Chronic isolation of adult rats decreases gene expression of catecholamine biosynthetic enzymes in adrenal medulla

Ljubica Gavrilović [1], Natasa Spasojević [1], Nikola Tanić [2], Sladjana Dronjak [1]

1. Institute of Nuclear Sciences "Vinča", Belgrade, Serbia,
2. Institute for Biological Research " Siniša Stanković", 11 000 Belgrade, Serbia

Correspondence to: Sladjana Dronjak, Ph.D.
Institute of Nuclear Sciences "Vinca", Lab.Mol. Biol. Endocrinol.,
P.O.B. 522-090, 11000 Belgrade, Serbia
TEL/FAX: 00381112455561;
E-MAIL: sladj@vin.bg.ac.yu

Submitted: 2008-07-07 Accepted: 2008-10-06 Published online: 2008-12-29

Key words: **social isolation; adrenal medulla; qRT-PCR; gene expression; protein levels; catecholamine biosynthetic enzymes**

Neuroendocrinol Lett 2008; **29**(6):1015–1020 **PMID:** 19112418 NEL290608A10

Abstract

OBJECTIVE: Isolation of adult animals represents a form of psychsocial stress that produces sympatho-adrenomedullar activation. The aim of this work was to investigate the changes in gene expression and protein levels of catecholamine biosynthetic enzymes: tyrosine hydroxylase (TH), dopamine-β-hydroxylase (DBH) and phenylethanolamine N-methyltransferase (PNMT) in the adrenal medulla of naive control and chronically (12 weeks) socially isolated adult Wistar rat males and the response of these animals to additional immobilization stress (2 h).

METHODS: TH, DBH and PNMT mRNA levels were quantified by quantitative real-time RT-PCR (qRT-PCR). TH, DBH and PNMT immunoproteins were assayed by Western Blot.

RESULTS: In chronically isolated rats, gene expression levels of catecholamine biosynthetic enzymes in the adrenal medulla were decreased, but only TH mRNA was significantly decreased. However, protein levels of TH, DBH and PNMT of these animals were elevated by 55%, 20% and 18%, respectively, in relation to the corresponding control. Naive control and chronically socially isolated rats exposed to additional 2-h-immobilization showed increased gene expression of the examined enzymes, the increase being greater in socially isolated rats as compared to the controls. Additional immobilization of naive controls did not affect TH, DBH and PNMT protein levels. In contrast, this stress produced increased TH, DBH and PNMT protein levels in long-term socially isolated rats.

CONCLUSION: We can conclud that psychosocial stress expressed a differential influence on gene expression and protein levels of catecholamine biosynthetic enzymes in the adrenal medulla of adult rats. The results indicate a possible adaptation of catecholamine-synthesizing system at the level of TH gene expression in adrenal medulla of chronically isolated animals.

ORIGINAL ARTICLE

Abbervations

CREB – cyclic-AMP response element binding protein
DBH – dopamine-β-hydroxylase
NGF – nerve growth factor
PKA – protein kinasa A
PKC – protein kinasa C
PNMT – phenylethanolamine N-methyltransferase
qRT-PCR – quantitative reverse-transcription polymerase chain reaction
TH – tyrosine hydroxylase

