



CHRONIC STRESS-INDUCED DEPRESSION-LIKE BEHAVIOUR OF RATS ACCOMPANIED BY MICROBIAL TRANSLOCATION THE BLOOD-BRAIN BARRIER AND PERSISTENT ACTIVATION THE INDUCIBLE NITRIC OXIDE SYNTHASE IN MITOCHONDRIA OF CORTICO-LIMBIC BRAIN

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Keywords: arginine, blood, brain, *Candida albicans*, chronic stress-induced depression, citrulline, gut, nitric oxide synthase, *Staphylococcus aureus*

Chronic stress may affect brain-gut axis and compromise blood-brain barrier (BBB) causing bacterial invasion and mounting nitric response that might be involved in stress-induced depression via direct impact on mitochondrial function in brain. The objectives of this study are to examine microbial composition in gut, blood and brain and assess a contribution of functionally different constitutive and inducible forms of nitric oxide synthase (cNOS and iNOS, respectively) to nitric oxide (NO) production in cytoplasm and mitochondria of cortico-limbic brain following chronic stress-induced depression. Depression-like behaviour of early adolescent male rats - resembling human depression - was developed by exposure to 2 weeks of chronic variable physical stress (CVS) (forced swimming, ether, restraint, cold, orthostatic shock and food deprivation). CVS induces in the rat gut a substantial enhancement the number of resident *Candida albicans* and manifestation of *Staphylococcus aureus* accompanied by a reduced number of obligate microbes immediately after CVS (stress group) and four days later (post-stress group). *S. aureus* and *C. albicans* were also detected in bloodstream and brain of CVS-treated rats. Simultaneously, the levels of L-arginine, L-citrulline and reactive nitrogen species were remarkably elevated in cytoplasm and mitochondria of the prefrontal cortex, striatum, hippocampus, and hypothalamus. We have found for the first time that CVS causes a persistent activation the iNOS in mitochondria of the mentioned brain regions, as well as in cytoplasm of the hypothalamus, while the cNOS activity was not significantly changed in mitochondria of the regions studied, with exception for the hypothalamic mitochondrial cNOS which reduced twice. We have also found a concurrent down-regulation the cNOS in cytoplasm of all the mentioned regions and a long-term up-regulation the iNOS in cytoplasm of the hypothalamus. CVS-induced opposite changes in the subcellular activity of distinct NOSs in cortico-limbic brain appear to be involved in the mitochondrial dysfunction leading to disturbances in neurotransmission, hemodynamic and energy impairment, pathophysiological pathways relevant to depression. We emphasize a role of gut microbiota in the persistent activation the mitochondrial iNOS in cortico-limbic brain and therefore the importance of origin the mitochondrial dysfunction involved in the pathological processes manifested during chronic stress-induced depression. This study would be helpful in understanding both brain-gut-microbiota communications and intracellular processes in the brain at depression, and therapeutic targeting of microbiome and nitric response might be included in future strategies in prevention and treatment of depression/anxiety.

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INTRODUCTION

Major depression is a common and sometimes fatal psychiatric disorder affecting up to 25 % of general population worldwide is a leading cause of disability worldwide.¹ A great body of evidence suggests a link for chronic stress with depression development.^{2,3} However, the mechanisms underlying the pathology of depression are still poorly understood, and at least one-third of cases are resistant to treatment with known antidepressants.⁴ Accumulating data suggest a bidirectional signalling between the gastrointestinal tract and the brain, the impact of the enteric microbiota on the brain gut-axis and a role for microbiota in the modulation of mood and behaviour reviewed elsewhere.^{5,6} Stress-induced disturbances in mucosal barrier function and mucin production are associated with increased microbial translocation the blood-brain barrier that may cause

depressive symptoms (anhedonia, anxiety-like behaviour, fatigue and somatic symptoms, e.g. mild cognitive impairment) by activating the cytokine network, oxidative and nitrosative stress pathways.⁷ The elevation of nitric oxide (NO) within the central nervous system is known to be associated with the pathogenesis of plenty of stress-induced diseases, including depression.⁸

Nitric oxide, a versatile molecule in signalling processes and unspecific immune defence is produced from L-arginine by functionally and pharmacologically distinguished forms of NO synthase (NOS): calcium-calmodulin-dependent constitutive NO synthases (cNOS), neuronal (nNOS) and endothelial (eNOS), involved in the regulation of neurotransmission and blood circulation, respectively, and calcium-calmodulin-independent inducible form (iNOS) contributed to the innate immune response.⁹ Recent findings suggest the presence in mitochondria constitutively active NOS (mtcNOS) which functionally couples with mitochondrial respiratory chain complex I, and NO produced by mtcNOS under physiological conditions reversibly inhibits oxygen

consumption and ATP synthesis.^{10,11} Mitochondria are involved in synaptic plasticity in the mature nervous system and abnormalities in mitochondria are associated with psychiatric and neurodegenerative diseases, suggesting a therapeutic potential for approaches that target mitochondrial mechanisms.^{12,13} Enhanced mitochondrial NO production with increased free radical generation, disrupted electron transport system and mitochondrial permeability transition have all been implicated in impaired mitochondrial function relevant to mental disorders.¹⁴ It is unknown, however, whether mitochondrial NOS make contributions to depression mechanism and how it is related to the iNOS and/or the cNOS. The studies cited in the review of Wegener and Volke¹⁵ demonstrate the effect of drugs modulating NO synthesis in anxiety and depression, affecting the different forms of NOS differently, but despite significant challenges in developing compounds which may differentially inhibit the 'right' form at the right place, NOS inhibition continue to be an interesting novel approach in the future development of antidepressants.

Chronic stress may compromise the blood-brain barrier (BBB) causing bacterial invasion and mounting nitric response that may affect mitochondria in brain tissues implicating in the pathology of depression. In the present study we aimed therefore to examine microbial composition of gut, blood and brain and assess the contribution of constitutive and inducible NOS forms to the production of NO in cytoplasmic and mitochondrial compartments of the major brain regions (hippocampus, hypothalamus, striatum and prefrontal cortex) following chronic stress-induced depression-like behaviour of rats.

