Biologia Serbica, 2017, 39(2): 43-51

DOI 10.5281/zenodo.1068961

Original paper

Psychophysical stress disturbs expression of mitochondrial biogenesis markers in hypothalamus and adenohypophysis

Isidora M Starovlah, Sava M Radovic, Maja A Marinovic, Tatjana S Kostic, Silvana A Andric*

Laboratory for Reproductive Endocrinology and Signaling (LaRES), Faculty of Sciences, University of Novi Sad, Novi Sad, Serbia

Received: 23 August 2017 / Accepted: 9 September 2017 / Published online: 30 November 2017

Summary. Although psychophysical stress is widespread in human society and a major contributor to a range of pathological conditions, it is not known if this form of stress regulates mitochondrial biogenesis in the hypothalamus or adenohypophysis, the main organs involved in compensatory specific response of the organism to stress (so called "fight or flight" response). Accordingly, this study was designed to evaluate the effects of acute and repeated psychophysical stress on a profile of mitochondrial biogenesis markers in the hypothalamus and adenohypophysis. Rats were either left undisturbed (freely moving, control group) or exposed to psychophysical stress by immobilization (IMO) for 2 h (acute, 1xIMO) or 2 h daily for 2 (repeated, 2xIMO) or 10 consecutive days (repeated, 10xIMO). Result suggest that all types of immobilization stress significantly increase expression of hypothalamic NRF1 (nuclear respiratory factor 1; acts on the genes for subunits of the OXPHOS encoded by the nuclear genome) as well as its downstream target TFAM (mitochondrial transcription factor A), the essential ubiquitous factors for mtDNA replication and expression. In the same samples, TFB1M (markedly enhance mtDNA transcription) significantly decreased, while the level of COX4 (mitochondrial complex IV cytochrome C oxidase) protein was reduced only in hypothalamuses isolated from repeatedly stressed rats. Independently of the type of stress, the level of PGC1 protein, the master regulator of mitochondrial biogenesis involved in transcriptional control of all processes related to mitochondrial homeostasis and integrator of environmental signals, remained unchanged. In adenohypophyses of the same animals, 10xIMO significantly increased expression of adenohypophyseal PGC1 as well as its downstream target TFB1M, while NRF1 and TFAM remained unchanged. Similarly to hypothalamuses, the level of COX4 protein was reduced in adenohypophyses isolated from repeatedly stressed rats. Our results provide new molecular insights into the relationship between stress and hypothalamo-adenohypophyseal axis, as well as mitochondrial biology.

Key words: adenohypophysis, hypothalamus, males, mitochondrial biogenesis markers, stress.

INTRODUCTION

Stress is defined as a complex of protective responses of the organism, generated in the process of evolution, which occur as a result of neuroendocrine and metabolic alterations in response to the impact of emergency or pathological factors. We are born in stress, we are dying in stress and stress has become a part of our everyday life. As a medical and scientific idea, stress was introduced and popularized by Selye. According to Selye: "Without stress, there would be no life" (Selye 1936). "Adaptability and resistance to stress are fundamental prerequisites for life, and every vital organ and function participates in them" (Selye 1995). In the modern era, stress is established as a unifying concept to understand the interaction of an organism with the environment and occurs when homeostasis is threatened or perceived to be so (Chrousos 2009). To sustain homeostasis, during stress, an orchestrated adaptive compensatory specific response of the organism (so called "fight or flight" response) is activated, including activation of the sympathoadrenal, sympathoneuronal systems and the hypothalamo-pituitary-adrenocortical (HPA) axis, but suppression of the hypothalamic-pituitary-gonadal (HPG) axis. It is very well established that the main hallmarks of stress are increased levels of circulating stress mediators/hormones, including corticotropin-releasing hormone (CRH), adrenocorticotropin (ACTH), glucocorticoids (cortisol in humans, corticosterone i rats and mice) and the catecholamines (Kvetnansky et al.

*Corresponding author, e-mail: silvana.andric@dbe.uns.ac.rs

1970; Pacák and Palkovits 2001; Manoli et al. 2007; Chrousos 2009; Gesquiere et al. 2011; Pitman et al. 2012) as well as a decrease of circulating androgens in males (Kostic et al. 2010; Gak/Radovic et al. 2015). It is well recognized that stress is a major contributor to a wide variety of psychosocial and physical pathological conditions in humans. Our lifestyle in modern society seems to be especially conducive for development stress-related disorders (Chrousos 2009; Gesquiere et al. 2011; Pitman et al. 2012).