INTRODUCTION

Stress disturbs homeostasis and may induce various disorders. Social isolation and acute environmental changes are risk factors in human depression and represent a lack of the social stimuli necessary to modulate adaptive responses to new situations (Ishida *et al*., 2003). Several authors have reported that socially isolated rats had enhanced responses to stressors (Plaznik *et al*., 1993; Bartolomucci *et al*., 2003; Weiss *et al*., 2004). For example, after 8 weeks of isolation, single housed rats continued to spend more time apparently attempting to escape while those housed in groups spent more time sleeping and feeding (Hurst *et al*., 1999). Catecholamines released from the adrenal medulla are substantial mediators of the stress response. During our previous studies, a potentiation of the sympatho-adrenomedullar system activity in socially isolated rats exposed to novel stressors has been observed (Gavrilovic and Dronjak, 2005; Gavrilović *et al*., 2005). Individually housed rats displayed a much higher increase of plasma norepinephrine and epinephrine to a novel stressor (*e.g.,* immobilization) when compared to the response of group-housed animals. Tyrosine hydroxylase (TH), a rate-limiting enzyme of catecholamine biosynthesis, dopamine-β-hydroxylase (DBH) which converts dopamine to norepinephrine and phenylethanolamine N-methyltransferase (PNMT) which catalyses conversion of norepinephrine to epinephrine are present in different types of tissues. It has been shown that their gene expression, protein level and activity could be changed by various stressors (Kvetnansky *et al*., 2003; 2006; Micutkova *et al*., 2003; Kubovcakova *et al*., 2006). Single immobilization was shown to lead to a dramatic induction of TH mRNA in the adrenal medulla that persisted for at least 12 h, while no significant induction of TH protein was observed (Nankova *et al*., 1994; Osterhout *et al*., 2005). In contrast, a repeated immobilization led to a prolonged induction of TH protein associated with the induction of TH mRNA (Kvetnansky *et al*., 1996; Nankova *et al*., 1999; 2000). Opposite to the response to immobilization, TH mRNA levels and activity in adrenal medula after prolonged cold stress were no longer different from the values found in control animals (Kvetnansky *et al*., 1971; 2002). In earlier studies, relative levels of TH, DBH and PNMT mRNAs were determined by a conventional RT-PCR analysis, the method commonly used for quantification of the transcription. Quantitative assay based on real-time PCR with TaqMan probes, currently the most sensitive method for the detection of low-abundance mRNA eliminates post-PCR processing of the PCR products (Bustin, 2000).

The aim of the present work was to investigate the changes in catecholamine biosynthetic enzymes TH, DBH and PNMT gene expression and protein levels in the adrenal medulla of naive control and long-term (12 weeks) socially isolated rats and the response of these animals to additional immobilization stress (2 h), using quantitative real-time RT-PCR (qRT-PCR) assay.

MATERIAL AND METHODS

Animals

Adult Wistar rat males 11 weeks old maintained under standard laboratory conditions with water and food *ad libitum* in the groups of four individuals *per* cage were used. The care was taken to minimize the pain and discomfort of the animals according to the recommendations of the Ethical Committee of the "Vinca" Institute, Belgrade, which are in accordance with the Guide for Care and Use of Laboratory Animals of the National Institute of Health, Bethesda, MD, U.S.A. One group of animals was subjected to social isolation with a single animal *per* cage for 12 weeks. After that, naive group-housed controls and the rats that suffered chronic isolation were exposed to acute immobilization stress for 2 h (Kvetnansky and Mikulaj, 1970). After 12 weeks of individual housing or 3 h after the termination of immobilization, the animals were decapitated, the adrenals excised, adrenal medulla dissected, frozen in liquid nitrogen and stored at 70 °C until analyzed.

RNA Isolation and cDNA synthesis

Total RNA was isolated using TRIZOL reagent (Invitrogen, CA, U.S.A.). Reverse transcription was performed using Ready-To-Go You-Prime Frst-Strand Bead (AP, Biotech) and pd $(N)_6$ primer according to manufacturer's protocol.

Real-time RT-PCR

TaqMan PCR reactions were carried out using Assay-on-Demand Gene Expression Products (Applied Biosystems) for TH (ID:Rn00562500-m1), DBH (ID:Rn 00565819-m1) and PNMT(ID:Rn 01495589-g1). The gene expression assays contained primers for amplification of target gene and TaqMan MGB (Minor groove binder) probe 6-FAM dye-labelled for the quantification. Reactions were performed in 25 µl reaction mixture containing 1× TaqMan Universal Master Mix with AmpErase UNG, 1× Assay Mix (Applied Biosystems) and cDNA template (10 ng of RNA converted to cDNA). PCR reactions were 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