Materials and Methods

N^G-monomethyl-L-arginine (NMMA) was obtained from Calbiochem (La Jolla, CA). All other reagents were purchased from Sigma Chemical Co (St Louis, MO).

Animals and study design

All procedures involving animals were approved by the respective Institutional Animal Care and Ethics Committee of the National Academy of Sciences the Republic of Armenia. For all experiments, groups ($n = 18/\text{group}$) of early adolescent, 35–48-day-old male Wistar rats weighing 100–140 g were used. Animals were housed at a constant temperature and humidity, and had unrestricted access to a standard diet and tap water. After overnight fasting, rats were subjected to chronic variable physical stress (CVS). Animals were divided into three groups: one group served as control and the other two as CVS-exposed groups studied immediately after CVS (stress group) and four days later (poststress group). Rats were sacrificed by a decapitation after behavioural testing.

Chronic variable stress. Animals underwent our CVS regimen for 14 days consisted some times of twice daily exposures to alternating stressors (forced swimming (FS), ether inhalation (EI), restraint stress (RS), cold stress (CS), orthostatic shock and food deprivation), once in the morning (8⁰⁰–12⁰⁰h) and once in the afternoon (13⁰⁰–18⁰⁰). In addition, animals were exposed to overnight food deprivation every night. Each stressor was applied

for 2–3 times and the same stressor was not applied continuously in 2 days so that animals could not predict the occurrence of stress. The CVS regimen included: 1st day – FS (20 min at 28°C); 2nd day – RS (2 h); 3rd day – EI and CS (4 h at 4 °C); 4th day – FS (20 min at 24 °C) and RS (2 h); 5th day – CS (4 h at 4 °C) and EI; 6th day – RS (4 h); 7 day - FS (20 min at 28 °C) and CS (3 h at 4 °C); 8th day - EI; 9th day – RS (4 h) and EI; 10th day - CS (5h at 4 °C); 11th day - FS (20 min at 24 °C) and EI; 12th day - food deprivation (24 h) and orthostatic shock (20 min); 13th day - EI and CS (5h at 4 °C); 14th day – RS (5 h).

FS was performed using a cylindrical container (height 50 cm, diameter 40 cm) filled with 30 cm water; temperature and time were varied under FS conditions. For the RS, rats were kept in plastic rodent restrainers completely restricted the lateral and front-to-back movements of the rat. Orthostatic stress was performed by placing rat in the plastic restraint tube upside down at a right angle to the horizontal surface.

Behavioural testing

Open field test. The rats were placed singly into an open field (diameter 1m, divided by 2 concentric circles into 16 equal sections on the floor of the arena) and observed in 5 min to measure locomotor activity (the number of sectors crossed with all paws (crossing), the number of rears (posture sustained with hind-paws on the floor), grooming (including washing or mouthing of forelimbs, hind-paws, body and genitals) (exploratory behaviour) and boluses (anxiety) counted manually/visually.¹⁶

Elevated plus-maze test. Immediately after the open field test the rats were placed singly into a common central platform (10 cm × 10 cm) of elevated plus-maze comprised two open and two closed arms (45cm x 10cm x 10cm) and elevated to a height of 80 cm above the floor. During a 5-min observation period, the following parameters were measured: number of open arms entries and number of closed arm entries. Percentage of the number of entries into the open arms of the total number of entries into all arms was calculated. Exploration (grooming and rearing) and risk assessment (number of hanging over the open arms) were also examined.¹⁷

At the end of each trial, the open field and elevated plus-maze were wiped clean with ethanol.

Microbiota. Each animal was opened aseptically; samples of feces from the lower part of the gut and brain washout were immediately placed into an anaerobic chamber for bacteriological analysis. Samples of blood were obtained from rats following decapitation. For identification light microscopy and/or the culturing method were used for all samples. Cultures were incubated in sucrose broth at 37 °C for 24 h (blood was diluted by 1:5 v/v). Thereafter, cultures were examined by microscopy and inoculated to the solid culture media, agar plates (Endo, sucrose, and blood agar) and incubated for 24 h; blood samples incubated for 5 days to facilitate a growth of microbes daily monitored. The characteristics (such as morphology and colour) of the colonies, as well as haemolysis, plasma-coagulation, aerobic fermentation of mannitol were examined for identification of microorganisms. Enterobacteriaceae and other isolates were identified by established procedures.^{18,19}

Isolation of cytoplasm and mitochondria. Brains were rapidly removed from the skulls, placed on cold plate, and prefrontal cortex, striatum, hippocampus and hypothalamus were dissected and homogenized in ice-cold 20 mM HEPES buffer pH 7.4, containing 0.25 M sucrose, (1:10, w/v) using Potter homogenizer (1500 rpm for 3 min). Homogenates were centrifuged at 3000 rpm for 10 min to remove nuclear fraction. Supernatants were collected, centrifuged at 15000 rpm for 20 min, and cytoplasm in the supernatants and mitochondria in the pellets were obtained. Mitochondria were washed twice with the above mentioned buffer.

Nitric oxide synthase assay. Total NOS activity was assessed by measuring nitrite (NO₂⁻) stable intermediate of NO, accumulating during a long-term incubation of samples (at 37 °C for 24 h) in 20 mM HEPES buffer, pH 7.4, containing 2 mM dithiothreitol and 3 mM MgCl₂·6H₂O, in the presence of 1.7 mM CaCl₂, 5.5 mM L-arginine·HCl, and NOS cofactors: 0.2 mM NADPH, 6 μM FAD, 5.5 μM FMN, 20 μM ((6R)-5,6,7,8-tetrahydro-L-biopterin dihydrochloride). The iNOS activity was determined in separate experiments with 5 mM EDTA included in the incubation medium. Control experiments were conducted in the presence of 5 mM NMMA, a non-selective inhibitor of NOS activity. The cNOS activity was calculated by subtracting the iNOS activity from the NOS total activity. Reaction was initiated by addition of samples to the incubation medium and terminated by deproteinization with 0.5 N NaOH and 10 % ZnSO₄. Following a centrifugation (15,000 x g, 3 min) the supernatants were sampled and analysed for nitrite content to determine the NOS activity expressed as nitrite produced in 24 h per mg of total protein.