Stress affects every level of the functional organization of life. As a response, organisms have developed the capacity to initiate a number of adaptive response pathways that attempt to reduce damage and maintain or reestablish cellular homeostasis (Manoli et al. 2007; Chrousos 2009). It is well known that many epidemiological studies strongly indicate stress-induced β2-adrenergic-receptor-mediated DNA damage as a molecular mechanism underlying ageing, tumorigenesis and neuropsychiatric conditions (Hara et al. 2011). On the cellular level, mitochondria are key components of the stress response, since they are exclusively responsible for meeting the enormous energy demands of the 'fight or flight response' by oxidizing large amounts of substrates available by stress hormone-induced mobilization from energy storages (Manoli et al. 2007; Chrousos 2009). Mitochondria are highly dynamic organelles that undergo frequent and rapid fusion and fission events, as well as mitochondrial biogenesis and mitophagy, to maintain extremely well-organized networks, in response to cellular demands and environmental imperatives (Archer 2013; Palikaras and Tavernarakis 2014; Villa et al. 2017). During episodes of damage or periods of intensified energy demand, cells renew, adapt, or expand their mitochondrial population by mitochondrial biogenesis. This is an extremely complex network of sophisticated and multistep interplay of cellular and molecular processes regulated spatiotemporally by nucleo-mitochondrial interactions dependent on the interplay between transcription factors (NRF1, NRF2, PPARs, ERRα, CREB, and others) and members of the PGC1 family of regulated co-activators (PGC1α, PGC1β, PRC). As a consequence, many genes that specify the mitochondrial transcription-translation-replication machinery, protein import-assembly apparatus and the respiratory chain are activated. All these processes are controlled by a complicated network of signaling pathways (Nisoli et al. 2003, 2005; Scarpulla, 2008; Canto et al. 2009; Scarpulla et al. 2012; Dominy and Puigserver 2013; Palikaras and Tavernarakis 2014). Signaling pathways involved in regulation of mitochondrial biogenesis convey environmental signals including temperature (Puigserver et al. 1998; Wu et al. 1999), exercise and energy deprivation (Puigserver and Spiegelman 2003; Archer 2013; Wentz 2013; Palikaras and Tavernarakis 2014), availability of growth factors and nutrients (Rodgers et al. 2005; Grasfeder et al. 2009).

Although hypothalamus and pituitary are involved in response to stress of whole organism and mitochondria are involved in response to stress on all levels of functional organization, there is no evidence about profile of mitochondrial biogenesis markers in hypothalamus or adenohypophysis of stressed organism (Manoli et al. 2007; Chrousos 2009). The focus of our study was on the effect of acute and repeated psychophysical stress, most common stress in human society and most cited biomedical subject, on mitochondrial biogenesis markers profile in hypothalamus and adenohypophysis. To do this, we studied serum hormonal profiles, as well as expression of transcripts and proteins for mitochondrial biogenesis after IMO was applied once (1xIMO), twice (2xIMO), or 10 times (10xIMO). Our results are the first to profile mitochondrial biogenesis markers in hypothalamus and adenohypophysis of stressed organism and provide new molecular insights into the relationship between stress and hypothalamo-adenohypophyseal axis, as well as mitochondrial biology.

MATERIALS AND METHODS

Materials

Antibodies against PGC1 (sc-13067, sc-5816), NRF1 (sc-33771), mtTFA/TFAM (sc-28200), TFB1M (sc-169583), COX4 (sc-69361) and ACTIN (sc-1616) were purchased from Santa Cruz Biotechnology (Heidelberg, Germany). The anti-rabbit and anti-goat secondary antibodies linked to horse-radish peroxidase were obtained from Kirkegaard & Pery Labs (Gaithersburg, MD, USA). Anti-testosterone-11-BSA serum №250 for RIA was kindly supplied by Gordon D. Niswender, while (1,2,6,7^{3}H(N)) labeled testosterone was from Perkin-Elmer Life Sciences (Waltham, Massachusetts, USA). Corticosterone (CORT) EIA Kit was purchased from Cayman (Ann Arbor, MI, USA), whereas adrenaline research ELISA Kit was from Labor Diagnostika Nord (Nordhorn, Germany). TRIzol® reagent for total RNA isolation was purchased from Thermo Fisher Scientific Inc. (Waltham, Massachusetts, USA) while High-Capacity cDNA Reverse Transcription kit for cDNA preparation was obtained from Applied Biosystems Inc. (Foster City, CA, USA). Power SYBR Green PCR Master Mix was purchased from Applied Biosystems (Foster City, CA, USA), while primers for real time PCR were obtained from Integrated DNA Technologies (Munich, Germany). All other reagents were of analytical grade.

All methods employed for experiments included in the present study were previously reported by our group in more detail (for all references please see Gak/Radovic et al. 2015), and are only briefly outlined here.

Animals and ethical issue

Three-month-old (250–270 g) male Wistar rats, bred and raised in the Animal Facility of the Faculty of Sciences, University of Novi Sad, Serbia, were used for experiments.

Animals were raised in controlled environmental conditions (22 ± 2 °C; 12 h light/dark cycle, lights on at 07^{00} h) with food and water *ad libitum*. All experimental protocols were approved (statement no. 01-201/3) by the local Ethical Committee on Animal Care and Use of the University of Novi Sad operating in accordance with the National Research Council publication Guide for the Care and Use of Laboratory Animals and NIH Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80 23, revised 1996, 7th edition; www.nap.edu/readingroom/books/labrats). All the experiments were carried out in the Laboratory for Reproductive Endocrinology and Signaling, Faculty of Sciences, University of Novi Sad (http://www.dbe.uns.ac.rs/en/nauka-eng/lares).

