Prefrontal Catecholaminergic Turnover and Antioxidant Defense System of Chronically Stressed Rats

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POPOVIĆ N., PAJOVIĆ S.B., STOJILJKOVIĆ V., PEJIĆ S., TODOROVIĆ A., PAVLOVIĆ I., GAVRILOVIĆ L. 2017. Prefrontal catecholaminergic turnover and antioxidant defense system of chronically stressed rats. Folia Biologica (Kraków) **65**: 43-54.

This study examined the effects of chronic restraint stress (CRS) on gene expression of tyrosine hydroxylase (TH), dopamine-β-hydroxylase (DBH), dopamine transporter (DAT), noradrenaline transporter (NET), vesicular monoamine transporter 2 (VMAT2), catechol-O-methyltransferase (COMT) and antioxidant enzymes such as superoxide dismutase (SOD1 and SOD2), catalase (CAT), glutathione peroxidase (GPx) and glutathione reductase (GR), as well as concentrations of dopamine (DA) and noradrenaline (NA), activities of monoamine oxidase (MAO A and MAO B) and activities of antioxidant enzymes (SOD1, SOD2, CAT, GPx and GR) in the rat prefrontal cortex (PFC). We found that CRS decreases gene expression of TH and DBH and concentrations of DA, which probably confirms the decrease of *de novo* synthesis of catecholamine**.** CRS increased protein levels of NET and VMAT2, which was followed by increased NA concentration. The increased activity of MAO A and MAO B, as well as increased protein levels of COMT probably indicate increased catecholamine degradation, which was followed by increased activity of SOD1, SOD2 and CAT, as well as decreased activity of GPx under stress conditions. Our findings confirm the increased prefrontal noradrenergic turnover in animals exposed to CRS. The molecular mechanisms by which CRS changes catecholaminergic turnover and the antioxidant defense system in the PFC may be very important for research on numerous psychiatric diseases caused by chronic stress.

Key words: Catecholamine, antioxidant enzymes, chronic restraint stress, prefrontal cortex, gene expresssion.

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Monoaminergic signaling is regarded as one of the key mechanisms for the modulation of brain functions. Both dopamine (DA) and noradrenaline (NA) are key to prefrontal cortex (PFC) function (ARNSTEN & GOLDMAN-RAKIC 1985). Chronic restraint stress (CRS) can exacerbate neurodegeneration and provoke anxiety and depressive-like behaviours in rats (LIU *et al.* 2013; WANG *et al.* 2014; FERRAZ *et al.* 2011). It is known that depression is caused by a functional deficiency of catecholamines, particularly NA, whereas mania is caused by a functional excess of catecholamines at synapses in the brain (SCHILDKRAUT 1965). The

monoamine hypothesis of depression states that depressive disorder is caused by insufficient signaling by monoamines (CARLSON 1988). This hypothesis has led to the development of several generations of selective and nonselective inhibitors of transporters of NA, with the goal of augmenting monoaminergic transmission (MONTOYA *et al.* 2016).

It is known that normal catecholaminergic turnover results from a balance among synthesis, degradation, release and reuptake of catecholamines. However, very little is known about the regulatory molecular mechanisms by which chronic stress

changes catecholaminergic turnover in the PFC. Therefore, it is very important to examine gene expression of key enzymes involved in catecholamine biosynthesis (tyrosine hydroxylase-TH and dopamine-β-hydroxylase-DBH), reuptake (dopamine transporter-DAT and noradrenaline transporter-NET), stored (vesicular monoamine transporters-VMATs) and degradation (monoamine oxidase-MAO and catechol-O-methyltransferase-COMT) in the PFC under stress conditions. In this work we investigated how CRS (2 hours × 14 days) affects the gene expression of TH, DBH, DAT, NET, VMAT2, and COMT as well as concentrations of catecholamines (DA and NA) and activity of MAO A and MAO B in the PFC. As a rise in catecholamine catabolism results in increased reactive oxygen species (ROS) production in the PFC, we also measured gene expression and activity of the antioxidant enzymes: superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and glutathione reductase (GR) in the PFC. In this study we applied CRS because LEVINSTEIN and SAMUELS (2014) found that CRS is an effective model for obtaining depressive-like symptoms in rodents.

In this study we presumed that CRS may induce the physiological maladaptations of catceholamine metabolism, as well as oxidative damage in the PFC. Because of the significant role of catecholamines in regulation of numerous brain functions, detection of the regulatory molecular mechanisms by which CRS changes catecholaminergic turnover and the antioxidant defense system in the PFC may by very important in research on numerous psychiatric diseases caused by chronic stress.

Materials and Methods

Animals and stress models

In this study Wistar male rats (11-week-old) were used. Animals were maintained under standard laboratory conditions with water and food *ad libitum and* kept three to four *per* cage. Care was taken to minimize the 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 registered "Serbian Society for the Use of Animals in Research and Education". Animals were divided into two groups: the control group (n=10) was not exposed to any treatment and the CRS group (n=10) consisted of animals exposed to treatment of chronic restraint stress. Restraint stress was performed by placing each animal in a 25 x 7 cm plastic bottle as described previously (GAMARO *et al.* 1999). Animals in these groups were exposed to 2h of restraint stress every day at random times during the light period of the light/dark cycle to avoid habituation during the experimental procedure of 14 days (KIM & HAN 2006). To reduce variance in the physiological parameters due to daily rhythms, the remaining animals were sacrificed at the same time point in the circadian cycle, between 9:00 and 11:00 a.m., i.e., one day after the last treatments. Animals were sacrificed under no-stress conditions by rapid decapitation. The prefrontal cortex was rapidly dissected, frozen in liquid nitrogen and stored at -70° C until analyzed.

