: role of inducible nitric oxide synthase," *Mol. Cell Biochem*., **270**, Nos. 1/2, 39-47 (2005).
20. M. Hsu, B. Srinivas, J. Kumar, et al., "Glutathione depletion resulting in selective mitochondrial complex I inhibition in dopaminergic cells is via an NO-mediated pathway not involving peroxynitrite: implications for Parkinson's disease," *J. Neurochem*., **92**, 1091-1103 (2005).

21. J. E. Duda, B. I. Giasson, Q. Chen, et al., "Widespread nitration of pathological inclusions in neurodegenerative synucleinopathies," *Am. J. Pathol*., **157**, 1439-1445 (2000).
22. B. I. Giasson, J. E. Duda, I. V. Murray, et al., "Oxidative damage linked to neurodegeneration by selective alpha-synuclein nitration in synucleinopathy lesions," *Science*, **290**, 985-989 (2000).
23. P. Klivenyi, O. A. Andreassen, R. J. Ferrante, et al., "Inhibition of neuronal nitric oxide synthase protects against MPTP toxicity," *NeuroReport*, **11**, 1265-1268 (2000).
24. Watanabe Y, Kato H, Araki T, "Protective action of neuronal nitric oxide synthase inhibitor in the MPTP mouse model of Parkinson's disease," *Metab. Brain Dis*., **23**, 51-69 (2008).
25. H. Yokoyama, S. Takagi, Y. Watanabe, et al., "Role of reactive nitrogen and reactive oxygen species against MPTP neurotoxicity in mice," *J. Neural Transm*., **115**, 831-842 (2008).
26. T. Alexander, C. E. Sortwell, C. D. Sladek, et al., "Comparison of neurotoxicity following repeated administration of L-dopa, D-dopa and dopamine to embryonic mesencephalic dopamine neurons in cultures derived from Fisher 344 and Sprague–Dawley donors," *Cell Transplant.*, **6**, 309-315 (1997).
27. F. Filloux and J. J. Townsend, "Pre- and postsynaptic neurotoxic effects of dopamine demonstrated by intrastriatal injection," *Exp. Neurol*., **119**, 79-88 (1993).
28. T. G. Hastings, D. A. Lewis, and M. J. Zigmond, "Role of oxidation in the neurotoxic effects of intrastriatal dopamine injections," *Proc. Natl. Acad. Sci. USA*, **93**, 1956-1961 (1996).
29. P. P. Michel and F. Hefti, "Toxicity of 6-hydroxydopamine and dopamine for dopaminergic neurons in culture," *J. Neurosci. Res*., **26**, 428-435 (1990).