Immobilization model of psychophysical stress

Rats were handled daily during a 3-week period of acclimation before experiments. Immobilization stress (IMO) was performed in the morning (from 08^{00} h to 10^{00} h) by the method of Kvetnansky (Kvetnansky et al. 1970) as described previously by our group (for references please see Gak/Radovic et al. 2015). Rats were divided into the following groups (each consisted of 5-8 animals depend of experiment): (1) Control group - freely moving (unstressed, C) rats; (2) 1xIMO - rats subjected to IMO for 2 h; (3) 2xIMO - rats subjected to repeated 2 h IMO for 2 consecutive days; (4) 10xIMO - rats subjected to repeated 2 h IMO for 10 consecutive days. At the end of IMO period all animals were quickly decapitated without anesthesia, trunk blood was collected and hypothalamuses and adenohypophyses (anterior pituitaries) were isolated and immediately stored at −80 °C. Serum samples were stored at −80 °C until they were assayed for adrenaline (ADR), corticosterone (CORT), and androgens (testosterone+dihydrotestosterone, T+DHT) levels. All experiments were repeated three times.

Hormone measurements

For serum androgen (T+DHT) levels all samples were measured in duplicate in one assay (sensitivity: 6 pg per tube; intra-assay coefficient of variation 5–8%). Androgens in serum are referred to as T + DHT, because the antitestosterone serum №250 showed 100% cross-reactivity with DHT (for references please see Gak/Radovic et al. 2015). For serum corticosterone levels, all samples were measured in duplicate, in a single assay by the corticosterone EIA Kit (www.caymanchem.com), with 30 pg/ml as the lowest standard significantly different from blank as described previously (for references please see Gak/Radovic et al. 2015). Levels of circulating adrenaline were also determined in duplicate (standard range of 0.45–45 ng/ml and detection limit of 3.9 pg/ml) using the adrenaline research ELISA Kit (www.ldn.de) as described previously (for references please see Gak/Radovic et al. 2015).

RNA isolation and cDNA synthesis

Total RNA samples from hypothalamuses and anterior pituitaries from control as well as stressed adult male rats were isolated using TRIzol® reagent following the protocol recommended by the manufacturer (www.thermofisher.com). Following DNase-I treatment, the first strand cDNA was synthesized according to the manufacturer's instructions (www.invitrogen.com). Quality of RNA and DNA integrity were checked using control primers for *Gapdh* as described previously (for references please see Gak/Radovic et al. 2015).

Real-time polymerase chain reaction and relative quantification

Relative gene expression was quantified by real time PCR using SYBR® Green-based chemistry from Applied Biosystems (www.appliedbiosystems.com) in the presence of specific primers (please see Supp. Table 1). *Gapdh* was also measured in the same samples and was used for variation corrections in RNA content between samples. Relative quantification of each gene was performed in duplicate, three times for each gene and twice for each of three independent *in vivo* experiments, as described previously by our group (for references please see Gak/Radovic et al. 2015).

Protein extraction and Western blot analysis

Western blot analysis was performed as described previously by our group (for references please see Gak/Radovic et al. 2015). Briefly, hypothalamuses and adenohypophyses were washed twice with ice-cold PBS and lysed in a 1 ml buffer containing 20 mM HEPES, 10 mM EDTA, 40 mM β-glycerophosphate, 1% tergitol, 2.5 mM $MgCl_2$, 1 mM dithiothreitol, 0.5 mM 4-(aminoethyl)-benzenesulfonyl fluoride hydrochloride, 20 μg/ml aprotinin, 20 μg/ml leupeptin, and a cocktail of phosphatase inhibitors (0.05 mM (-)-P-bromotetramisol oxalate, 10 μM cantharidin, and 10 nM microcystinLR; pH 7.5). Protein concentrations were estimated by the Bradford method using BSA as a standard (Bradford 1976). For Western blot analysis, equal amount of proteins were mixed (1:1 v/v) with the SDS-PAGE loading buffer, denatured for 5 min at 95 °C, and loaded on 16% SDS-PAGE gels. All gels were analyzed by one-dimensional SDS-PAGE, using a discontinuous buffer system, and proteins were transferred to a PVDF membrane, Immobilon-P (www.millipore.com), using a wet transfer, according to the manufacturer's recommendation. Blocking was done with 3% BSA, for 2 hours at room temperature. Antibodies against PGC1 (sc-13067, sc-5816), NRF1 (sc-33771), mtTFA/TFAM (sc-28200), TFB1M (sc-169583), COX4 (sc-69361) and ACTIN (sc-1616) were purchased from Santa Cruz Biotechnology (Heidelberg, Germany). For more details please

see Supp. Table 2. The anti-rabbit and anti-goat secondary antibodies linked to horseradish peroxidase were obtained from Kirkegaard & Pery Labs (Gaithersburg, MD, USA). The immunoreactive bands were analyzed as two-dimensional images using Image J (version 1.6.0_24; https://imagej.nih.gov/ij/download.html). The integrated optical density of images was used to quantify both area and the intensity of the immunoreactive bands (version 1.6.0_24; https://imagej.nih.gov/ij/download.html).

Statistical analysis

Results represent group mean ± SEM values of individual variation from three independent experiments. Results from each experiment were analyzed by Mann–Whitney's unpaired nonparametric two-tailed test (for two-point data experiments), or by one-way ANOVA for group comparison, followed by Student–Newman–Keuls multiple range test. Linear correlations were calculated using the program GraphPad Prism 5 (GraphPad Software Inc, La Jolla, CA, USA).