RNA isolation and cDNA synthesis

Total RNAs were isolated from prefrontal cortex tissue by using the TRIZOL reagent (Invitrogen, USA). After the isolation of mRNA, DNA-ase treatment was applied with DNAse I (Fermentas, Lithuania). The concentration of total mRNA was measured in triplicates on a spectrophotometer (Pharmacia, GeneQuantII BioTech, USA). The quality of mRNA was checked on an agarose gel. Reverse transcription was performed using Ready-To-Go You-Prime First-Strand Bead (Amersham Biosciences, UK) and pd $(N)_6$ Random Hexamer (Amersham Biosciences, UK) primer according to the manufacturer's protocol. 12 μl of sample containing 1500 ng mRNA was incubated for 10 min at 65°C. Then a 21 μl reverse transcriptase with pd (N) 6 primer (final 0.2 μg) was added per sample and incubated 1 hour at 37°C.

Real-time RT-PCR

TH, DBH, DAT, NET, VMAT2, CuZn SOD (SOD1), Mn SOD (SOD2), CAT, GPx, and GR mRNA levels were quantified by quantitative real-time RT-PCR, as described previously by GAVRILOVIĆ *et al.* (2013). TaqMan PCR assays were carried out using Assay-on-Demand Gene Expression Products (Applied Biosystems, USA) for TH (Rn00562500_m1), DBH (Rn00565819_m1), DAT (Rn00562224_m1), NET (Rn00580207_m1), VMAT2 (Rn00564688_m1), SOD1 (Rn00566938_m1), SOD2 (Rn00690587_g1), CAT (Rn00560930_m1), GPx (Rn00577994_g1) and GR (Rn01482159_m1). The gene expression assays contained primers for amplification of the target gene and the TaqMan MGB (Minor Groove Binder) probe 6-FAM dye-labeled for the quantification. Reactions were performed in a 25 μl reaction mixture containing 1x TaqMan Universal Master Mix with AmpErase UNG, 1x Assay Mix (Applied Biosystems, USA) and cDNA template (10 ng of RNA converted to cDNA). PCR was carried out in the ABI Prism 7000 Sequence Detection System at 50°C for 2 min, 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. The experimental threshold was calculated based on the mean baseline fluorescence signal from cycle 3 to 15 plus 10 standard deviations. The point at which the amplifica-

tion plot crossed this threshold defined as Ct, represents the cycle number at this point and is inversely proportional to the number of target copies present in the initial sample. Each sample was run in triplicate and the mean value of each Ct triplicate was used for further calculations. The reference gene (endogenous control) was included in each analysis to correct for the differences in inter-assay amplification efficiency and all transcripts were normalised to cyclophyline A (Rn00690933_m1) expression. The reaction mixture for endogenous control gene amplification consisted of 1x TaqMan Universal Master Mix with AmpErase UNG (Applied Biosystems, USA), 1x Assay (6-FAM dye-labeled MGB probes) and cDNA (10 ng of RNA converted to cDNA). The levels of expression of cyclophyline A in samples under different treatments were checked by additional experiments that confirmed that the chosen reference gene was not regulated. Before quantification, validation experiments were performed to determine the similar amplification efficiency of endogenous control and each target gene. We tested cyclophyline A and demonstrated that its efficiency of amplification was approximately equal to all assays used for target genes. Briefly, serial dilutions of cDNA were prepared and amplified by real-time PCR using specific primers and fluorogenic probes for target and endogenous control genes. Quantification was done using the $2^{-\Delta\Delta Ct}$ method according to LIVAK and SCHMITTGEN (2001). The results obtained were analyzed by the RQ Study Add On software for the 7000 v 1.1 SDS instrument (ABI Prism Sequence Detection System, Applied Biosystems, USA) with a confidence level of 95% (P<0.05). The relative expression of the target gene was normalized to cyclophyline A and expressed in relation to the calibrator, i.e. the control sample. Due to individual differences among animals, one sample from the control group with the expression value closest to the mean of all samples in this group and with the lowest measurement error was chosen as a calibrator. The results are reported as a fold change relative to the calibrator and normalized to cyclophyline A using the equation: $N_{sample} = 2^{-\Delta\Delta Ct}$.

Prefrontal cortex tissue homogenization and measurment of the protein concentration

The prefrontal cortex 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), described by STICH (1990).

Western blot analysis

TH, DBH, DAT, NET, VMAT2, COMT, CuZn SOD (SOD1), Mn SOD (SOD2), CAT, GPx, and GR proteins were assayed by Western blot analy-sis as described previously by GAVRILOVIĆ et al. (2013). The samples were boiled in denaturing buffer according to LAEMMLI (1970), for 5 min at 95°C. Fifteen micrograms of protein extract from prefrontal cortex was separated by 10% SDS-polyacrylamide gel electrophoresis and then transferred to a supported nitrocellulose membrane (Hybond™ C Extra, Amersham Biosciences, UK). 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. Protein molecular mass standards (PageRuler™ Plus Prestained Protein Ladder, Fermentas) were used for calibration. Antibodies used for quantification of specific proteins were as follows: for TH (ab51191, Abcam, USA), for DBH (ab63939, Abcam, USA), for DAT (ab18548 Abcam, USA), for NET (ab41559, Abcam, USA), for VMAT2 (ab70808 Abcam, USA), for SOD1 (SOD-101, Stressgen, USA), for SOD2 (SOD-110, Stressgen, USA), for CAT (Calbiochem, Germany), GPx (sc-30147 Santa Cruz Biotechnology, USA) and GR (sc-32886, Santa Cruz Biotechnology, USA), and for β-actin (ab8227, Abcam, USA). After washing, the membranes were incubated in the secondary anti-mouse and anti-rabbit (dilution 1:5000, Amersham ECL™ Western Blotting Analysis System, UK) antibodies conjugated to horseradish peroxidase. A secondary antibody was then visualized by the Western blotting enhanced chemiluminescent detection system (ECL, Amersham Biosciences, UK). The membranes were exposed to ECL film (Amersham Biosciences, UK). Densitometry of protein bands on ECL film was performed by Image J analysis PC software. The result was expressed in arbitrary units normalized in relation to β actin.