Measurement of nitrite. Protein-free samples, obtained by deproteinization of brain tissue samples with 0.5 N NaOH and 10% ZnSO₄ and following centrifugation (vide supra) were analysed for nitrite (a stable intermediate of NO) using colorimetric technique based on diazotization. Samples were mixed in equal parts with Griess-Ilosvay reagent (1:1 mixture of 0.17 % sulfanilic acid and 0.05 % α-naphthylamine in 12.5 % acetic acid), and measured at 546 nm. The measured nitrite content was corrected with the background values found in the samples without tissue.

Measurement of L-arginine. Protein-free supernatants obtained by deproteinization of the samples with 0.5 N NaOH and 10% ZnSO₄ and following centrifugation (vide supra) were tested for L-arginine by the method of Akamatsy & Watanabe²⁰ with our modification. Briefly: a sample of supernatant diluted with distilled water (1:1, v/v) was added in a ratio 3:1 (v/v) to a mixture of equal amounts of 0.4 % 8-oxychinoline in 96 % ethanol, 5 % sulfosalicylic acid in 0.01 M glycine buffer, 2.5 % NaOH, and then added 1 % solution of sodium hypobromite stirred and the absorbance was measured at 525 nm spectrophotometrically.

Measurement of L-citrulline. L-citrulline was measured by the modified method of Moore & Kauffman.²¹ Briefly: brain tissue homogenates were deproteinated with 10 % TCA, centrifuged at 15,000 x g,

5 min, and supernatants were added in a ratio 1:2 (v/v) to a mixture of equal amounts of 9.6 % H₂SO₄ and DAMO reagent (5 mM diacetylmonoxime (DAMO), 0.9 mM thiosemicarbazide, and 0.025 mM FeCl₃) heated in a boiling water bath for 10 min, cooled to the room temperature and the absorbance was measured at 490 nm spectrophotometrically.

Protein was determined by the method of Lowry, using crystalline bovine serum albumin as standard.²²

Statistical analysis. Data are expressed as the mean ± S.E.M. All data were analysed using a one-way analysis of variance (ANOVA) followed by post hoc Holm-Sidak method) (SigmaStat 3.5 for Windows). Differences are considered significant at *P* < 0.05.

RESULTS

We have developed a rat model of CVS-induced depression (presented in *Materials and Methods*). Depression-like behaviour of rats was observed immediately after CVS (stress group) and four days later (post-stress group) (Tabl. 1, 2). The results of behavioural testing show that CVS-exposed rats display a decreased locomotor activity in the open field, as well as a reduced total number of entries into all arms in elevated plus-maze, that also probably related to motor activity.²³ This symptom characterizes depressive illness - mimicking human psychomotor retardation.²⁴ CVS caused a decrease in number of rearing and grooming relevant to exploratory behaviour and may indicate a “refractory loss of interest” another major symptom of depression.²⁵

Table 1. Rat behavioural activity in the open field following chronic variable stress

Groups	Locomotor activity	Rearing	Grooming	Defecation boli
Control	16.6 ± 2.8	6.8 ± 0.8	2.8 ± 0.6	2.9 ± 0.5
Stress	1.3 ± 0.4***	1.2 ± 0.3***	1.0 ± 0.2***	2.3 ± 0.4***
Poststress	6.1 ± 1.6***	1.5 ± 0.4***	1.5 ± 0.5***	2.8 ± 0.3#
	<i>p</i> ' < 0.001	<i>p</i> ' = 0.11	<i>p</i> ' = 0.002	<i>p</i> ' < 0.001

M ± SD (n=18). # *p* > 0.05, * *p* < 0.05, ** *p* < 0.01, *** *p* < 0.001 (compared to control); *p*' (compared to stress group).

In addition rats exposed to stressful stimuli showed aborted acts of grooming, freezing in the closed arm and remarkable drop in the number of hanging from an enclosed arm in risk-assessment in the elevated plus-maze. These symptoms indicate also a development of anxiety resembling human anxiety/depression.

Microbiome changes in the rat model of CVS-induced depression

As shown in Tables 3 and 4, gut microbiota in CVS-exposed rats showed significantly higher (compared to control) number of *Candida albicans* and *Staphylococcus aureus*, the latter was only detected in the gut of stressed animals. At the same time the numbers of *Lactobacilli* and *Bifidobacteria* were reduced in both CVS-groups, as well as the number of *E. coli* that dropped drastically, and *E. coli* only represented by single colonies of lactose-negative strains.

Table 2. Rat behavioural activity in the elevated plus-maze following chronic variable stress

Groups	Rearing	Griming	Number of entries into the open arms	Total number of entries	Number of hanging over the open arms	Defecation boli
Control	6.3 ± 0.5	1.9 ± 0.2	1.6 ± 0.8	2.9 ± 0.5	4.9 ± 0.7	1.5 ± 0.4
Stress	2.3 ± 0.4***	3.3 ± 0.3***	0.2 ± 0.1**	1.0 ± 0.2***	0.5 ± 0.2***	0.3 ± 0.1***
Poststress	2.1 ± 0.2***	0.25 ± 0.05**	0.25 ± 0.1**	1.2 ± 0.5***	0.75 ± 0.3***	1.1 ± 0.3***
	$p^*=0.13$	$p^*<0.001$	$p^*=0.75$	$p^*=0.16$	$p^*=0.11$	$p^*<0.001$

M ± SD (n=18). # $p > 0.05$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ (compared to control); p^* (compared to stress group).