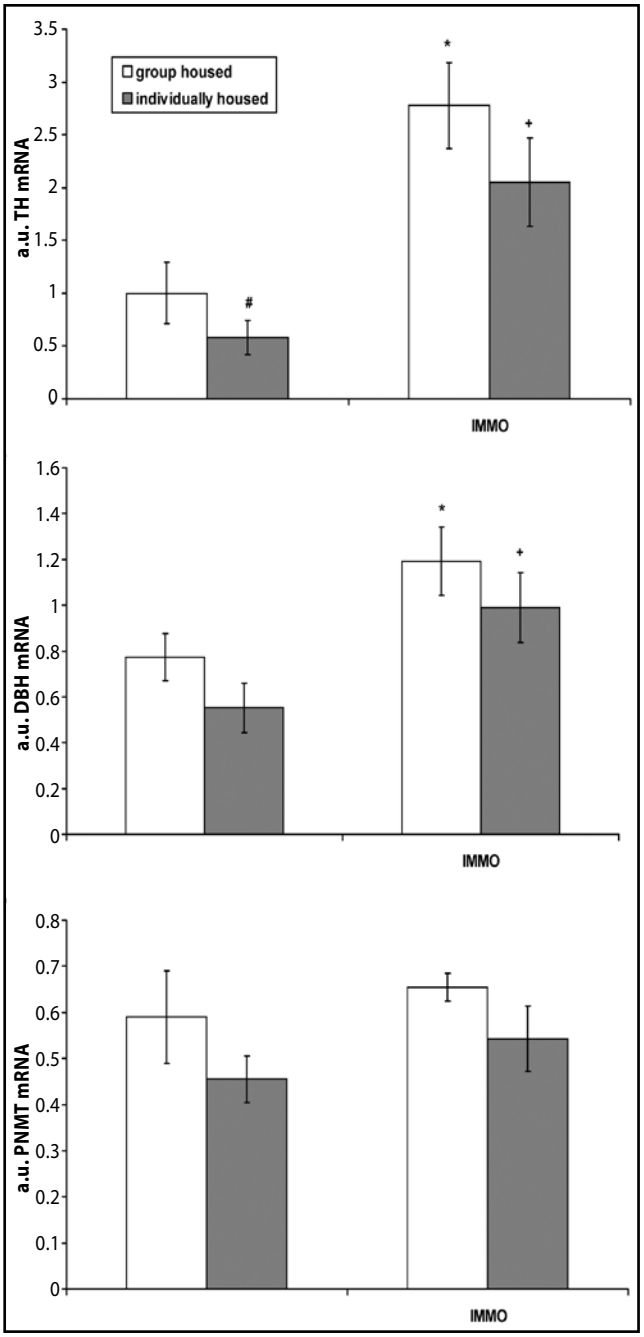

Figure 1. Effects of immobilization stress on adrenomedullary tyrosine hydroxylase (TH), dopamine-β-hydroxylase (DBH) and phenylethanolamine N-methyltransferase (PNMT) mRNA levels in group- and individually housed adult Wistar rat males. The values are means ±S.E.M. of 5–7 rats. Statistical significance: *$p<0.05$ 2 h immobilization *vs.* group-housed control (***t***-test); + $p<0.05$ long-term isolation *vs.* long-term isolation and additional 2-h-immobilization (***t***-test); # $p<0.05$ individually housed *vs.* group-housed control (***t***-test). The final result was expressed as fold change relative to the calibrator and normalized to GAPDH.

15 sec 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 amplification plot crosses 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 triplicates and the mean value of each Ct triplicate was used for further calculations. A reference, endogenous control, was included in each analysis to correct differences in the inter-assay amplification efficiency and all transcripts were normalized to GAPDH (glyceraldehyde-3-phosphate dehydrogenase) (ID:Rn 99999916-s1) expression. For the quantification, a validation experiments were performed to determine the relevant endogenous control for each target gene. We tested GAPDH and demonstrated that the efficiency of amplification was approximately equal for endogenous control gene and all target genes. Namely, serial dilutions of cDNAs were made and amplified by real-time PCR using specific primers and fluorogenic probes for target and endogenous control genes. Reaction mixture for endogenous control genes amplification was 1× TaqMan Universal Master Mix with AmpErase UNG (Applied Biosystems), 1× Assay (6-FAM dye-labelled MGB probes) and cDNA (10 ng of RNA converted to cDNA). The levels of expression of GAPDH in samples under different treatment were checked by additional experiments which confirmed that chosen reference gene was not regulated. Quantification was done using $2^{-\Delta\Delta Ct}$ method according to Livak and Schmittgen (2001). The obtained results were analyzed by RQ Study Add ON software for 7000 v 1.1 SDS instrument (ABI Prism Sequence Detection System) with a confidence level of 95% ($p \leq 0.05$). Relative expression of the target gene was expressed in relation to the calibrator, *i.e.* 1 control sample. Due to individual differences among animals, the sample of the control groups with the expression value close to the mean of all samples in the group and with the lowest error of measurement was used as a calibrator. The final result was expressed as fold change relative to the calibrator and normalized to GAPDH using the equation: $N_{sample} = 2^{-\Delta\Delta Ct}$.