Catecholamine measurement

Prefrontal cortex tissues were homogenised in 0.01 N HCl in the presence of EDTA and sodium metabisulfite. Catecholamine concentration in the prefrontal cortex fractions 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). Concentrations were normalized to 1 g of tissues in homogenate. Values were expressed as ng of catecholamine per g of tissues.

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), described by ZHOU and PANCHUK-VOLO-

SHINA (1997). 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. Fluorescence was measured with a fluoremeter using excitation at 560 ± 10 nm and fluorescence detection at 590 ± 10 nm. Monoamine oxidase activity was expressed as U/mg of protein.

Antioxidant enzyme activities

SOD, CAT, GPx, and GR activity was determined using previously described methods by STOJILJKOVIĆ *et al.* (2009).

Assay of SOD activity

Total SOD activity was measured using the Oxis Bioxytech® SOD-525™ Assay (Oxis International, Inc., Portland, OR, USA). The method is based on the SOD-mediated increase in the rate of autoxidation of reagent 1 (5,6,6a,11b-tetrahydro-3,9,10-trihydroxybenzo (c) fluorene, R1) in aqueous alkaline solution, yielding a chromophore with maximum absorbance at 525 nm. The kinetic measurement of the change in absorbance at 525 nm was conducted. One SOD-525 activity unit was defined as the activity that doubles the autoxidation rate of the control blank. CuZnSOD activity was measured as described above after pretreating samples with ethanol-chloroform reagent (5/3 vol/vol), which inactivates MnSOD. MnSOD activity; the calculation involved subtracting CuZn-SOD activity from total SOD activity.

Assay of CAT activity

CAT activity was determined by the method of BEUTLER (1982). The reaction is based on the rate of H_2O_2 degradation by catalase contained in the examined samples. The reaction was performed in an incubation mixture containing 1 M Tris-HCl, 5 mM EDTA, pH 8.0, and monitored spectrophotometrically at 230 nm. One unit of CAT activity is defined as 1 μmol of H_2O_2 decomposed per minute under the assay conditions.

Assay of GPx activity

GPx activity was assessed using the Oxis Bioxytech GPx-340 Assay (Oxis International, Inc., Portland, OR, USA), based on the principle that oxidized glutathione (GSSG) produced upon reduction of an organic peroxide by GPx is immediately recycled to its reduced form (GSH) with concomitant oxidation of NADPH to NADP+. The oxidation of NADPH was monitored spectrophotometrically as a decrease in absorbance at 340 nm. One GPx-340 unit is defined as 1 μmol of NADH oxidized per minute under the assay conditions.

Assay of GR activity

Activity of GR was measured using the Oxis Bioxytech GR-340 Assay (Oxis International, Inc., Portland, OR, USA). The assay is based on the oxidation of NADPH to NADP+ during the reduction of oxidized glutathione (GSSG), catalyzed by a limiting concentration of glutathione reductase. The oxidation of NADPH was monitored spectrophotometrically as a decrease in absorbance at 340 nm. One GR-340 unit is defined as 1 μmol of NADH oxidized per minute under the assay conditions.

Data analysis

The data are presented as means $\pm$ S.E.M. Differences of gene expression (mRNA and protein levels) of TH, DBH, DAT, NET, VMAT2, COMT, SOD1, SOD2, CAT, GPx, and GR; concentration of DA and NA, as well as activity of enzymes (MAO A, MAO B, SOD1, SOD2 CAT, GPx, and GR) between control and CRS animals in the PFC were analyzed by the *t*-test. Statistical significance was accepted at P<0.05. Correlations of mRNA levels, protein levels, catecholamine levels and enzyme activity were analyzed by the Pearson test, using the Sigma Plot v10.0 (with SigmaStat integration). The data conformed with statistical test requirements.

Results

Changes in DA and NA concentrations in the prefrontal cortex

We found that in prefrontal cortex CRS significantly decreased the concentration of DA by 47% (P<0.01, *t*-test, Fig. 1a) and increased NA concentration by 49% (P<0.05, *t*-test, Fig. 1b), compared with control animals.

A significant positive correlation was found between DA and NA concentration in the prefrontal cortex (Pearson R=0.678; P<0.05) of control animals. However, in CRS animals we recorded a significant negative correlation between DA and NA concentration in the prefrontal cortex (Pearson R=-0.664; P<0.05).

Changes in TH and DBH gene expression in the prefrontal cortex

Animals exposed to CRS showed decreased levels of TH mRNA by 12% (P<0.05, *t*-test, Fig. 2a), TH protein by 34% (P<0.01, *t*-test, Fig. 2b), DBH mRNA by 30% (P<0.05, *t*-test, Fig. 2c) and levels of DBH protein by 55% (P<0.01, *t*-test, Fig. 2d) in the prefrontal cortex, compared with control animals.

In control animals we recorded a significant positive correlation between levels of TH protein and DA concentration in the prefrontal cortex
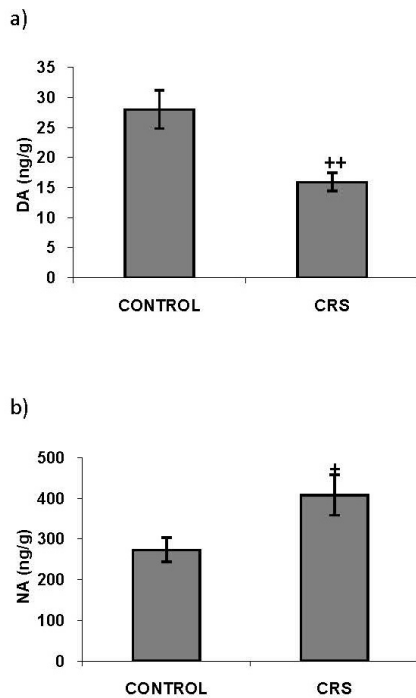
a)

b)

Fig. 1. Effects of chronic restraint stress (CRS) on the concentration in the prefrontal cortex: a – of dopamine (DA); b – of noradrenaline (NA). The values are means ± S.E.M. of 10 rats. Statistical significance: +P<0.05, ++P<0.01 animals exposed to chronic restraint stress vs. control animals (t-test). The final result for concentration of DA and NA was expressed as ng per gram of tissue (ng/g).

