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DIFFERENCES IN THE FUNCTIONAL ACTIVITY AND REDOX HOMEOSTASIS BETWEEN THE LEFT AND RIGHT ADRENAL GLAND OF RATS EXPOSED TO CHRONIC ISOLATION STRESS

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The aim of this study was to examine whether there are differences in adrenomedullary function in respect to the left and right sides in chronic stress conditions. We investigated how chronic stress isolation (CSI 12 weeks) affected the protein levels of key enzymes involved in adrenaline (A) synthesis (phenyl ethanolamine N-methyltransferase - PNMT), storage (vesicular monoamine transporters 2 - VMAT2) and degradation (catechol-O-methyltransferase - COMT), as well as the concentrations of A as an index for adrenomedullary function in the left and right adrenal medulla. Also, we examined the concentrations of malondialdehyde (MDA), protein levels of nuclear factor κB (NF-κB), and activity of catalase (CAT) in the left and right adrenal medulla. The investigated parameters were quantified by Western blot analysis, assay of enzymatic activity, and CAT Research ELISA kits. We found that CSI produced significantly increased levels of PNMT protein, and VMAT2 protein, as well as increased concentrations of A in the right adrenal medulla. However, we recorded that CSI increased protein levels of COMT and NF-κB, as well as the concentrations of MDA in the left adrenal medulla. Also, CSI decreased the activity of CAT only in the left adrenal medulla. Based on these results, it may be concluded that adrenomedullary function is different in respect to the left and right sides in chronic stress conditions.

Keywords: adrenomedullary function, adrenaline, antioxidant status, chronic stress isolation, rats

INTRODUCTION

Many studies have shown that most even organs are functionally different in respect to the left and right sides [1]. Cerebral lateralization is one of the well-known asymmetries

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[2]. For example, Chesterman *et al.* [3] reported asymmetry in the hippocampal structure and function. In addition, literature data indicate that chronic stress induced differences in function of most paired organs. For example, it is known that there are differences in the expression of catecholamine synthesizing enzyme in the left and right hippocampus of rats exposed to long-term stress isolation [4].

Chronic stress isolation *(CSI)* represents a very strong psychosocial stress [5,6], which can induce increased concentration of adrenaline (A) in the plasma [7] and changed activity of the antioxidant defense system [8,9]. It is known that CSI is a major risk factor for neuropsychiatric disorders such as depressive and anxiety disorders [10], as well as that psychological stress is a risk factor for Parkinson's disease [11]. Mandala et al. [12] found that the right hemisphere dysfunction is characteristic of depressive disorders. Also, the right brain hemisphere plays a dominant role in the control of both hypothalamo-pituitary-adrenal (HPA) axis and autonomous functions essential for survival [13,14]. In addition, it is known that the left adrenal glands received more cerebral neural inputs than the right ones [15]. Interestingly, Mehta et al. [16] have found a pheochromocytoma only in the left adrenal medulla in Parkinson's disease patients. In addition, the literature data have shown that CSI induced hypertrophy only of the left adrenal medulla [17]. However, very little is known about differences in adrenomedullary function in respect to the left and right sides in chronic stress conditions. Therefore, in this study, we investigated how 12 weeks CSI affected key enzymes involved in A biosynthesis (phenyl ethanolamine N-methyltransferase - PNMT), storage (vesicular monoamine transporters 2 - VMAT2) and degradation (catechol-O-methyltransferase - COMT), as well as the concentration of A as an index for adrenal function in left and right adrenal medulla. In addition, numerous studies have confirmed that reactive oxygen species (ROS) and mitochondrial dysfunction play an important role in the development of diseases including pheochromocytomas and Parkinson's disease [18,19,20]. Malondialdehyde (MDA) is a frequently used biomarker of oxidative stress in many health problems. Also, it is known that nuclear factor $\varkappa$B (NF-$\varkappa$B), a ubiquitously expressed protein complex regulating the transcription of many genes, is more readily activated in oxidative environments [21]. ROS, which can oxidize cellular macromolecules, are neutralized by the antioxidant defense system (AO). For example, chronic stress increases the activity of catalase (CAT) in the brain [22]. For this reason, as an additional aim of the study was how CSI affected the concentrations of MDA, protein levels of NF-$\varkappa$B and the activity of CAT in the left and right adrenal medulla. In this study we presumed that chronic psychosocial stress induced differences in the functional activity and redox homeostasis between the left and right adrenal gland.