Western blot analysis

Adrenal medulla were homogenized in 0.05 M sodium phosphate buffer (ph 6,65). Subsequently, the protein concentration was dtermined according to Stich (1990). Fifteen microgram of protein extract from adrenal medulla was separated by 10% SDS-polyacrylamide gel electrophoresis and then transferred to a supported nitrocellulose membrane (Hybond™ C Extra, Amersham Bioscience). 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 TBST at room temperature on a shaker. For measuring of TH protein levels a monoclonal primary antibody against mouse TH (monoclonal

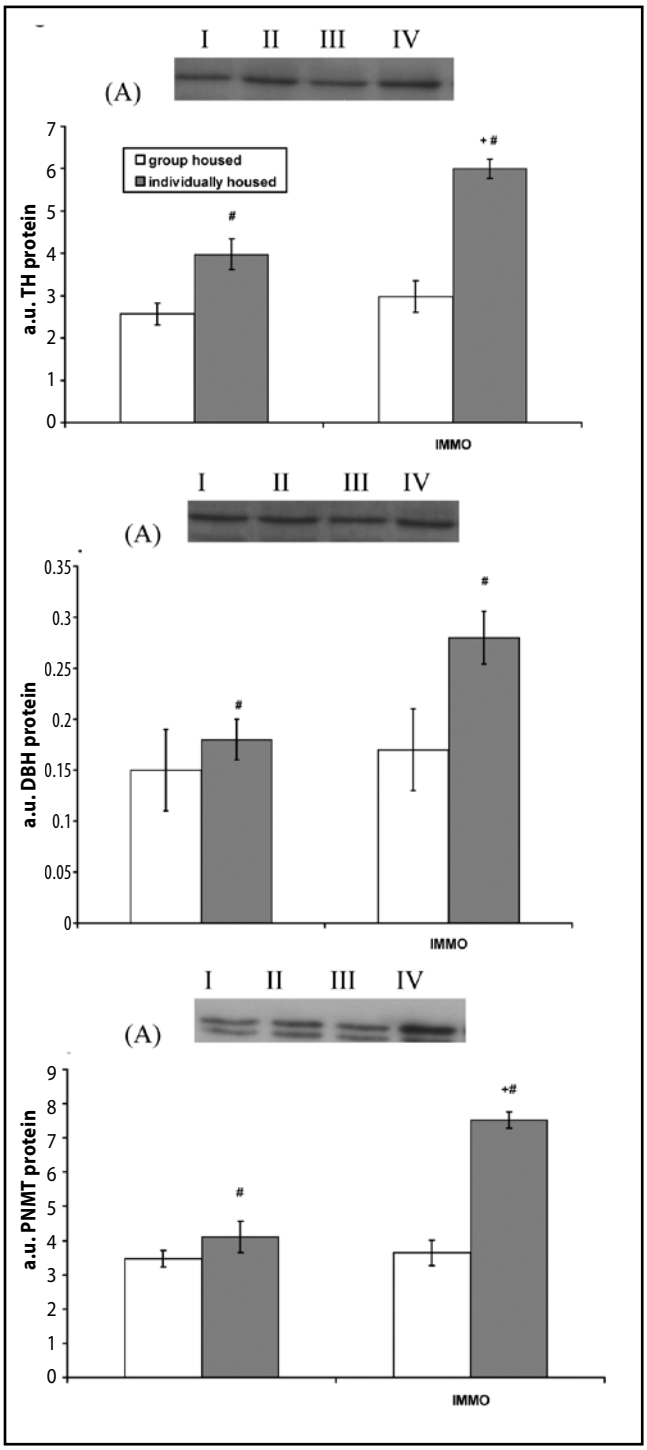

Figure 2. Effects of immobilization stress on adrenomedullary tyrosine hydroxylase (TH), dopamine-β-hydroxylase (DBH) and phenylethanolamine N-methyltransferase (PNMT) protein levels in group- and individually housed rats. (A) Distribution of TH, DBH and PNMT proteins in the adrenal medulla of group houded I, individually housed II, group housed+immobilization III and individually housed+immobilization IV rats mesaured by Western blot analysis. The values are means ±S.E.M. of 5-7 rats. Statistical significance: + $p<0.05$ long-term social isolation and additional 2-h-immobilization *vs.* long-term social isolation (***t***-test); # $p<0.05$ individually housed *vs.* group-housed control (***t***-test). The final result was expressed in arbitrary units normalized in relation to GAPDH.