(Pearson R=0.704; P<0.05). In addition, a significant positive correlation was found between reduced levels of TH protein and DA concentration (Pearson, R=0.668; P<0.05) in the prefrontal cortex of animals exposed to CRS. Also, a significant positive correlation was found between DBH protein level and NA level in the prefrontal cortex (Pearson R=0.728; P<0.05) of control animals. However, in CRS animals we recorded a significant negative correlation between the reduced DBH protein level and increased NA level in the prefrontal cortex (Pearson, R=-0.645; P<0.05).

Changes in DAT, NET and VMAT2 gene expression in the prefrontal cortex

CRS provoked decreased levels of NET mRNA by 37% (P<0.05, t-test, Fig. 3a) and VMAT2 mRNA by 20% (P<0.05, t-test, Fig. 3c), compared with control animals. However, CRS significantly increased levels of NET protein by 25% (P<0.05, t-test, Fig. 3b) and VMAT2 protein by 12% (P<0.05, t-test, Fig. 3d), as well as decreased levels of DAT protein by 26% (P<0.05, t-test, Fig. 3e), compared with control animals.

In control animals we did not find a significant correlation between levels of NET protein and NA concentration, ors VMAT2 protein and NA con-
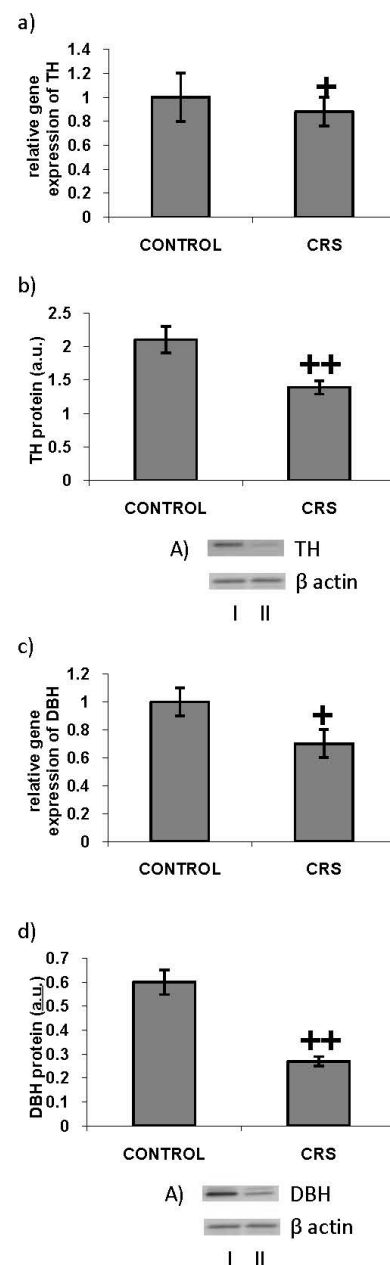
a)

b)

c)

d)

Fig. 2. Effects of chronic restraint stress (CRS) in the prefrontal cortex: a – on tyrosine hydroxylase (TH) mRNA; b – on TH protein levels; c – as well as dopamine-ß-hydroxylase (DBH) mRNA levels; d – on DBH protein levels. The values are means ± S.E.M. of 10 rats. Statistical significance: +P<0.05, ++P<0.01 animals exposed to chronic restraint stress vs. control animals (t-test). Levels of mRNA were expressed as fold change relative to the calibrator and normalized to cyclophyline A, and protein levels were expressed in arbitrary units normalized in relation to β actin. A – Distribution of TH, DBH and β-actin proteins in the prefrontal cortex of control animals (I), animals exposed to CRS (II).

centration in the prefrontal cortex. However, a significant positive correlation was found between the levels of NET protein and NA concentration (Pearson, R=0.782; P<0.01), as well as VMAT2 protein and NA concentration (Pearson, R=0.709; P<0.05) in the prefrontal cortex of animals exposed to CRS.

a)

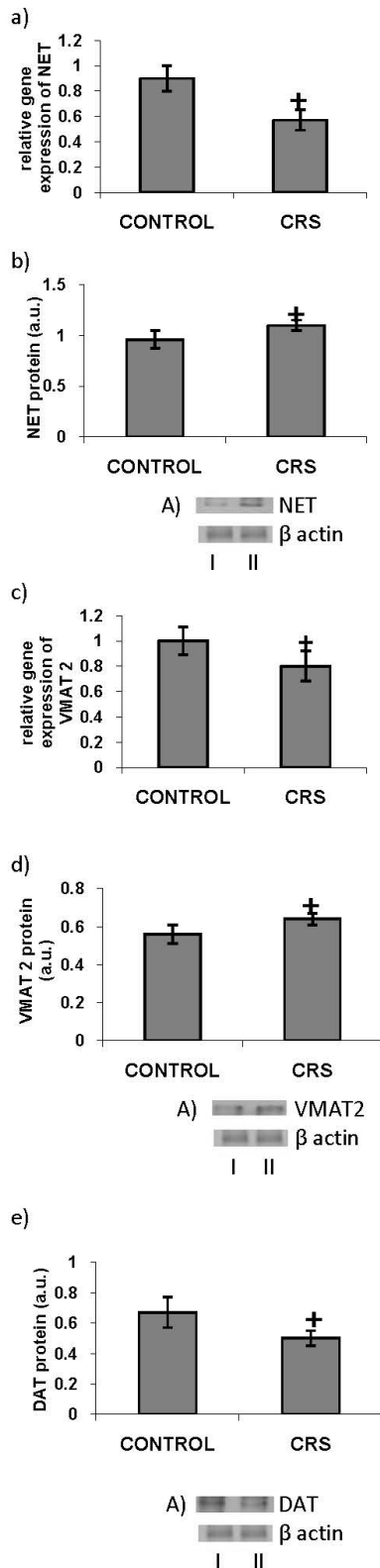## Changes in MAO A and MAO B activity in the prefrontal cortex

CRS treatment significantly increased enzymatic activity of MAO A by 58% (P<0.05, *t*-test, Fig. 4a), and MAO B by 68% (P<0.05, *t*-test, Fig. 4b), compared with control animals.