RESULTS

In order to mimic psychophysical stress, adult male rats were exposed to a previously established model of immobilization stress (IMO) (for references see Kvetnansky et al. 1970 and Gak/Radovic et al. 2015).

To confirm the effect of acute as well as repeated immobilization stress, concentrations of stress hormones (adrenaline and corticosterone) and androgens (T+DHT) were measured in serum obtained from undisturbed control as well as stressed animals. As shown previously (for references see Kostic et al. 2010 and Gak/Radovic et al. 2015) immobilization was followed with common "hallmarks" of stress: increased levels of circulating stress hormones (adrenaline and corticosterone) were accompanied by a decrease of androgens (T+DHT) levels (Fig. 1).

Psychophysical stress by immobilization alters the expression profile of mitochondrial biogenesis markers in hypothalamus

To determine whether the profile of expression of mitochondrial biogenesis markers is changed in hypothalamuses of stressed rats, the expression of transcripts and proteins for several main mitochondrial biogenesis markers were examined in hypothalamuses isolated from control as well as stressed male rats. The results show (Fig. 2) that psychophysical stress by immobilization altered expression profile of transcripts (Fig 2A) as well as proteins (Fig. 2B) for main markers of mitochondrial biogenesis and functionality in hypothalamus.

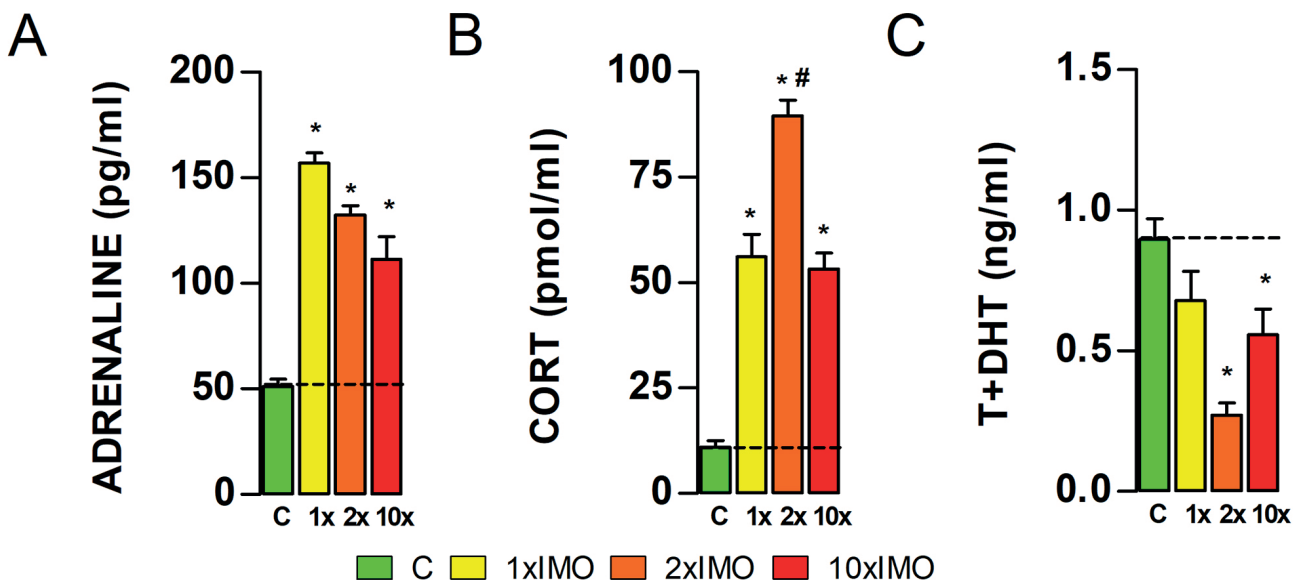
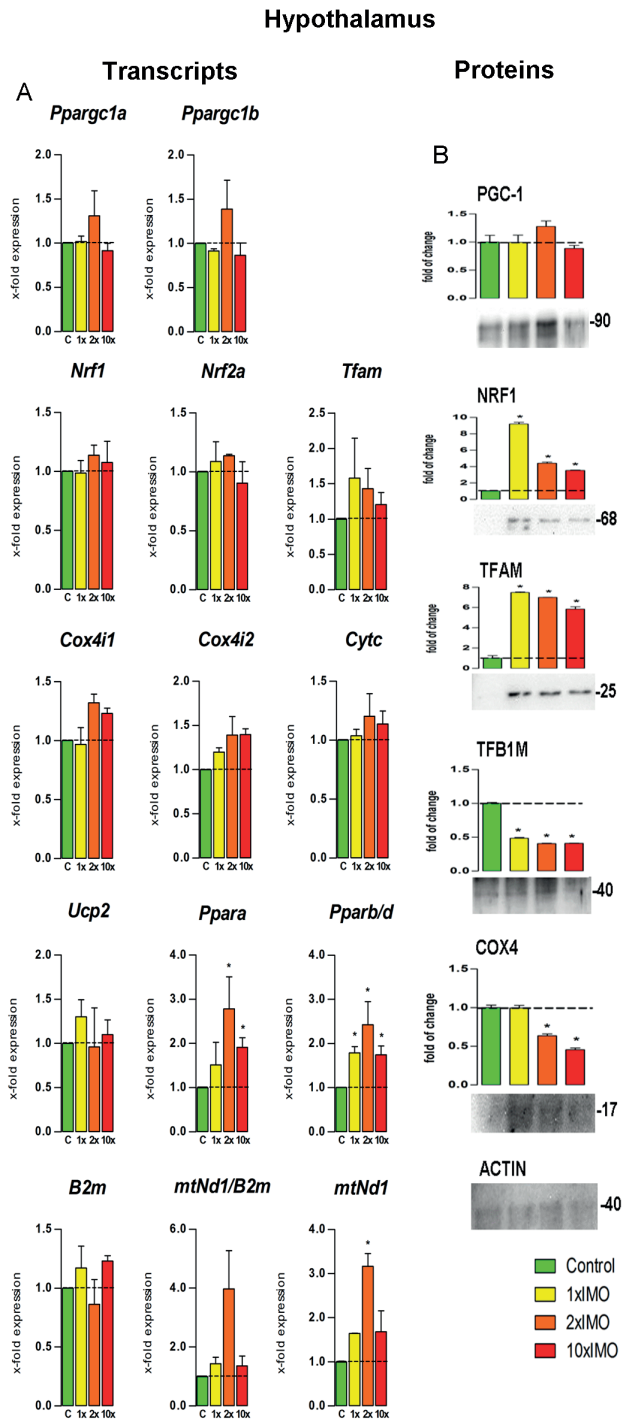