antibody against TH from mouse-mouse hybrid cells, clone 2/40/15, dilution 1:5000), for measuring of DBH protein levels a anti-dopamine-β hydroxylase (N-terminal) antibody, human (dilutation 1:1000, Sigma) and for measuring of PNMT protein levels a polyclonal ant-PNMT primary antibody, rabbit (dilutation 1:1000, Protos Biotech Corporation, New York, USA) were used. After the membrane was washed, it was further incubated in the secondary anti-mouse antibody conjugated to horseradish peroxidase (dilution 1:5000, Amersham Bioscience). Secondary antibody was then visualized by the Western blotting enhanced chemiluminiscent detection system (ECL, Amersham Bioscience).

Statistical analysis

The results are reported as means ± S.E.M. Differences of gene expression and protein levels of catecholamine biosynthetic enzymes TH, DBH and PNMT in the adrenal medulla, were analyzed by one-way ANOVA. To test the effects of long-term isolation and acute immobilization stress compared to group-housed control, as well as the effects of acute immobilization stress in comparison with long-term isolation-pretreated animals, Student's ***t***-test was used.

RESULTS

One-way ANOVA analysis revealed significant variations of TH ($F=10.79$, $p<0.05$) and DBH ($F=4.19$, $p<0.05$) mRNA levels, as well as of TH ($F=80.6$, $p<0.05$), DBH ($F=17.58$, $p<0.05$) and PNMT ($F=80.6$, $p<0.05$) protein levels under the examined stress conditions. Chronic individual housing of adult Wistar rat males produced a decrease in TH, DBH and PNMT mRNA levels in the adrenal medulla, but only TH mRNA was significantly decreased ($p<0.05$, ***t***-test) in comparison with the group-housed controls. The exposure of group-housed rats to those exposed to acute immobilization for 2 h led to increased ($p<0.05$, ***t***-test) mRNA levels of TH and DBH by 177% and about 54%, respectively, while mRNA level of PNMT was increased only by 11%. Chronically socally isolated rats exposed to additional immobilization stress displayed a more significant rise in mRNA level ($p<0.05$, ***t***-test) of TH and DBH by some 253% and 79%. At the same time mRNA level of PNMT was increased only by 19% and the difference was stastistically insignificant as compared to the corresponding group-housed control (Fig. 1).

Protein levels of TH, DBH and PNMT were higher in long-term isolated rats by 55%, 20% and 18%, respectively, as compared to those found in the control group-housed animals. Acute immobilization (2 h) did not

change protein level of these enzymes in group-housed control animals. In contrast, this stress led to protein level increase of both TH and PNMT in long-term individually housed animals, while the increase of DBH protein level was statistically insignificant (Fig. 2).