Table 3. Gut obligate microbiota in the rat model of CVS-induced depression

Microflora	Control	Stress CFU per g feces	Poststress
<i>Bifidobacteria</i>	(2.4±0.4)×10 ¹⁰	(1.8±0.6)×10 ⁷	(5.4±1.2)×10 ⁷
Anaerobic cocci	(4.1±0.3)×10 ¹⁰	(3.5±0.6)×10 ¹⁰	(4.5±0.9)×10 ¹⁰
Other anaerobes	(1.9±0.8)×10 ³	(2.1±0.5)×10 ³	(1.4±0.4)×10 ³
<i>Lactobacilli</i>	(6.3±0.8)×10	5.2±0.4	8.7±0.9
<i>E. coli</i>	(3.04±0.2)×10 ⁵	Single colonies	Single colonies
<i>Streptococcus fecalis</i>	(4.5±0.6)×10 ⁶	(5.5±0.9)×10 ⁶	(3.9±0.7)×10 ⁶
<i>Clostridium perfringens</i>	10 ³	10 ⁵	10 ⁵

Values are means ± S.D.; $P < 0.05$ vs. control (native rats). Note: for stressed rats data concern to lactose-negative strains of *E. coli*

Table 4. Gut resident microbiota in the rat model of CVS-induced depression

Microflora	Control	Stress CFU per g feces	Poststress
Haemolytic <i>E. coli</i>	ND	ND	ND
<i>Staphylococcus aureus</i>	ND	(7.2±0.9)×10 ⁷	(4.8±0.7)×10 ⁵
<i>Clebsiellapneumoniae</i>	ND	Single colonies	ND
<i>Candida albicans</i>	(1.8±0.3)×10 ³	(5.6±0.8)×10 ⁶	(2.6±0.4)×10 ⁵

Values are means ± S.D.; $P < 0.05$ vs. control (native rats). ND-non-detectable

In the bloodstream of the stress group, the number of *S. aureus* and *C. albicans* gained to $(1.9 ± 0.4) × 10^2$ and $(2.5 ± 0.6) × 10^5$ (CFU/ml), respectively, and single colonies the mentioned microbes were detected in brain. The microbial pattern of the blood for rats from post-stress was similar to stress-group, while in the brain a growth of *S. aureus* and *C. albicans* increased up to $(2.1 ± 0.5) × 10$ and $(3.5 ± 0.4) × 10^3$ (CFU/ml), respectively. The changes in the microbe compositions in gut and translocation the BBB observed should impact the metabolism and underlying physiological processes. We have found remarkable changes in the L-arginine-

dependent generation of NO responsible for efficient immune response and cytotoxicity of host cells to kill the invading pathogens.

NO synthase activity and reactive nitrogen species subcellular levels in the rat brain regions following CVS-induced depression-like behaviour

As shown in Fig 1, after CVS in the PFC of rats from stress group a ten-fold drop in the cNOS activity was observed in cytoplasm, and a simultaneous approximately thrice increase in the iNOS activity and of a 3.3-, and 1.5-fold in the nitrite content in cytoplasm and mitochondria as respectively compared to control. In post-stress group the activity of cytosolic cNOS increased, but not recovered to control values, while the iNOS activity decreased in cytoplasm, but in mitochondria continued to grow. Of note, the cNOS was not significantly changed in the PFC mitochondria in both CVS-exposed groups. The nitrite content also increased in the PFC by 4.2-, and 4.9 times in cytoplasm and mitochondria respectively compared with the control. Changes similar to the PFC subcellular nitregeric response observed were detected in limbic brain.

As shown in Figures 2-4, in cytoplasm of the striatum, hippocampus and hypothalamus of stress group of a 4.7-, 3.7-, and 2-fold decrease in the cNOS activity, and of 1.8-, 2.6-, and 2-fold decrease in that of poststress group respectively compared to control. Changes in the cNOS activity in mitochondria were negligible, except of a 3-2-fold decrease in the hypothalamic mtcNOS activity. Contrary to cNOS, the iNOS was markedly activated in cytoplasmic compartments of striatum, hippocampus and hypothalamus. Concurrently, the nitrite levels increased with time, and in post-stress group in cytoplasm and mitochondria up to 7.8-, and 6.5-fold in the striatum, and 11.2-, and 9.9-fold in the hypothalamus respectively compared to control rats. In stress group the RNS content was also elevated in the hippocampus up to 9 times in cytoplasm, and 8.1 times in mitochondria, while in post-stress group it decreased, but was however higher of control values.

L-arginine and L-citrulline subcellular levels in the rat brain regions following CVS-induced depression-like behaviour

As shown in Fig. 5, in the PFC from stress-group the L-arginine and L-citrulline levels were increased of 8.8-,

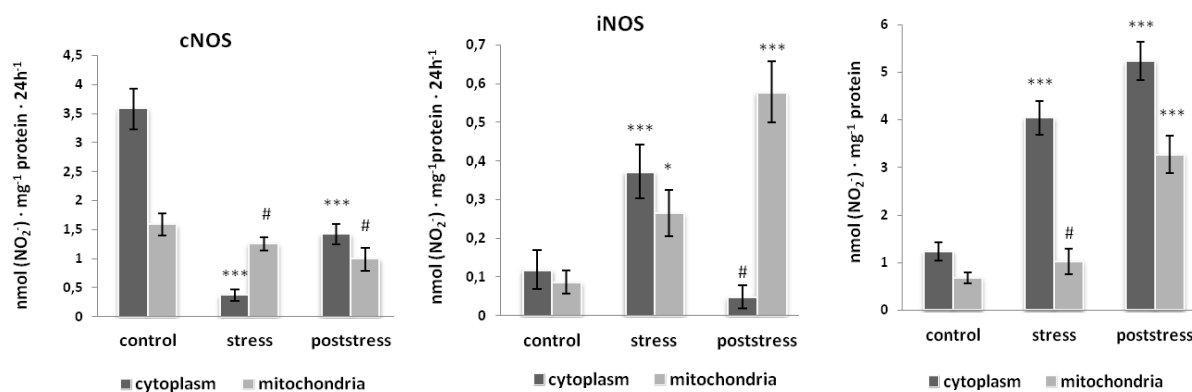


Figure 1. Subcellular changes in the constitutive (cNOS) and inducible (iNOS) NOS activities and the nitrite levels in the rat prefrontal cortex following CVS. Data are expressed as $M \pm SEM$, $n=18$, statistical comparisons made by one way ANOVA followed by the Holm-Sidak test. Differences are considered significant if $p < 0.05$. # $p > 0.05$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