Changes in COMT protein level in the prefrontal cortex

CRS increased the protein level of COMT by 59% (P<0.05, *t*-test, Fig. 5), compared with control animals.
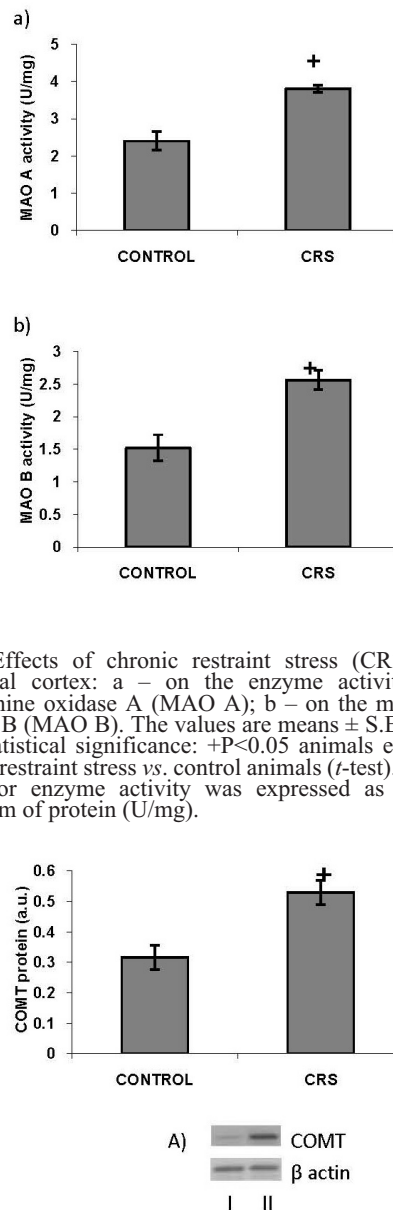
b)

c)

d)

a)

b)

Fig. 4. Effects of chronic restraint stress (CRS) in the prefrontal cortex: a – on the enzyme activity of the monoamine oxidase A (MAO A); b – on the monoamine oxidase B (MAO B). The values are means ± S.E.M. of 10 rats. Statistical significance: +P<0.05 animals exposed to chronic restraint stress *vs*. control animals (*t*-test). The final result for enzyme activity was expressed as units per milligram of protein (U/mg).

e)

Fig. 3. Effects of chronic restraint stress (CRS) in the prefrontal cortex: a – on NA transporter (NET) mRNA; b – on NET protein levels; c – vesicular monoamine transporter 2 (VMAT2) mRNA levels; d – VMAT2 protein levels; e – as well as DA transporter (DAT) protein levels. The values are means ± S.E.M. of 10 rats. Statistical significance: +P<0.05 animals exposed to chronic restraint stress *vs*. control animals (*t*-test). Levels of mRNA were expressed as fold change relative to the calibrator and normalized to cyclophyline A, and protein levels were expressed in arbitrary units normalized in relation to β actin. A – Distribution of NET, VMAT2, DAT and β-actin proteins in the prefrontal cortex of control animals (I), animals exposed to CRS (II).

Fig. 5. Effects of chronic restraint stress (CRS) on catechol-O-methyltransferase (COMT) protein levels in the prefrontal cortex. The values are means ± S.E.M. of 10 rats. Statistical significance: +P<0.05 animals exposed to chronic restraint stress *vs*. control animals (*t*-test). The result was expressed in arbitrary units normalized in relation to β actin. A – Distribution of COMT and β-actin proteins in the prefrontal cortex of control animals (I), animals exposed to CRS (II).
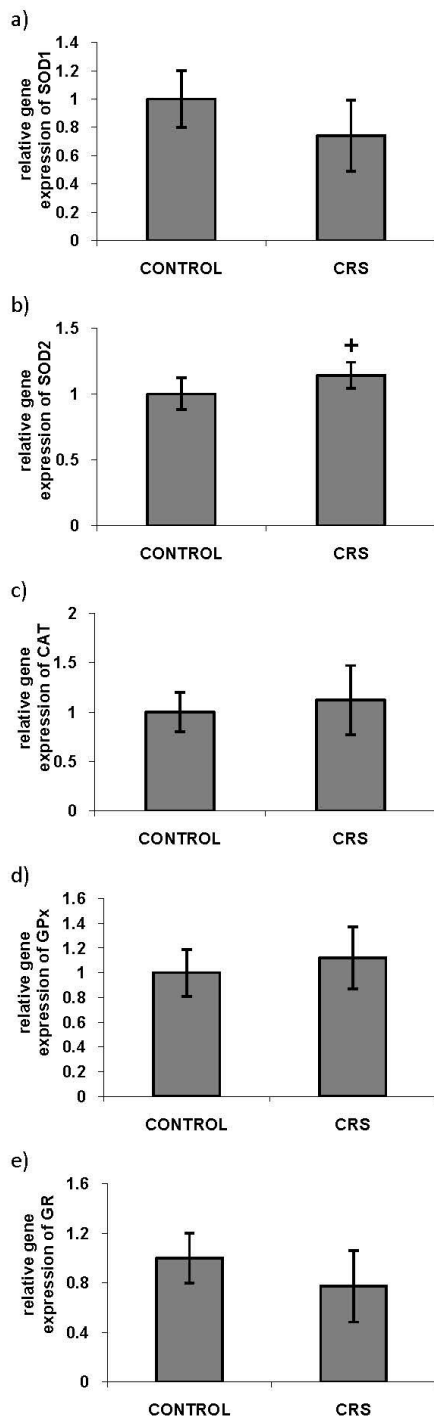
Fig. 6. Effects of chronic restraint stress (CRS) in the prefrontal cortex mRNA levels: a – on CuZn superoxide dismutase (SOD1); b – Mn superoxide dismutase (SOD2); c – catalase (CAT); d – glutathione peroxidase GPx (GPx); e – glutathione reductase (GR). The values are means ± S.E.M. of 10 rats. Statistical significance: +P<0.05 animals exposed to chronic restraint stress *vs*. control animals (*t*-test). The final result was expressed as fold change relative to the calibrator and normalized to cyclophyline A.