Fig. 1. Immobilization was associated with common "hallmarks" of stress: increased circulating stress hormones (adrenaline and corticosterone) and decreased androgen hormones. A-C: Stress-induced increase of circulating adrenaline **(A)** and corticosterone **(B)** levels were accompanied with a decrease of androgens (T+DHT) levels **(C)**. In this and following figures, rats were left undisturbed (Control) or subjected to immobilization stress (IMO), once for 2 hours (1xIMO), twice for 2 hours IMO for 2 consecutive days (2xIMO), 2 hours IMO for 10 consecutive days (10xIMO). At the end of the IMO period, trunk blood was collected for hormonal analysis and hypothalamus and anterior pituitary were isolated. Androgen (T+DHT) levels were determined by RIA, while adrenaline and corticosterone levels were determined by EIA. (Data bars are mean ± SEM values of five to seven independent *in vivo* experiments. Statistical significance was set at level $P < 0.05$: * vs. control group; # vs. 1xIMO group.)

With respect to transcription profiles in hypothalamus (Fig. 2A), only repeated IMO significantly increased transcript levels for for *Ppara* (peroxisome proliferator-activated receptors alpha) in hypothalamus, whereas level of *Ppard* increased independently of the type of the stress. In addition, a significant increase of the transcript for *mtNd1* was recorded in hypothalamuses isolated from 2xIMO rats. *mtNd1* encodes for the core subunit of the mitochondrial membrane respiratory chain NADH dehydrogenase (Complex I) that is believed to belong to the minimal assembly required for catalysis. However, mitochondrial DNA shown as the relative abundance of *mtNd1* (a mitochondrial-encoded gene) vs. *B2m* (β2-macroglobin, a nuclear-encoded gene), remained unchanged. Although changes to the transcription profile of mitochondrial biogenesis markers were evident, there were no significant changes in the level of transcripts for *Ppargc1a, Ppargc1b, Nrf1, Nrf2, Tfam,* nor transcripts for genes encoding other mitochondrial functionality markers *Cox4i1, Cox4i2, Cyt c, Ucp2* (Fig. 2A).

With respect to protein profiles in the hypothalamus (Fig. 2B), the expression of PGC1 was not significantly changed in hypothalamuses of stressed rats. PGC1 is a master regulator involved in transcriptional control of all processes related to mitochondrial homeostasis and an integrator of environmental signals (Dominy and Puigserver, 2013). However, all types of immobilization stress significantly increased the expression of hypothalamic NRF1 (nuclear respiratory factor 1). NRF1 is a downstream-target of PGC1 that acts on genes encoding subunits of the oxidative phosphorylation (OXPHOS), and is encoded by nuclear genome (Dominy and Puigserver, 2013; Palikaras and Tavernarakis, 2014). In parallel, increased expressions of NRF1-downstream target TFAM (mitochondrial transcription factor A), an essential ubiquitous factor for mtDNA replication and expression were registered. In the same samples, expression of protein for another NRF1-downstream target, TFB1M (markedly enhance mtDNA transcription) significantly decreased, while the level of COX4 (mitochondrial complex IV cytochrome C oxidase) protein was reduced only in hypothalamuses isolated from repeatedly stressed rats (Fig. 2B).