Our results may be very important in the research of the right and left adrenal gland susceptibility to chronic psychosocial stress, as well as their functional differences in chronic stress conditions for the purpose of prevention of neuroendocrine diseases caused by chronic stress.

MATERIALS AND METHODS

Animals and stress models

Wistar male rats (11-week-old) were under standard laboratory conditions with water and food *ad libitum*. The experimental protocol was previously described in more details by Gavrilović *et al.* [23]. Animals were divided into two groups: **CONTROL group** (n=20) was not exposed to treatment and **CSI group** (n=20) consisted of animals exposed to social isolation. Animal models of CSI consisted of rats that were subjected to social isolation, with a single animal per cage for 12 weeks. The visual and olfactory communication among the isolated rats was reduced to a minimal level. The Ethical Committee for the care and use of laboratory animals of the Institute of Nuclear Sciences "Vinča" has approved the planned experiment (opinion number 12/14). The study was completed in 2021. Care was taken to minimize the pain and discomfort of the animals according to the recommendations of the Ethical Committee of the Institute of Nuclear Sciences "Vinča", which follows the guidelines of the registered "Serbian Society for the Use of Animals in Research and Education". After 12 weeks of individual housing, the animals were decapitated, the adrenals excised, right and left adrenal medulla dissected, frozen separately in liquid nitrogen and stored at -70 ºC until analyzed [23].

Homogenization and measurment of the concentration of protein

Ten adrenal medullas (left and right) were homogenized in 0.05 M sodium phosphate buffer (pH 6.65). Subsequently, the protein concentration was determined using BCA method (Thermo Scientific Pierce, USA).

Western blot analysis

Antibodies used for the quantification of specific proteins were as follows: for PNMT the polyclonal ant-PNMT primary antibody, rabbit (Protos Biotech Corporation, USA), for VAMT (ab70808 Abcam, Abcam, USA), for NF-κB (ab16502, Abcam, USA), for COMT (ab126618 Abcam, USA), and for β-actin (ab8227, Abcam, USA). After washing, the membranes were incubated in the secondary anti-rabbit (Amersham ECL™ Western Blotting Analysis System, UK) antibodies conjugated to horseradish peroxidase. Secondary antibodies were then visualized by the Western blotting enhanced chemiluminiscent detection system (ECL, Amersham Biosciences, UK). The result was expressed in arbitrary units normalized in relation to β actin, which is in accordance with the protocol of Gavrilović et al. [24].

Malondialdehyde measurement

Malondialdehyde concentration in the adrenal medulla was determined using Spectrophotometric Assay for Malondialdehyde BIOXYTECH® MDA-586 (OXIS

Health Products, Inc.,USA) according to the manufacturer's protocol. The MDA-586 method is based on the reaction of a chromogenic reagent, Nmethyl-2-phenylindole, with MDA at 45° C. Malondialdehyde concentration was expressed as μM/mg of protein.

Assay of CAT activity

CAT activity was determined by the method of Beutler [25]. One unit of CAT activity was defined as 1 μmol of H_2O_2 decomposed per minute under assay conditions [26].

Adrenaline measurement

Ten adrenal medullas (left and right) were homogenized in 0.01 N HCl. Adrenaline concentration in the adrenal medulla was determined using 3-CAT Research ELISA kits (Labor Diagnostica Nord, Nordhorn, Germany) according to the manufacturer's protocol. Absorbance was determined at 450 nm using a microplate reader (Stat Fax 2100). Values were expressed as ng of A per g of tissues.