Changes in SOD 1, SOD 2, CAT, GPx and GR gene expression and enzyme activities in the prefrontal cortex

The animals exposed to CRS showed unchanged levels mRNA and protein of SOD1 (Fig. 6a, 7a), CAT (Fig. 6c, 7c), GPx (Fig. 6d, 7d) and GR (Fig. 6e, 7e), as well as significantly increased levels of SOD2 mRNA by 18% (P<0.05, *t*-test, Fig. 6b) and SOD2 protein level by 20% (P<0.05, *t*-test, Fig.7b) compared with control animals.

CRS treatment significantly increased the enzyme activities of SOD1 by 20% (P<0.05, *t*-test, Fig. 8a), SOD2 by 100% (P<0.01, *t*-test, Fig. 8b), CAT by 12% (P<0.05, *t*-test, Fig. 8c), as well as significantly decreased enzyme activities of GPx by 15% (P<0.05, *t*-test, Fig. 8d), while GR activity remained unchanged (Fig. 8e).

Discussion

The results show that CRS affects the molecular mechanisms for catecholaminergic turnover and antioxidant defense system in rat PFC. The potentially negative physiological adaptations after CRS were recorded as decreased DA and increased NA concentrations in the PFC. In addition, we recorded a significant negative correlation between the levels of DA and NA in the PFC of chronically stressed animals. A significantly decreased concentration of DA and increased concentration of NA were observed in the PFC in many pathophysiological conditions. For example, a significantly decreased concentration of DA and increased concentration of NA were observed in the PFC in aggressive rats (PATKI *et al*. 2015). Also, a decreased concentration of DA was observed in many psychiatric disorders. It is known that several antidepressants increase DA levels in the PFC (TANDA *et al*. 1994). Also, raising the DA level in patients with Parkinson's disease with l-3,4-dihydroxyphenylalanine improves their working memory deficit (LANGE *et al*. 1992). In addition, we have detected decreased TH mRNA expression in the PFC of chronically stressed animals which is in accordance with results of QIU *et al*. (2015). These authors found decreased TH mRNA expression in the PFC of chronically stressed animals. Importantly, reduced protein levels of TH positively correlated with a reduced concentration of DA in the PFC of chronically stressed animals. This finding probably confirms the decrease of *de novo* synthesis of DA in the PFC. It is possible that excessive stimulation by chronic stress could provoke monoamine cell desensitization, resulting in decreased DA synthesis in the PFC which is in accordance with our previous results (GAVRILOVIĆ *et al*. 2013) showing that chronic stress induced adrenomedular cell desensitization and reduced
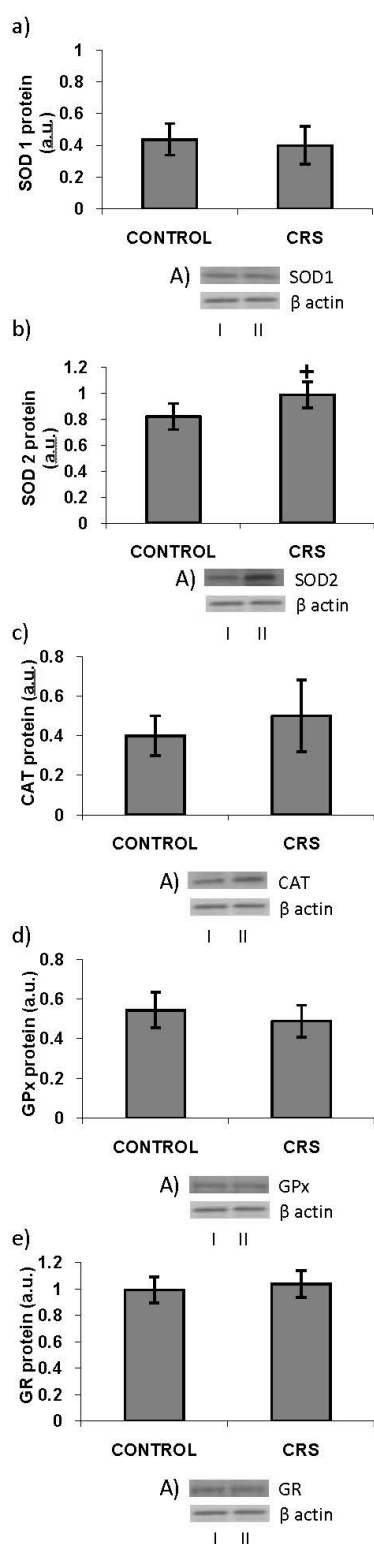
Fig. 7. Effects of chronic restraint stress (CRS) protein levels in the prefrontal cortex: a – on CuZn superoxide dismutase (SOD1); b – Mn superoxide dismutase (SOD2); c – catalase (CAT); d – glutathione peroxidase GPx (Gpx); e – glutathione reductase (GR). The values are means ± S.E.M. of 10 rats. Statistical significance: +P<0.05 animals exposed to chronic restraint stress *vs*. control animals (*t*-test). The result was expressed in arbitrary units normalized in relation to β actin. A – Distribution of SOD1, SOD2, CAT, GPx, GR and β-actin proteins in the prefrontal cortex of control animals (I), animals exposed to CRS (II).
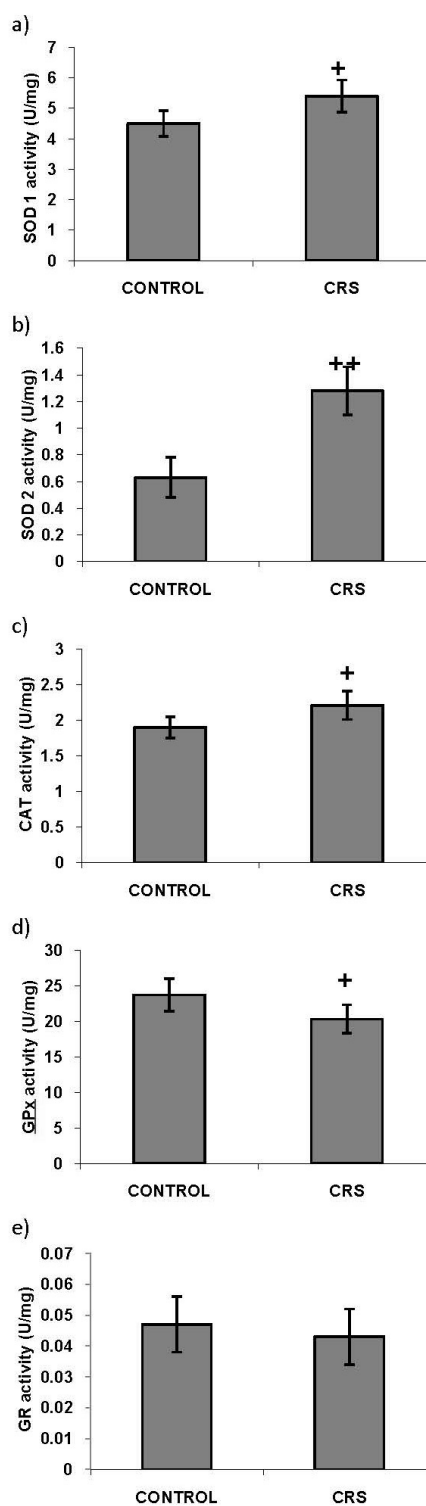
Fig. 8. Effects of chronic restraint stress (CRS) enzyme activities in the prefrontal cortex: a – on CuZn superoxide dismutase (SOD1); b – Mn superoxide dismutase (SOD2); c – catalase (CAT); d – glutathione peroxidase GPx (GPx; e – glutathione reductase (GR). The values are means ± S.E.M. of 10 rats. Statistical significance: +P<0.05, ++P<0.01 animals exposed to chronic restraint stress *vs*. control animals (*t*-test). The final result for enzyme activity was expressed as units per milligram of protein (U/mg).