Psychophysical stress by immobilization altered expression of mitochondrial biogenesis markers in adenohypophysis

To determine whether the profile of expression of mitochondrial biogenesis markers is changed in adenohypophyses of stressed rats, the expression of transcripts and proteins

Fig. 2. Psychophysical stress by immobilization disturbs the profile of mitochondrial biogenesis markers in hypothalamus. Expression of transcripts (A) and proteins (B) for the main mitochondrial biogenesis markers in hypothalamus from control and rats exposed to acute or repeated IMO. Relative quantification of transcription was determined by RQ-PCR using specific primers (see Supp. Table 1). Western blot was performed using specific antibodies (see Supp. Table 2). Representative blots are shown as panels, while pooled data from scanning densitometry normalized on ACTIN (internal control) values are shown as bars on the top of the blots. Normalized data shown are mean ± SEM from triplicate determination. Data bars are mean ± SEM values from independent experiments. Statistical significance was set at level $P < 0.05$: * vs. control group.

for main mitochondrial biogenesis markers were examined in adenohypophyses isolated from control as well as stressed male rats. The results show (Fig. 3) that psychophysical stress by immobilization altered the expression profiles of transcripts (Fig. 3A) as well as proteins (Fig. 3B) for the main markers of mitochondrial biogenesis and functionality in adenohypophysis.

With respect to transcription profiles in adenohypophysis (Fig. 3A), all types of IMO significantly decreased transcripts for *Ppargc1b*. *Ppargc1b* encodes PGC1β protein, an important player in regulation on nuclear transcriptional activities including constitutive non-adrenergic-mediated mitochondrial biogenesis (Scarpulla 2008; Scarpulla et al. 2012; Dominy and Puigserver 2013; Palikaras and Tavernarakis 2014). In parallel, in the same samples, significant decreases were registered in the level of one PGC1β downstream target, *Ucp3*; which encodes the mediator of regulated proton leak and controller of the production of superoxide and other downstream reactive oxygen species (Krauss et al. 2005). In contrast, a significant increase in the level of transcripts for *Ppard* in the adenohypophysis was observed. Other markers of mitochondrial biogenesis and functionality such as *Ppargc1a*, *Nrf1, Nrf2, Tfam*, *Cox4i1, Cox4i2, Cyt c, Ppara, Ucp2,* remained unchanged. Although a significant increase in the level of transcripts for *mtNd1* was detected in adenohypophyses isolated from rats exposed to 10xIMO, mitochondrial DNA relatilve expression remained unchanged independently of the type of stress (Fig. 3A).

Related to protein profile in adenohypophysis, only 10xIMO significantly increased the expression of adenohypophyseal PGC1, whereas all types of IMO significantly increased the level of protein for TFB1M, a downstream target of PGC1. In the same samples of adenohypophyses, the level of NRF1 and TFAM proteins remained unchanged. Similarly to hypothalamuses, the level of COX4 protein was reduced in adenohypophyses isolated from repeatedly stressed rats.

DISCUSSION

In both humans and animals psychophysical stress triggers systemic physiological responses that vary in nature and magnitude. The "fight or flight" response is a universal mechanism of extraordinary physiological and pathophysiological significance. In order to respond to stress, organisms employ complex, multifactorial processes involving an elaborate neuroendocrine, cellular and molecular infrastruc-