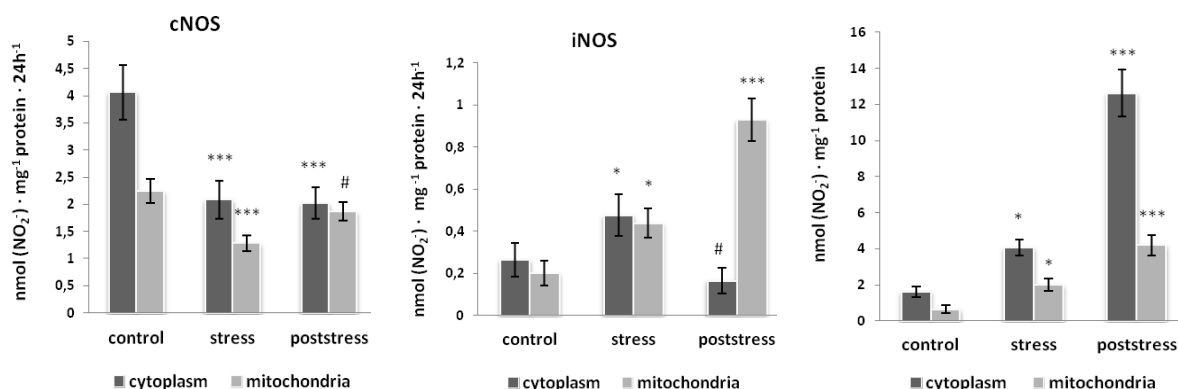


Figure 2. Subcellular changes in the constitutive (cNOS) and inducible (iNOS) NOS activities and the nitrite levels in the rat striatum following CVS. Data are expressed as $M \pm SEM$, $n=18$, statistical comparisons made by one way ANOVA followed by the Holm-Sidak test. Differences are considered significant if $p < 0.05$. # $p > 0.05$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

and 5.1-fold in cytoplasm, and of 5.9-, and 4.4-fold in mitochondria as compared to control. In the post-stress group the levels of L-arginine were by 2.6-, and 8.8 times higher in cytoplasm and mitochondria of the PFC respectively compared to control. L-citrulline content was reduced in the cytoplasm, but remaining higher upon control values by 2.4 times, while in mitochondria it continued to grow and was by 8.2 times higher than that of under physiological circumstances. In the striatum of stressed rats in both cytoplasm and mitochondria the L-arginine content increased by about twice in stress group, then 6 times in post-stress group; the L-citrulline level also increased by approximately 9 and 10 times respectively in stress and post-stress groups (Fig. 6). We have found that L-arginine content was increased of 2.9-, and 15.1-fold in cytoplasm and mitochondria of the hippocampus from stress group, and of a 9.3-, and 5.0-fold from post-stress group respectively compared to control. L-citrulline concentrations were also elevated in the hippocampus up to about 12-, and 5 times in cytoplasm and mitochondria respectively in poststress group (Fig. 7). In the cytoplasm and mitochondria of the hypothalamus of about a 10-, and 11-fold increase in the L-arginine level, and of 9- and 15-fold increase in the L-

citrulline levels were observed in the post-stress group (Fig. 8).

DISCUSSION

A matter of special relevance is that blood-brain barrier (BBB) structure or function may be affected, as is the case after stress exposure in animal models of depression or in humans with depression.²⁶ In our rat model of CVS-induced depression we have found in the digestive tract enhanced growth of such opportunistic and pathogenic bacteria and fungi as *S. aureus* and *C. albicans* that may markedly decrease the number of the beneficial bacteria in gut. Clinical cultures of *C. albicans* can cause desquamation of small fragments peptidoglycan layers of cell wall and total destruction of Lactobacilli cytoplasmic contents and growth reducing the number of beneficial bacteria in the gut flora.²⁷ Of interest, association of *C. albicans* with *S. aureus* occurs of 13-46 % in patients with intestinal dysbiosis.²⁸ Pathogenic microbes start digesting food in their own way producing large amounts of various toxic substances, which get absorbed into the blood stream, carried to the brain and cross the BBB causing different neurological and psychiatric

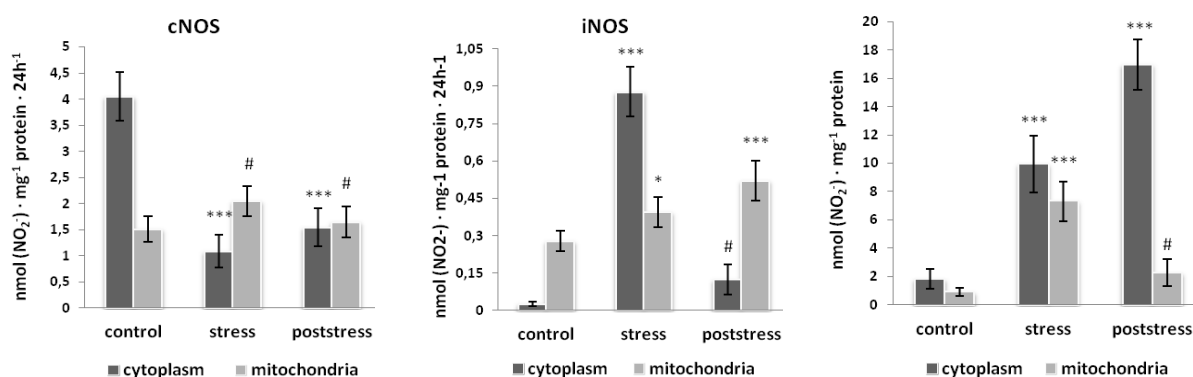


Figure 3. Subcellular changes in the constitutive (cNOS) and inducible (iNOS) NOS activities and the nitrite levels in the rat hippocampus following CVS. Data are expressed as $M \pm SEM$, $n=18$, statistical comparisons made by one way ANOVA followed by the Holm-Sidak test. Differences are considered significant if $p < 0.05$. # $p > 0.05$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