catecholamine synthesis. In addition, although gene expression of DBH enzyme decreased, we have found an increased concentration of NA in the PFC of chronically stressed animals. These findings suggest the possibility of a decrease of conversion of neurotransmitter DA to NA in the PFC of the animals exposed to CRS. This is supported by a significant negative correlation between the levels of DBH protein and NA in the PFC. It is known that PFC is an integral part of stress sensitive limbic circuitry, receiving afferents from different limbic regions including hippocampus, amygdala, ventral tegmental area (VTA) and locus coeruleus (LC). Our findings strengthen the idea that LC, SN, VTA and amygdala participate in the NA response to CRS in the PFC, which is in accordance with the results of JEDEMA and GRACE (2003), who have confirmed that the excitability of LC neurons is increased after chronic stress. Clinical *post-mortem* brain studies have shown increased TH protein expression in the LC of patients with major depression (ZHU *et al*. 1999). Many authors have confirmed that amygdala plays an important role in changing catecholaminergic turnover in the PFC during stress. For example, lesions to the amygdala prevent an increase in DA and NA release that occurs in the PFC in response to a psychological stressor (GOLDSTEIN *et al*. 1996). Optimal functioning of the PFC occurs when moderate levels of NA are present, activating the α2-adrenergic receptors and strengthening the preferred network inputs (HAINS & ARNSTEN 2008; ARNSTEN 2009). During periods of stress, there is an excessive release of NA into the PFC, activating the α1- and β-adrenergic receptors, leading to a collapse of network functioning and impairment of working memory (HAINS & ARNSTEN 2008; ARNSTEN 2009). The monoaminergic hypothesis of depression has been revised to include changes in the sensitivity of noradrenergic receptors (LOPEZ-MUNOZ & ALAMO 2009). Stress and posttraumatic stress disorder (PTSD) are associated with highly elevated levels of NA in the PFC and impaired cognitive function (HAINS & ARNSTEN 2008; ARNSTEN 2009). Antagonists of the α1- and β-adrenergic receptors are useful in treating PTSD and stress-induced impairment of cognitive flexibility (HAINS & ARNSTEN 2008; ARNSTEN 2009).

Temporal and spatial dynamics of NA transmission are potently regulated by reuptake through the NA transporter (NET) (BLAKELY & BAUMAN 2000). In this study we found that CRS significantly increased protein levels of NET and levels of NA, which probably confirm the uptake of neurotransmitter NA via NET in the PFC. This is indicated by a significant positive correlation between the levels of NET and NA in the PFC of chronically stressed animals. The higher protein levels of NET suggest that NET can be upregulated in response to a heightened demand for uptake of NA in conditions provoked by CRS. According to the literature, the monoamine hypothesis of mood disorders states that depression is caused by a functional deficiency of NA. Our result indicates that stress-induced changes in degradation of nonvesicular NA may play a role in the decrease of monoaminergic transmission. Inhibition of the activity of the NET can prolong the duration during which these neurotransmitters are available in the synaptic cleft (MONTOYA *et al*. 2016). For example, drugs blocking NET effectively treat mood disorders and attention deficit hyperactivity disorder, likely via an action within the PFC (FRAZER 2000; BYMASTER *et al*. 2002; MICHELSON *et al*. 2003).