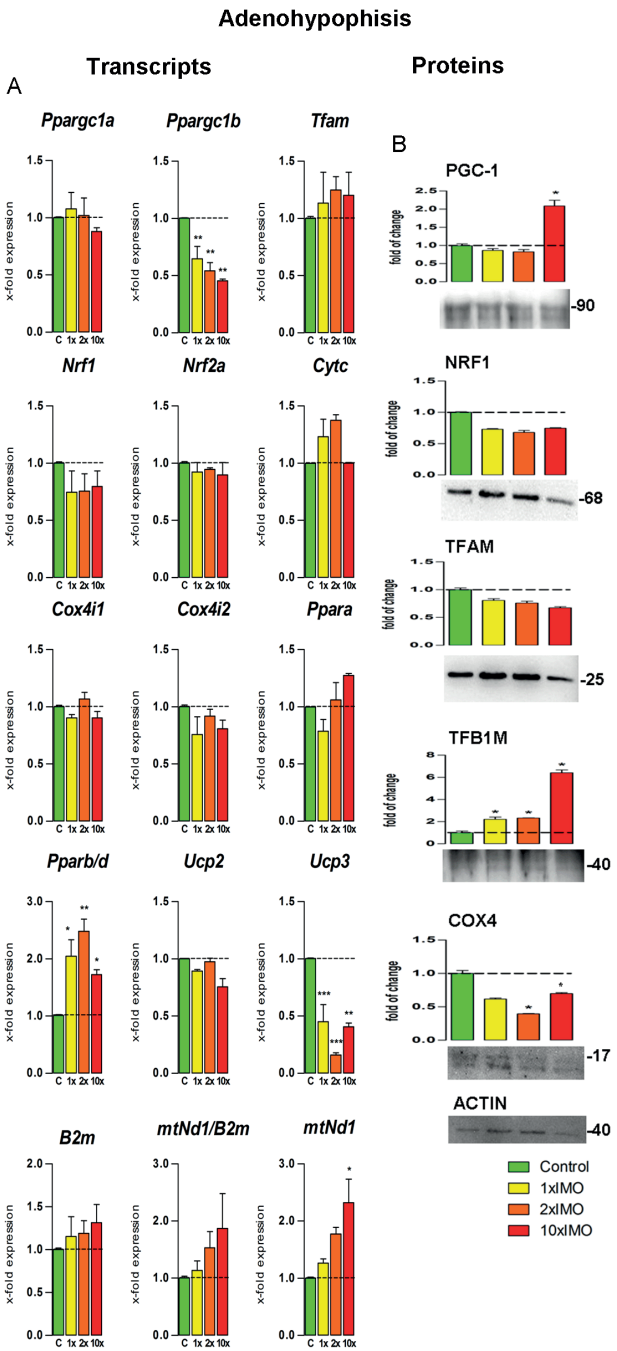


Fig. 3. Psychophysical stress by immobilization disturbs the profile of mitochondrial biogenesis markers in the anterior pituitary. Expression of transcripts (A) and proteins (B) for the main mitochondrial biogenesis markers in the anterior pituitary from control and rats exposed to acute or repeated IMO. Relative quantification of transcription was determined by RQ-PCR using specific primers (see Supp. Table 1). Western blot was performed using specific antibodies (see Supp. Table 2). Representative blots are shown as panels, while pooled data from scanning densitometry normalized on ACTIN (internal control) values are shown as bars on the top of the blots. Normalized data shown are mean ± SEM from triplicate determination. Data bars are mean ± SEM values from independent experiments. Statistical significance was set at level $P < 0.05$(*), $P < 0.01$(**), $P < 0.001$(***): vs. control group.

ture (Pacák and Palkovits 2001; Sapolsky 2005; Manoli et al. 2007; Chrousos 2009; Pitman et al. 2012; Picard et al. 2015). Here we show, according to the best of our knowledge for the first time, that profiles of the expression of transcripts and proteins representing the main markers of mitochondrial biogenesis are disturbed in hypothalamuses and adenohypophyses of stressed male rats. By this expressional scenario, it is possible that the hypothalamuses and adenohypophysis work to preserve and maintain basal function during periods of stress.

Given the crucial role of mitochondria in cell physiology, it is obvious that these organelles are among the first responders to various stressors challenging homeostasis of the cell and organism (Manoli et al. 2007; Chrousos 2009). Mitochondria are highly dynamic organelles undergoing frequent and rapid fusion and fission events, as well as mitochondrial biogenesis and mitophagy, to maintain an extremely well-organized network in response to cellular demands and environmental imperatives (Archer 2013; Palikaras and Tavernarakis 2014; Villa et al. 2017). The form-function dynamic of the mitochondrial network is maintained by the bidirectional relationship of the mitochondria itself and with other organelles including the nucleus and endoplasmic reticulum. Several important mitochondrial capabilities include active travel on dynein and kinesin tracks, fusion, fission, biogenesis, autophagy, coupling with the endoplasmic reticulum, coupling with the nucleus and oxygen-sensing (Archer 2013; St-Pierre et al. 2006). It has been clearly shown that mitochondria can regulate complex, whole-body, physiological responses, impacting stress perception at the level of both the cell and organism. Our study in adult rats demonstrates that mitochondrial biogenesis markers, as specific cellular components that maintain the integrity of life homeostasis via energy transformation and intracellular signaling, are involved in an integrated response to psychophysical stress.