Data analysis

The data were presented as means ± S.E.M. Differences of protein levels of PNMT, VMAT, COMT and NF- κB; concentration of A and MDA, as well as and CAT activity between control and CSI animals in the right and left adrenal medulla were analyzed by t-test. Statistical significance was accepted at $p<0.05$.

RESULTS

The animals exposed to CSI showed increased protein levels of PNMT by 36% (p=0.002, Figure 1a), and VMAT2 44% (p=0.010, Figure 1b), as well as increased concentration of A by 16% (p=0.012, Figure 1d) in the right adrenal medulla, compared with the control animals. In CSI animals we recorded significant increased protein levels of COMT by 37% (p=0.009, Figure 1c) and NF-κB by 25% (p=0.032, Figure 2c) in the left adrenal medulla, compared with the control animals. In addition, we found that CSI produced significantly increased concentration of MDA by 35% (p=0.004, Figure 2a) and decreased activities of CAT by 15% (p=0.047, Figure 2b) in the left adrenal medulla, compared with the control animals.

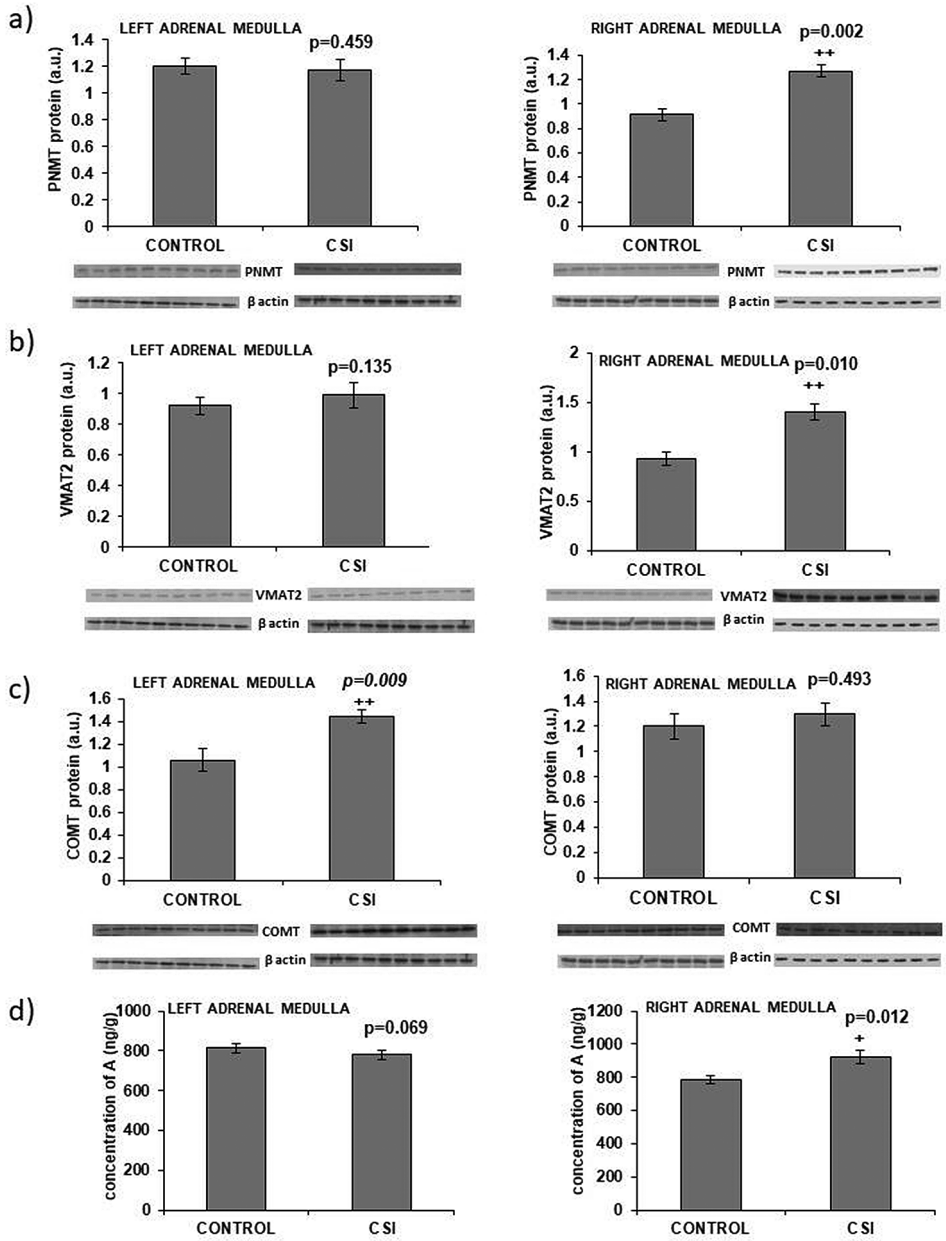

Figure 1. The effects of chronic social isolation (CSI) on protein levels of phenyl ethanolamine N-methyltransferase (PNMT**) [a]**, vesicular monoamine transporters 2 (VMAT2) **[b]**, and catechol-O-methyltransferase (COMT) **[c]**, as well as on the concentrations of adrenaline (A) **[d]** in the left and right adrenal medulla. The values are means ± S.E.M. of 10 rats. Statistical significance: +p<0.05, ++p<0.01 animals exposed to chronic social isolation vs. control animals (t-test). The levels of protein were expressed in arbitrary units normalized in relation to β actin, and the concentration of A was expressed as ng per gram of tissue (ng/g).