The vesicular monoamine transporter 2 (VMAT2) is responsible for the uptake of monoamines from the cytoplasm into the intracellular storage vesicles and is expressed in neurons that are implicated in the regulation of mood and behavior (SCHWARTZ *et al*. 2003). ZUCKER *et al*. (2002) found that significant elevation in VMAT2 density was observed in the platelets of untreated major depressed patients compared to healthy control subjects. An important result in this work was that CRS increases protein levels of VMAT2 and that increased levels of VMAT2 coincide with increased levels of NA in the PFC of chronically stressed rats. We recorded a significant positive correlation between the levels of VMAT2 and NA in the PFC of chronically stressed rats. It is known that VMATs play a critical role not only in sorting, storing and releasing of monoamines, but also in fine-tuning neuronal and endocrine informational output (EIDEN 2000). Repeated stress increased expression of VMAT2 levels in many NA synthesizing cells indicating an adaptation to prolonged exposure to a strong stressor, which could provide a mechanism to facilitate utilization of the enhanced noradrenergic capacity (TILLINGER *et al*. 2010).

Importantly, CRS treatment significantly increased enzyme activity of MAO A and MAO B, as well as levels of COMT protein in the PFC. These findings suggest the possibility of increased degradation of catecholamine in the PFC of animals exposed to CRS, which may result in deregulation of redox balances and mitochondrial damage. Our results are in accordance with the reports of BORTOLATO *et al*. (2008) who found that stress-induced activation of MAO may cause oxidative damage to the mitochondrion. Also, immobilization stress (IMO) generated oxidative stress in the rat brain (ZAIDI & BANU 2004). A correlation between ROS overproduction and increased MAO activity has been proven (HERKEN *et al*. 2007). Increased ROS levels in the CNS have been associated with the development of a number of neuropsychiatric diseases (HALLIWELL & CROSS 1994). It is known that there is direct involvement

of oxidative stress in anxiety-like behavior in rodents (MASOOD *et al*. 2008). Also, oxidative stress plays a critical role in the degeneration of DA neurons in Parkinson's disease (PRASAD *et al*. 1999). HU *et al*. (2011) found that the activity of mitochondrial MAO-A is elevated throughout the brain during major depression. In addition, increased MAO B activity in the astrocytes causes Parkinsonian manifestations (MALLAJOSYULA *et al*. 2008). The monoamine hypothesis of mood disorders states that depression is caused by insufficient signaling by monoamines (CARLSON 1988). The inhibition of the activity of the MAO can prolong the duration during which neurotransmitters are available in the synaptic cleft (MONTOYA *et al*. 2016). The literature data confirm that important therapeutic strategies for several neuropsychiatric disorders are inhibition of MAO and/or increase of monoamine neurotransmitter levels. For example, YOUDIM *et al*. (2006) showed that selective MAO A inhibitors have been used as antidepressants and for treatment of other common neurodegenerative diseases. In addition, an increase in DA levels is caused by MAO B inhibitors (CESURA *et al*. 1992). Also, the literature data confirm that inhibitors of COMT are commonly used in patients with Parkinson's disease (MÜLLER 2015).

In the present study the animals exposed to CRS showed unchanged levels of mRNA and protein of SOD1, CAT, GPx, and GR, as well as significantly increased gene expression of SOD2. However, CRS treatment significantly increased the enzyme activities of SOD1, SOD2 and CAT, and significantly decreased enzyme activity of GPx, while GR activity remained unchanged. Discrepancies between mRNA levels and activities may be related to differences in mRNA stability or translational efficiency (GOMI & MATSUO 2001). GARCÍA-LÓPEZ *et al*. (2007) suspect that it is possible that the expression of antioxidant enzyme mRNAs was initially up-regulated and then down-regulated. Regulation of expression might act on individual mRNAs to block their translation and thereby lead to their degradation (GARCÍA-LÓPEZ *et al*. 2007). Message degradation may be the primary target of regulation. These findings are considered to be mechanisms of adaptation (GARCÍA-LÓPEZ *et al*. 2007). Our data suggest high readiness of antioxidant enzymes (SOD1 and CAT) in the PFC to repair or prevent damage by reactive oxygen species in chronically stressed animals. Our results, together with above mentioned data, show that the exaggerated enzyme activity of antioxidant enzymes (SOD1, SOD2 and CAT) in the PFC may be an important adaptive phenomenon of the antioxidant defense system in chronically stressed rats. The increased activity of SOD during stress regime is an indicator of a relative increase in super-

oxide radical production, which could stimulate the second line of defense including CAT (FRIDOVICH 1995). Treatment with antidepressants significantly decreased the activities of SOD and CAT in depressive patients (ABDALLA & BECHARA 1994; BILICI *et al*. 2001; KHANZODE *et al*. 2003). An important result in this study is that CRS treatment significantly decreased enzyme activity of GPx in rat PFC. Our result is in accordance with the reports of NILAKANTAN *et al*. (2005). These authors have found that NO or NO-derived products inhibit GPx enzyme activity. Down regulation of endogenous antioxidant defense is induced by lipid peroxidation, contributing to a role of chronic stress in depression (CHE *et al*. 2015).

Conclusion

In this study we found that CRS decreased gene expression of TH, DBH, DAT and concentrations of DA, and increased protein levels of NET and VMAT2 as well as concentrations of NA in the PFC. These results indicate: the decrease of *de novo* synthesis of catecholamine, the decrease of conversion of neurotransmitter DA to NA and increased noradrenergic capacity in the PFC in conditions provoked by CRS. In addition, the increased activity of MAO A and MAO B, as well as increased protein levels of COMT indicate a possible increase of catecholamine degradation, which is followed by increased activity of SOD1, SOD2 and CAT and decreased activity of GPx in chronic stress conditions. Our findings confirm increased prefrontal noradrenergic turnover in animals exposed to CRS. Based on our results, it could be speculated that the detected molecular mechanisms by which CRS changes catecholaminergic turnover and the antioxidant defense system in the PFC may be very important in research on numerous psychiatric diseases caused by chronic stress.

Acknowledgments

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