To meet the energy demands of the cell and compensate for cell damage, PGC1 mediates a complex process of mitochondrial biogenesis as the master regulator involved in transcriptional control of all processes related to mitochondrial homeostasis; integrator of environmental signals (Scarpulla 2008; Scarpulla et al. 2012; Dominy and Puigserver 2013); and controller of fusion mediator mitofusin-2 (Archer 2013; Palikaras and Tavernarakis 2014; Villa et al. 2017). Results from the present study show that expression of PGC1 significantly increased in adenohypophyses of 10xIMO rat, but not in hypothalamuses of stressed rats. To the best of our knowledge, there is no published evidence concerning the effects of psychophysical stress on PGC1 expression in the hypothalamus or pituitary. However, it has been shown that PGC1 transcriptional coactivators suppress reactive oxygen species and neurodegeneration (St-Pierre et al. 2006), that exercise training increases mitochondrial biogenesis in the brain (Steiner et al. 2011) and that mitochondrial biogenesis and PGC1α are regulated by cellular stress (Wenz 2013). Also, PGC1β coordinates mitochondria function in the brain and ablation of PGC1β prevents mTOR dependent endoplasmic reticulum stress response in neural cells (Camacho et al. 2012). Since it has been shown that PGC1α is required for the induction of many ROS-detoxifying enzymes (St-Pierre et al. 2006), it is possible that in the hypothalamus and adenohypophysis PGC1 could function in a similar manner. Activated PGC1α/β binds to transcription factors (including NRF1, NRF2, TFAM) to regulate nuclear gene encoding mitochondrial proteins. After transcription and translation, the newly synthesized mitochondrially-destined proteins are imported into the organelle via the translocase of the outer and inner membrane complexes; and become incorporated into the OXPHOS enzyme complexes once imported into the organelle (Dominy and Puigserver, 2013; Palikaras and Tavernarakis 2014). It is possible that PGC1 also enhances the transcription of additional mitochondrially-destined proteins: such as for example cholesterol transporters and OXPHOS enzyme complexes. In fact, it was recently shown that PRKA, CREB and PGC1α are involved in the regulation of OXPHOS in cell transition from the replicating to the quiescent state (Signorile et al. 2014). Our results show increased expression of NRF1, TFAM in hypothalamuses and TFB1M in adenohypophyses of stressed rats, in agreement with findings that glucocorticoids stimulate mitochondrial biogenesis in skeletal muscle (Weber et al. 2002). Also, results of our study show that acute as well as repeated stress significantly increase expression of transcripts for *Ppard* in hypothalamus and adenohypophysis; in support of findings suggesting that stress stimulates brain PPARγ (García-Bueno et al. 2008). Emerging data suggest that PPAR-β/δ activation has a potential neuroprotective role in ischemic stroke (García-Bueno et al. 2008), so increased *Ppard* transcription in hypothalamuses and adenohypophyses of stressed rats maybe an adaptive stress response. Activation of the hypothalamic-pituitary-adrenal stress axis induces cellular oxidative stress (Spierss et al. 2015). On the other hand, mitochondrial dysfunctions alter the hypothalamic -pituitary adrenal axis, sympathetic adrenal-medullary activation and catecholamine levels, as well as circulating metabolites and hippocampal gene expression responses to stress. It has been demonstrated that the mitochondrial energetics and redox balance are modulators of key pathophysiological perturbations previously linked to disease (Picard et al. 2015). Accordingly, it is possible that acute stress could also activate a so called "energy cleaning program" since PGC1, a protein required to produce new mitochondria, also protects against the resulting oxidative damage (Finkel 2006). It is possible that by sensing the intracellular energy status, the mitochondrial functional state and ROS production, the stress network regulates mitochondrial biogenesis in hypothalamus and adenohypophysis by coordinating information flow along its convergent, divergent and multiply branched signaling pathways. This is especially important in light of the micro-heterogeneity of brain mito-

chondria; which distinguish intra-synaptic light and heavy mitochondria in pre-synaptic compartments and non-synaptic mitochondria of neuronal perikaryon in post-synaptic compartment (Villa et al. 2017). According to the above, it is possible that stress-induced coordinated expression of both nuclear and mitochondrial genomes contributes to the expansion of the organelle network, and enhances the process of stress-regulated mitochondrial biogenesis as a mechanism that contributes to the establishment of a new adaptive response to maintain homeostasis and prevent loss of function.

Giving the importance of hypothalamus and adenohypophysis for stress response, as well as the role of mitochondria in stress response and metabolic syndrome, we anticipate our result to be a starting point for future investigations; such as on mitochondrial biogenesis in adrenal gland, brown/white adipose tissues or the cardiovascular system of stressed organisms. These studies are important, since stress is a constant factor in life and has become one of the most significant health problems in modern societies (Sapolsky 2005; Manoli et al. 2007; Chrousos 2009; Pitman et al. 2012). It has been shown that social rank is a factor in stress characteristics (Sapolsky 2005; Gesquiere et al. 2011), where higher-ranking (alpha) males exhibit much higher levels of stress hormones than second-ranking (beta) males, suggesting that being at the very top is stressful (Gesquiere et al. 2011). In addition, stress and emotional brain networks foster eating behaviors that can lead to obesity and activation of a neural-stress-response network, stressors bias cognition toward increased emotional activity and degraded executive function (Dallman et al. 2010). On the other hand, several lines of evidence support a primary role of mitochondrial impairment in the pathophysiology of stress-related disorders (Manoli et al. 2007; Chrousos 2009; Pitman et al. 2012; Archer 2013; Palikaras and Tavernarakis 2014; Picard et al. 2015; Villa et al. 2017). Therefore, we suggest that pharmacological interventions that activate mitochondrial biogenesis markers and consequently stimulate mitochondrial biogenesis in hypothalamus and adenohypophysis might offer a new approach for treatment of energy-disturbance-related disorders or aging as well as the prevention of metabolic syndrome.

CONCLUSIONS

Acute and repeated psychophysical stress, the most common stress in human society, disturbs the expression profiles of mitochondrial biogenesis markers in hypothalamus and adenohypophysis by affecting the expression of NRF1, TFAM, TFB1M in hypothalamuses and TFB1M in adenohypophyses of stressed rats, while COX4 was affected in both endocrine organs, but only in rats exposed to repeated stress. Our results are the first to show the profile of mitochondrial biogenesis markers in the hypothalamus and adenohypophysis of stressed organisms. The present study provides new molecular insight into the relationship between stress and the hypothalamo-adenohypophyseal axis, as well as mitochondrial biology.

ACKNOWLEDGMENTS

We thank Professor Gordon Niswender (Colorado State University) for supplying antibodies for RIA analysis and financial support by the Autonomic Province of Vojvodina (Grant No. 2551) and the Serbian Ministry of Science and Technological Development (Grant No. 173057).

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Supporting information

Additional supporting information may be found in the online version of this article at the publisher's web-site:

Table S1. The primers sequences used for real time PCR analysis.

Table S2. The characteristics of the antibodies used for western blot analysis.