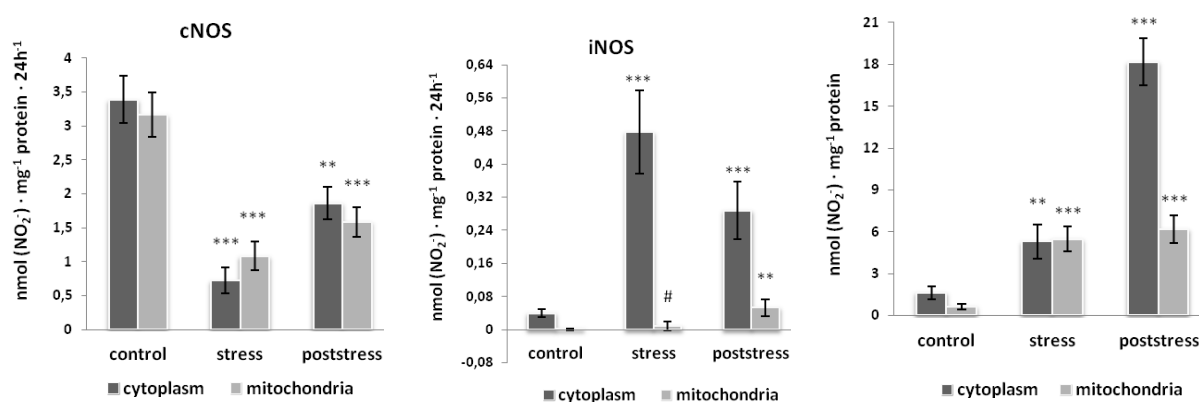


Figure 4. Subcellular changes in the constitutive (cNOS) and inducible (iNOS) NOS activities and the nitrite levels in the rat hypothalamus following CVS. Data are expressed as $M \pm SEM$, $n=18$, statistical comparisons made by one way ANOVA followed by the Holm-Sidak test. Differences are considered significant if $p < 0.05$. # $p > 0.05$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

symptoms.²⁹ Stress at the level of the CNS can also affect gut function and lead to perturbations of the microbiome. Chronic stress modulates the barrier function of the follicle associated epithelium, and in animal models exhibiting damage to the mucosal epithelium, indigenous bacteria translocate intercellular between the epithelial cells to directly access the blood.^{30,31} Our results show that both *S. aureus* and *C. albicans* appear in the bloodstream, penetrate the BBB and persist in the brain for up to 4 days after CVS. Recently, the ability of *S. aureus* to penetrate the CNS has been demonstrated.³² It has also long been known that *C. albicans* may cause certain manifestations occurred with such frequency as to suggest the diagnosis of yeast induced illness-mental depression, anxiety, and the abnormalities in brain function can be cleared completely with anti-yeast therapy.^{33,34} A method for treatment or prophylaxis of a bipolar disorder disease state, by administration of an anti-fungal composition is successfully used for depression treatment.³⁵

NO production by iNOS is essential for host defence against many pathogens, and microbial invasion observed in CVS-exposed rats may induce innate immune response by an up-regulation the iNOS in brain. Importantly, *S.*

aureus has been demonstrated to elicit immune functions in both microglia and astrocytes on the level of responses to Toll-like receptors-2 (TLRs)³⁶, which involve innate immune effector molecules including iNOS.³⁷ LPS from bacterial translocation is responsible, at least in part, for the TLR-4 activation found in the brain after chronic mild stress exposure which leads to the release of inflammatory mediators in the CNS and may play role in the depression-like behaviour of rats.³⁸ Similarly, depression-like behaviour of rats might be developed via microbe-induced enhancement of nitergic response with intracellular shift in the activities of different NOS forms in cortico-limbic brain.

In the present investigation we have shown for the first time a CVS-induced persistent activation of the brain mitochondrial iNOS probably contributed to the enhanced citrulline and RNS intracellular levels observed and could lead to persistent mitochondrial dysfunction and development depression-like behaviour. Our data are supported by findings that the chronic stress-induced depression-like behaviour is accompanied by an activation of iNOS in the hippocampus and its inhibition prevents depression.³⁹

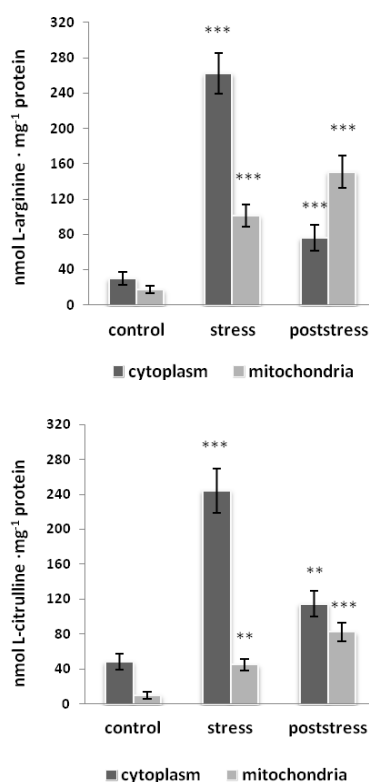


Figure 5. Subcellular changes in the levels of L-arginine and L-citrulline in the rat prefrontal cortex following CVS. Data are expressed as $M \pm SEM$, $n=18$, statistical comparisons made by one way ANOVA followed by the Holm-Sidak test. Differences are considered significant if $p < 0.05$. # $p > 0.05$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