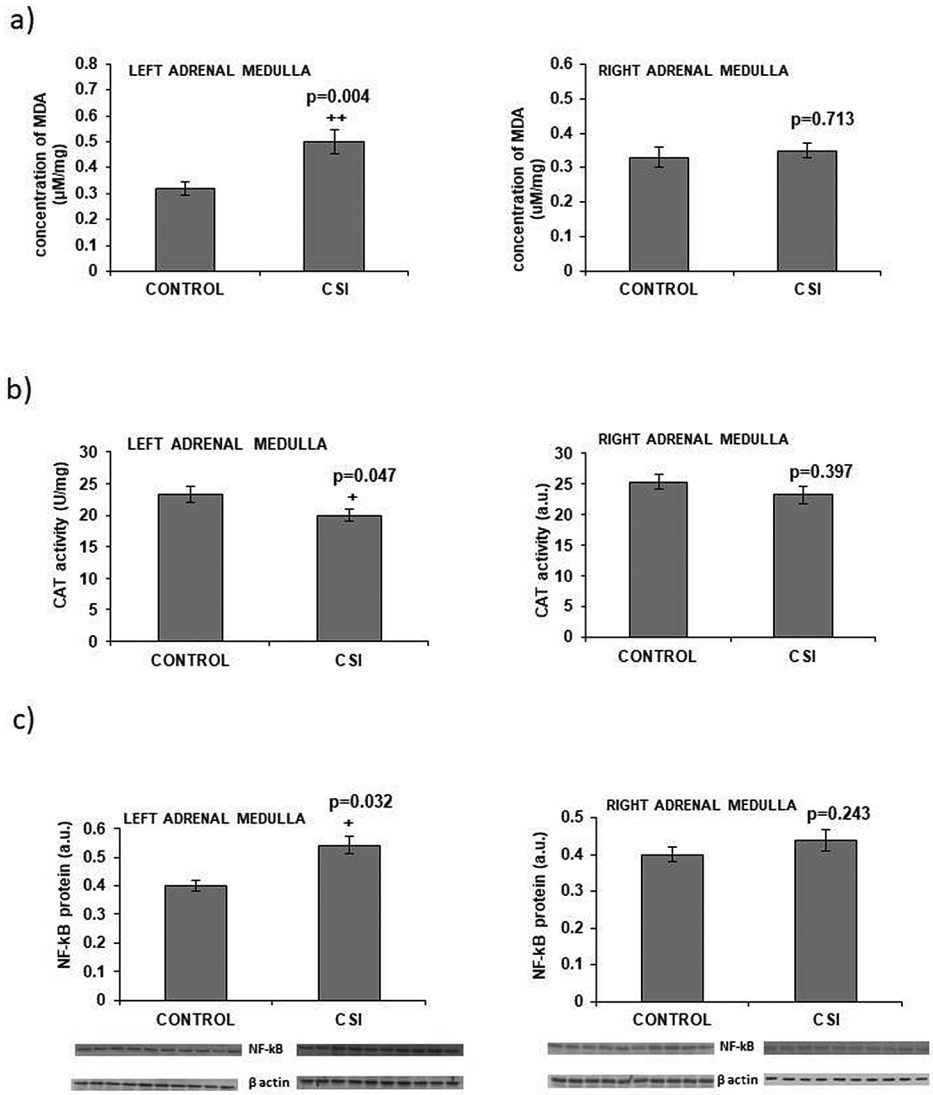

Figure 2. The effects of chronic social isolation (CSI) on the concentrations of malondialdehyde (MDA) **[a]**, and enzyme activity of catalase (CAT) **[b]**, as well as protein levels of nuclear factor κB (NF-kB) **[c]** in the left and right adrenal medulla. The values are means ± S.E.M. of 10 rats. Statistical significance: +p<0.05, **++**p<0.01 animals exposed to chronic social isolation *vs.* control animals (t-test). The final result for concentration of MDA was expressed as µM/mg protein. The levels of protein were expressed in arbitrary units normalized in relation to β actin, and the enzyme activity was expressed as units per milligram of protein (U/mg).

DISCUSSION

The regulation of adrenomedullary function such as catecholamine release was under complex and multifactorial control, including both hormonal and neural regulatory

processes [15]. The key observation in the present study was that chronic psychosocial stress significantly increased the levels of A in chromaffin cells of right adrenal medulla. In addition, we found that CSI produced significantly increased protein levels of PNMT a „rate-limiting"enzyme in the synthesis of A only in the right adrenal medulla, which confirms differences in the synthesis of A between the left and right adrenal medulla. The increased protein levels of PNMT as well as increased concentration of A are an important adaptive phenomenon of catecholamine system in chronic stress conditions, which is necessary to maintain A synthetic capacity during periods of sustained A secretion. Our results indicate that the right adrenal