DISCUSSION

It has been found recently that housing conditions affect the response of rats to chronic exposure to stress. Current findings demonstrate that social isolation may affect the ability of the adrenal medulla to synthesise catecholamines at the level of gene expression and protein level of catecholamine synthesising enzymes. Our results showed that chronic individual housing of the animals acted significantly decreasing TH mRNA levels and negligibly decreasing DBH and PNMT mRNA levels in the adrenal medulla. Due to a large standard error and limited sample size, the observed decrease of mRNA level of both DBH and PNMT was statistically insignificant. Selective depression of the TH mRNA level by stress is not without precedence in the literature for the catecholamine synthesising enzymes. For example, a single immobilization stress was shown to elevate TH mRNA level without affecting that of DBH (McMachon *et al*., 1992). Also, exposure to microgravity resulted in the inhibition of expression and specific activity of TH, but not of PNMT (Lelkes *et al*., 1994). It is a question how could we explain stress-related reduction of TH mRNA observed throughout the present study? Stress can activate a number of signaling pathways such as intracellular calcium, adenylyl cyclase and PKC (Zigmond *et al*., 1989; Thomas *et al*., 1997; Rolli *et al*., 1999). Also, it was shown that PKC-dependent phosphorylation induces TH gene transcription resulting in enhanced levels of TH mRNA (Stachowiak *et al*., 1990; Vyas *et al*., 1990). As early as in 1990, Gizang-Ginsberg and Ziff (1990) established that the elevation of TH activity by NGF was mediated by inducing *fos* and *jun* protooncogene transcripts, which act at the AP1 binding site in the 5′-flanking region of the TH gene. On the other hand, TH activation might also occur entirely independently of AP1, *e.g., via* cAMP, mediated by the binding protein (CREB) that activates gene transcription after phosphorylation by PKA. Recently, it was found that the signaling pathways mediating induction of TH and DBH gene expression by stress are tissue-specific (Sabban *et al*., 2004). Therefore, it could be hypothesised that the depression of TH expression in the adrenal medulla upon chronic social isolation observed here, might be attributed to the interference of psychological stress with some of the second messengers involved in the synthesis of catecholamines. Tai *et al*. (2007) reported that different kinds of stress influenced transcriptional mechanism. They found that seven daily immobilizations for 120 min significantly elevated both PNMT mRNA and PNMT protein levels, but protein stimulation did not attain highly elevated levels expected based on mRNA changes. These authors suggested initiation of adrenergic desensitisation to prolonged and repeated immobilization stress and/or dissociation of transcriptional and post-transcriptional regulatory mechanisms. The question arises is what might be the physilogical relevance of decreased TH gene expression in the adrenal medulla. The data from the available literature show that many of chronic stressors act by elevating gene expression of catecholamine synthesising enzymes (Tumer, 2002; Erdem *et al*., 2002). This sustained elevation in expression of catecholamine biosynthetic enzymes may be invilved in the increased risk of many diseases connected with stress (Micutkova *et al*., 2003). Thus, we suggest that decreased TH gene expression protects the organism from extraproduction of catecholamines during chronic psychosocial stress. Interestingly, we found that TH, DBH and PNMT protein levels were markedly elevated in individually housed rats. Similarly, Kvetnansky *et al*. (2002) recently observed that the levels of TH mRNA did not significantly differ from the control after long-term cold stress, while TH protein level was significantly elevated. They explained the dissociation between adrenal TH mTNA level and TH protein level after long-term cold exposure by the differences in their half-life. Thus, in chronically isolated rats, adrenal TH mRNA is reduced but prepared to be quickly elevated whenever it might be necessary, whereas TH protein remained increased.

Acute immobilization produced a significant increase in TH, and DBH mRNA levels in group-housed rats. Chronically isolated rats additionally exposed to immobilization displayed an exaggerated response comparing to control group-housed rats. However, PNMT mRNA level showed a certain increase but statistically insignificant in both groups. The levels of TH, DBH and PNMT protein were unchanged in group-housed animals exposed to immobilization. However, this kind of stress significantly elevated TH, DBH and especially PNMT protein levels in long-term isolated rats. These results corroborate recent data of Kvetnansky *et al*. (2002) who demonstrated that long-term stressed rats exposed to novel stressors exhibited exaggerated responses of adrenal gene expression of catecholamine biosynthetic enzymes. This could mean that prior experience may condition physiological systems to "expect" a problem and therefore, be more ready to respond to a subsequent stressor. These responses might be affected by the mechanisms acting directly at the level of transcriptional regulation in the adrenal medulla.

Based on these results, it may be concluded that psychosocial stress has a different influence on gene expression and protein levels of catecholamine biosynthetic enzymes in the adrenal medulla of adult rat males. The results indicate a possible adaptation of catecholamine-synthesizing system at the level of TH gene expression in adrenal medulla of chronically isolated animals.

Acknowledgement

This work was supported by the Ministry for Science, Technology and Environmental Protection of Serbia, Contract No. 143044B.