It should be noted that we found cNOS and iNOS activities in cytoplasmic and mitochondrial compartments of the rat brain regions under physiological circumstances. Other authors findings^{40,41} also show that antibodies against the eNOS, as well as the nNOS or iNOS, showed positive immunoblotting in brain mitochondria and submitochondrial membranes, and iNOS is expressed constitutively at low levels in mouse motoneurons and Schwann cells could be another source of NO. The iNOS can produce NO for prolonged periods and contribute to excessive NO and a deleterious effects mediated by a potent oxidant, peroxynitrite which is readily formed from superoxide and NO produced together during the stress.⁴² Moreover, exposure of astrocytes in culture to low concentrations of NO over the 4-6 h induces an increased rate of superoxide generation unless changes in oxygen consumption, lactate or ATP content suggesting that NO-mediated partial mitochondrial electron transport chain (ETC) inhibition may initially cause oxidative stress rather than ATP depletion, and this may subsequently induce irreversible changes in ETC function providing the basis for a cycle of damage and mitochondrial dysfunction through a decrease in mitochondrial respiratory control and loss of membrane potential, probably mediated by free radical production.⁴³ In addition, human recombinant MnSOD and CuZnSOD were both inactivated when exposed to simultaneous fluxes of superoxide and NO.⁴⁴

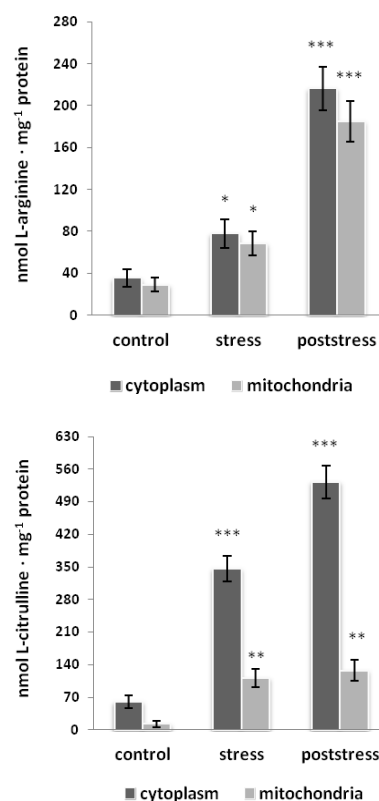


Figure 6. Subcellular changes in the levels of L-arginine and L-citrulline in the rat striatum following CVS. Data are expressed as $M \pm SEM$, $n=18$, statistical comparisons made by one way ANOVA followed by the Holm-Sidak test. Differences are considered significant if $p < 0.05$. # $p > 0.05$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

We have also found a CVS-induced elevation of the L-arginine levels in the cellular compartments of all the brain regions. Arginine could facilitate the activation of iNOS which in contrast with the cNOS is a high-output form strongly dependent on the presence of L-arginine and the availability of the intracellular arginine have proved is a rate-limiting factor in NO synthesis.⁴⁵ Argininosuccinate synthetase (ASS) and argininosuccinate lyase together can recycle the NOS reaction by-product, L-citrulline to L-arginine and are expressed constitutively in neurons, but hardly colocalise with each other or with NOS in the same neuron. Therefore, trafficking of citrulline and arginine between neurons necessitates transport capacities in these cells which are fulfilled by well-described carriers for cationic and neutral amino acids. In cultured astrocytes transcription and protein expression of arginine transport system y (+) and of ASS are up-regulated concomitantly with induction of iNOS. Any attempt to estimate the contributions of arginine transport and synthesis to substrate supply for NOS has to consider competition for arginine between NOS and arginase.⁴⁶ The two forms of arginase (AI and AII) are coexpressed in brain, AI has a cytoplasmic localization, whereas AII is present in the mitochondrial matrix, and these two arginases can access the cytoplasmic arginine pool and modulate iNOS function by means of substrate quenching.⁴⁷ The competition between iNOS and arginase for arginine can

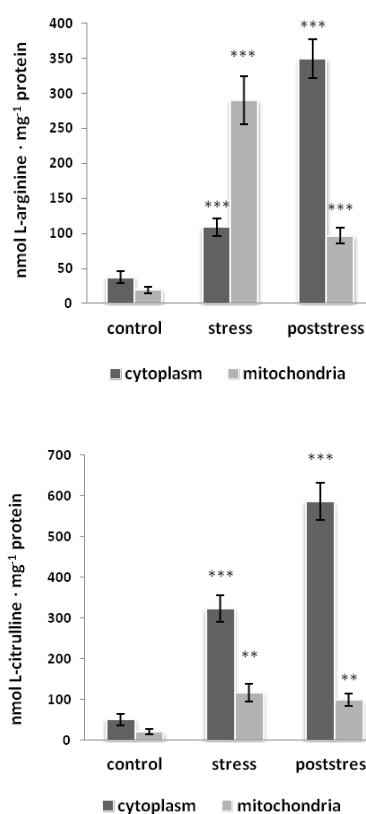


Figure 7. Subcellular changes in the levels of L-arginine and L-citrulline in the rat hippocampus following CVS. Data are expressed as $M \pm SEM$, $n=18$, statistical comparisons made by one way ANOVA followed by the Holm-Sidak test. Differences are considered significant if $p < 0.05$. # $p > 0.05$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

thus contribute to the outcome of several parasitic and bacterial infections.⁴⁸ Conversion of arginine to ornithine and urea via the arginase pathway can support the growth of bacterial and parasitic pathogens, at least via iNOS inhibition by urea.⁴⁹ In turn, N^G-hydroxy-L-arginine, a first intermediate in NO synthesis and a potent endogenous inhibitor of arginases, can accumulate sufficiently in iNOS-expressing cells to inhibit arginase activity.⁵⁰ Recently, it has been reported a significant decrease in the arginase activity and manganese level and elevated level of nitrite in plasma of patients with bipolar disorder.⁵¹ The iNOS might be contributed to an increase in the L-arginine levels in brain following CVS via inhibition of arginase in both mitochondria and cytoplasm.