medulla is the main source of A in the circulation in response to chronic social isolation stress. In addition, our result demonstrates differences in the level of enzyme involved in A storage. We found that CSI produced significantly increased protein levels of VMAT2 in the right adrenal medulla, which confirms the increased storage of A only in the right adrenal medulla. The increased level of A makes the right adrenal medulla more ready to respond to a novel acute stressor that may occur later. However, we found that CSI treatment significantly increased protein levels of COMT only in the left adrenal medulla. Our finding suggests the possibility of increased degradation of A in the left adrenal medulla.

Although it is not a direct indicator of oxidative stress, we found a significantly increased MDA level in the left adrenal medulla. This finding suggests elevated levels of lipid peroxidation, which could be mediated by CSI-induced oxidative stress only in the left adrenal medulla. It is known that high levels of ROS deplete the antioxidant capacity. An important result in this work is that CSI treatment significantly decreased the activity of CAT in the left adrenal medulla. Lower CAT activity during the detoxication of ROS in the left adrenal medulla as a response to CSI found in our study may provoke irreversible processes that can result in reducing AO potential in the left adrenal medulla. Our results are in line with the results of Che et al. [27] who found that MDA, a lipid peroxidation product, is increased, but antioxidant capacity, catalase activity is decreased in chronic stress condition.