REFERENCES

1 Bartolomucci A, Palanza P, Sacerdote P, Ceresini G, Chirieleison A, Panerai AE, *et al* (2003). Individual housing induces altered immuno-endocrine responses to psychological stress in male mice. Psychoneuroendocrinol. **28:** 540–548.
2 Bustin SA (2000). Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays. J Mol Endocrinol. **25:** 169–193.
3 Erdem SR, Demirel HA, Broxson CS, Nankova BB, Sabban EL, Tumer N (2002). Effect of exercise on mRNA expression of select adrenal medullary catecholamine biosynthetic enzymes. J Appl Physiol. **93:** 463–468.
4 Gavrilovic L, Dronjak S (2005). Activation of rat pituitary-adrenocortical and sympatho-adrenomedullary system in response to different stressors. Neuroendocrinol Lett. **26**: 481–486.
5 Gavrilovic L, Spasojevic N, Dronjak S (2005). Novel stressors affected catecholamine stores in socially isolated normotensive and spontaneously-hypertensive rats. Auton Neurosci Basic Clin. **122**: 38–44.
6 Gizang-Ginsberg E, Ziff EB (1990). Nerve growth factor regulates tyrosyne hydroxylase gene transcription through a nucleoprotein complex that contains c-fos. Genes Dev. **4:** 477–491.
7 Hurst JL, Barnard J, Tolladay U, Nevison CM, West CD (1999). Housing and welfare in laboratory rats: effects of cage stocking density and behavioural predictors of welfare. Anim Behav. **58**: 563–586.
8 Ishida H, Mitsui K, Nukaya H, Matusumoto K, Tsuji K (2003). Study of active substances involved in skin dysfunction induced by crowding stress. I. Effect of crowding and isolation on some physiological variables, skin function and skin blood perfusion in hairless mice. Biol Pharm Bull. **26:** 170–181.
9 Kubovcakova L, Micutkova L, Bartosova Z, Sabban EL, Kriyanova EL, Kvetnansky R (2006). Identification of phenylethanolamine N-methyltransferase gene expression in stellate ganglia and its modulation by stress. J Neurochem. **97:** 1419–1430.
10 Kvetnansky R, Mikulaj L (1970). Adrenal and urinary catecholamines in rat during adaptation to repeated immobilization stress. Endocrinol. **8**: 1868–1874.
11 Kvetnansky R, Gewirtz GP, Weise VK, Kopin IP, (1971). Catecholamine-synthesizing enzymes in the rat adrenal gland during exposure to cold. Am J Physiol. **220:** 928–931.
12 Kvetnansky R, Nankova B, Hiremagalur B, Viskupic E, Vietor I, Rusnak M, *et al* (1996). Induction of adrenal tyrosine hydroxylase mRNA by single immobilization stress occurs even after splanchnic transection and in the presence of cholinergic antagonists. J Neurochem. **66:** 138–146.
13 Kvetnansky R, Jelokova J, Rusnak M, Dronjak S, Serova L, Nankova BB, *et al* (2002). Novel stressors exaggerate tyrosine hydroxylase gene expression in the adrenal medulla of rats exposed to long-term cold stress. In: McCarty R, Aguilera G, Sabban E, Kvetnansky R, editors. Stress: Neural, Endocrine and Molecular Studies. Taylor-Francis, London and New York. p. 121–128.
14 Kvetnansky R, Rusnak M, Dronjak S, Krizanova O, Sabban EL (2003). Effect of novel stressors on tyrosine hydroxylase gene expression in the adrenal medulla of repeatedly immobilized rats. Neuroch. Res. **28**: 625–630.
15 Kvetnansky R, Kubovcakova L, Tilinger A, Micutkova L, Krizanova O, Sabban EL (2006). Gene expression of phenylethanolamine N-methyltransferase in cotricotropin-releasing hormone knockout mice during stress exposure. Cell Mol Neutobiol. **26:** 735–754.
16 Lelkes PI, Ramos EM, Crick DM, Liu J, Unsworth B (1994). Microgravity decreases tyrosine hydroxylase expression in rat adrenals. The FASEB J. **8:** 1177–1182.
17 Livak KJ, Schmittgen TD (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2 (-Delta Delta C(T)) Method. Methods. **25**: 402–408.