It should be noted that arginine and lysine, a potent inhibitor of arginase⁵² suppress glutamatergic neuronal activity *in vivo* directly via block the ion channels or indirectly through activation of non-glutamate receptors or via adjacent inhibitory interneurons.⁵³ Elevated cytoplasmic arginine levels in brain regions may affect a creatine/phosphocreatine pool, because creatine transporters, localized on inner and outer mitochondrial membrane fractions are inhibited by arginine (and to some extent also by lysine) but not by other creatine analogues and related compounds.⁵⁴ Moreover, NO may potentially de-energise mitochondria via inhibition of creatine kinase, as well as such mitochondrial enzymes as aconitase and cytochrome c oxidase.⁵⁵ Acute arginine

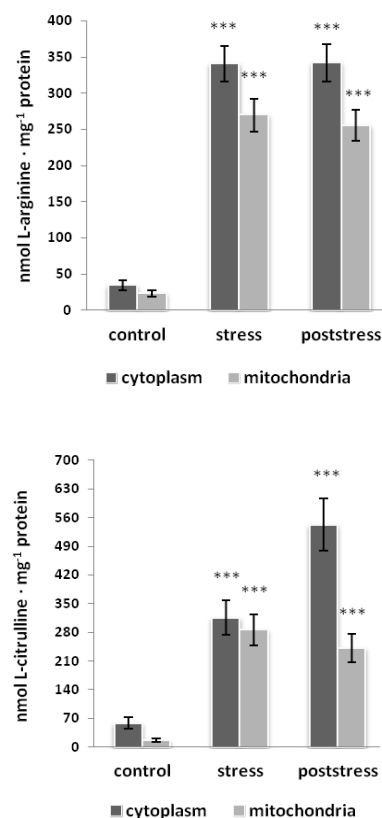


Figure 8. Subcellular changes in the levels of L-arginine and L-citrulline in the rat hypothalamus following CVS. Data are expressed as $M \pm SEM$, $n=18$, statistical comparisons made by one way ANOVA followed by the Holm-Sidak test. Differences are considered significant if $p < 0.05$. # $p > 0.05$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

administration induces oxidative stress and compromises energy metabolism in rat hippocampus and the inhibition of Na(+),K(+)-ATPase activity caused by this amino acid was probably mediated by NO and/or its derivatives ONOO(-) and/or other free radicals.⁵⁶ Notably, the reduction of creatine kinase activity in cerebellum of rats caused by arginine was probably mediated by NO and/or its derivatives peroxynitrite and other reactive oxygen species.⁵⁷ All these processes leading to mitochondrial dysfunction and energy impairment are involved in the pathophysiology of some psychiatric disorders like depression, bipolar disorder etc.¹³

At the same time, CVS down-regulated the hypothalamic mtNOS and cytoplasmic cNOS in all the brain regions studied. It is not excluded also that iNOS-derived NO may inhibit cNOS through a formation of stable inhibitory ferrous nitrosyl complexes in case of iNOS it appears weak.^{58,59} Inhibition of cNOS, i.e. nNOS and eNOS could affect central release of neurotransmitters and messengers and cerebral haemodynamics, respectively.^{9,60} Pharmacologic studies suggest that administration the nNOS inhibitors could reduce locomotor activity in rodents.⁶¹ Moreover, NO produced by nNOS inhibits neural progenitor cells neurogenesis in the subgranular zone of the dentate gyrus (generate neurons in the hippocampus),⁶² whereas, NO from iNOS may stimulate neurogenesis.⁶³ Pathogen recognition by a particular TLR results in a cascade of events starting with the activation of the nuclear

(transcription) factor-kappaB (NF-κB) signalling system and resulting in increased cytokine production and T cell activation.⁶⁴ cNOS-derived NO stabilizes inhibitory complex NF-κB thereby reducing the release of proinflammatory cytokines, while iNOS/NO counteracted the process.⁶⁵ Opposite changes in the activity of distinct NOSs following CVS-induced depression, reflected differences in their contribution to the pathological processes involved in depression.

Conclusion

Our findings indicate that depression-like behavior of rats exposed to CVS is accompanied by significant changes in microbial composition of the gut, a reduced number of obligate beneficial microbes and enhanced growth of opportunistic pathogens *S. aureus* and *C. albicans* and their translocation the BBB that might be a likely cause of compromised L-arginine metabolism in cytoplasmic and mitochondrial compartments of the brain regions involved in chronic stress response. We have shown for the first time that CVS-induced depression-like behaviour of rats accompanied with a persistent activation the iNOS in mitochondria of the cortico-limbic brain regions, and in cytoplasm of the hypothalamus, while the cNOS activity in mitochondria was not significantly changed, with exception for the hypothalamic mtcNOS which reduced twice. We have found a concomitant down-regulation cNOS in cytoplasm of all the brain regions studied and a long-term up-regulation iNOS in cytoplasm of the hypothalamus in CVS-treated rats. Opposite changes in the subcellular activity of distinct NOSs appear to be involved in the metabolic perturbation in L-arginine metabolism and mitochondrial dysfunction leading to disturbances in neurotransmission, hemodynamic and energy impairment, pathophysiological pathways relevant to depression. We emphasize a role of gut microbiota in the persistent activation the mitochondrial iNOS in cortico-limbic brain and therefore the importance of origin the mitochondrial dysfunction involved in the pathological processes manifested during chronic stress-induced depression. This study would be helpful in understanding both brain-gut-microbiota communications and related intracellular processes in the brain at depression, and therapeutic targeting of microbiome and nitric response might be included in future strategies in prevention and treatment of depression/anxiety.

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Received: 17.01.2013.

Accepted: 15.02.2013.