Lingappan [28] found that the early phase of oxidative stress is associated with activation of the NF-κB pathway. In our experiment we found the increased protein levels of NF-kB indicating dysregulation of the redox balance in the left adrenal medulla in animals exposed to CSI. The literature data have shown that NF-κB inhibitors can be successfully used in the treatment of pheochromocytoma [29]. A greater understanding of the NF-κB signaling and oxidative stress may lead to the development of therapeutic strategies for the treatment of diseases for which oxidative stress has an etiologic role.

CONCLUSIONS

Based on our results, it may be concluded that the adrenomedullary activity and antioxidant status in the right and left adrenal medulla are different in conditions of

chronic psychosocial stress. Detecting a regulatory molecular mechanism by which CSI changes functional activity and redox homeostasis in the left and right adrenal gland in conditions provoked by chronic psychosocial stress is important in the prevention of neuroendocrine diseases caused by chronic stress.

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Author's contributions

LjG participated in the experiment, carried out the molecular genetic studies, participated in the statistical analysis, designed and wrote manuscript. VS helped to draft the statistical analysis, helped to draft the manuscript. SP helped to draft the statistical analysis and helped to draft the manuscript. VST participated in the experiment. DN participated in the experiment. SBP helped to draft the manuscript.

Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Ethical Statement

The Ethical Committee for the care and use of laboratory animals of the Institute of Nuclear Sciences "Vinča", Belgrade, Serbia has approved the planned experiment. The opinion of the Ethical Committee (number 12/14) is that the use of laboratory animals is ethically justified for conducting the experiment. The Ethical Committee of the "Vinča" Institute of Nuclear Sciences "Vinča", Belgrade, Serbia, follows the guidelines of the Serbian Society for the Use of Animals in Research and Education.

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RAZLIKE U FUNKCIONALNOJ AKTIVNOSTI I REDOKS HOMEOSTAZI IZMEĐU LEVE I DESNE NADBUBREŽNE ŽLEZDE PACOVA IZLOŽENIH HRONIČNOM STRESU IZOLACIJE

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Cilj ove studije bio je da ispita da li postoji razlika u funkciji srži leve i desne nadbubrežne žlezde u hroničnom stresu. Ispitivali smo kako hronični stres izolacije (CSI 12 nedelja) utiče na nivo ključnih enzima koji učestvuju u sintezi adrenalina (A) (fenil etanolamin N-metiltransferaze-PNMT), u skladištenju A (vezikularnog monoaminskog transportera-VMAT2), u razgradnji A (katehol-O-metil transferaze COMT), kao i na koncentraciju A, kao pokazatelja funkcije srži leve i desne nadbubrežne žlezde. Takođe, isopitivali smo i koncentraciju malondialdehida (MDA), nivo proteina nuklearnog faktora kB (NF-kB) i aktivnost katalaze (CAT) u levoj i desnoj srži nadbubrežne žlezde hronično stresiranih pacova. Ispitivani parametri su kvantifikovani Western blot analizom, testom za enzimsku aktivnost i ELISA kitom za ispitivanje kateholamina. Pronašli smo da CSI značajno povećava nivo PNMT proteina i VMAT2 proteina, kao

i koncentraciju A u srži desne nadbubrežne žlezde. Međutim, zabeležili smo da CSI povećava nivo proteina COMT i NF-kB, kao i koncentraciju MDA u srži leve nadbubrežne žlezde. Takođe, CSI smanjuje CAT samo u levoj srži nadbubrežne žlezde. Na osnovu naših rezultata, možemo zaključiti da postoji razlika u funkciji srži leve i desne nadbubrežne žlezde u uslovima hroničnog stresa.