18 McMahon A, Kvetnansky R, Fukuhara K, Weise VK, Kopin IJ, Sabban EL (1992). Regulation of tyrosine hydroxylase and dopamine β-hydroxylase mRNA levels in rat adrenals by single and repeated immobilization stress. J Neurochem. **58:** 2124–2130.
19 Micutkova L, Rychkova N, Sabban EL, Krizanova O, Kvetnansky R (2003). Quantitation of changes in gene expression of norepinephrine biosynthetic enzymes in rat stellate ganglia induced by stress. Neurochem Int. **1323**: 1–8.
20 Nankova B, Kvetnansky R, McMahon A, Viskupic E, Frankle G, Fukuhara K, *et al* (1994). Induction of tyrosine hydroxylase gene expression by a nonneural nonpituitary-mediated mechanism in immobilization stress. Proc Natl Acad Sci. U.S.A **91:** 5937–5941.
21 Nankova BB, Tank AW, Sabban EL (1999). Transient or sustained transcriptional activation of the gene encoding rat adrenomedullary catecholamine biosynthetic enzymes by different durations of immobilization stress. Neurosci. **94:** 803–808.
22 Nankova BB, Riviken M, Kelz M, Nestler EJ, Sabban EL (2000). Fos related antigen 2: potential mediator of the transcriptional activation in rat adrenal medulla evoked by repeated immobilization stress. J Neurosci. **20**: 5647–5653.
23 Osterhout CA, Sterling CR, Chikaraishi DM, Tank AW (2005). Induction of tyrosine hydroxylase in the *locus coeruleus* of transgenic mice in response to stress or nicotine treatment: lack of activition of tyrosine hydroxylase promoter activity. J Neurochem. **94**: 731–741.
24 Plaznik, A, Palejko W, Stefanski R, Kostowski W (1993). Open field behavior of rats reared in different social conditions: the effects of stress and imipramine. Pol J Pharmacol. **45:** 243–252.
25 Rolli M, Kotlyarov A, Sakamoto KM, Gaestel M, Neininger A (1999). Stress-induced stimulation of early growth response gene-1 by p38/stress-activated protein kinase 2 is mediated by a cAMP-responsive promoter element in a MAPKAP kinase 2-independent manner. J Biol Chem. **274:** 19559–19564.
26 Sabban EL, Nankova BB, Serova LI, Kvetnansky R, Liu X (2004). Molecular regulation of gene expression of catecholamine biosynthetic enzymes by stress: sympathetic ganglia *versus* adrenal medulla. Ann N Y Acad Sci. **1018:** 370– 377.
27 Stachowiak MK, Hong JS, Viveros OH (1990). Coordinate and differential regulation of phenylethanolamine N-methyltransferase, tyrosine hydroxylase and proenkephalin mRNA by neural and hormonal mechanisms in cultured bovine adrenal medullary cells. Brain Res. **510**: 277–288.
28 Stich TM (1990). Determination of protein covalently bound to agarose supports using bicinchoninic acid. Ann Biochem. **191**,: 343–346.
29 Tai TC, Claycomb R, Siddall BJ, Bell RA, Kvetnansky R, Wong DL (2007). Stress-induced changes in epinephrine expression in the adrenal medulla *in vivo*. J Neurochem. **101:** 1180–1118.
30 Thomas G, Haavik J, Cohen P (1997). Participation of a stress-activated protein kinase cascade in the activation of tyrosine hydroxylase in chromaffin cells. Eur J Biochem. **247:** 1180–1189.
31 Tumer, N., 2002. Regulation of tyrosine hydroxylase with age: modulation by cold and exercise. In: McCarty R, Aguilera G, Sabban E, Kvetnansky R, editors. Stress: Neural, Endocrine and Molecular Studies. Taylor-Francis, London and New York. p. 195–203.
32 Vyas S, Biguet NF, Mallet J (1990). Transcriptional and post-transcriptional regulation of tyrosine hydroxylase gene by protein kinase C EMBO J. **9**: 3707– 3712.
33 Weiss IC, Pryce CR, Jongen-Relo AL, Nanz-Bahr NI, Feldon J (2004). Effect of social isolation on stress-related behavioural and neuroendocrine state in the rat. Behav Brain Res. **152:** 279–295.
34 Zigmond RE, Schwartachild MA, Rittenhouse AR (1989). Acute regulation of tyrosine hydroxylase by nerve activity and by neurotransmitters *via* phosphorylation. Ann Rev Neurosci. **12:** 415–465.