Original Article

Changes of Hippocampal Noradrenergic Capacity in Stress Condition

(chronic restraint stress / noradrenaline / hippocampus / rats)

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Abstract. This study aimed to investigate the effects of chronic restraint stress (CRS) on the protein levels of dopamine-β-hydroxylase (DBH), noradrenaline transporter (NET), vesicular monoamine transporter 2 (VMAT2) and brain-derived neurotrophic factor (BDNF), as well as the concentration of noradrenaline (NA) in the rat hippocampus. The investigated parameters were quantified by Western blot analyses and ELISA kits. We found that CRS increased the protein levels of DBH by 30 %, VMAT2 by 11 %, BDNF by 11 % and the concentration of NA by 104 %, but decreased the protein levels of NET by 16 % in the hippocampus of chronically stressed rats. The molecular mechanisms by which CRS increased the hippocampal NA level are an important adaptive phenomenon of the noradrenergic system in the stress condition.

Introduction

The hippocampus is a limbic structure of the brain, which is very sensitive to chronic stress (Eichenbaum, 2004; Tse et al., 2014). It is known that numerous diseases are related to the long-term adaptive response to stress. For example, stress has a major impact upon neurodegenerative diseases and mental disorders (Esch et al., 2002). In this study, we applied chronic restraint stress (CRS) because CRS can exacerbate neurodegeneration and provoke anxiety and depressive-like behaviours in rats (Ferraz et al., 2011; Liu et al., 2013; Wang et al., 2014). Many studies have shown that monoaminergic signalling is the key mechanism for the modulation of brain functions (Arnsten and Goldman-Rakic, 1985). Therefore, monitoring of the changes of monoamine level is important in the research of numerous diseases caused by chronic stress. However, data about the regulatory molecular mechanisms by which CRS changes noradrenergic capacity in the hippocampus are rather limited. Because of the significant role of noradrenaline (NA) in the regulation of numerous hippocampal functions and hippocampal sensitivity to stress, in this work we investigated how CRS (2 h × 14 days) affected the protein levels of the key enzymes involved in NA synthesis (dopamine-β-hydroxylase-DBH), reuptake (noradrenaline transporter-NET), and storage (vesicular monoamine transporters-VMAT2), as well as the concentration of NA in the rat hippocampus. In addition, it is known that brain-derived neurotrophic factor (BDNF) modulates the activity of monoaminergic systems in the rat brain (Siuciak et al., 1996). Therefore, an additional aim of the study was to investigate how CRS affected the protein levels of BDNF. Detection of the regulatory molecular mechanisms by which CRS changes the NA level in the hippocampus may be very important in the research of numerous diseases caused by chronic stress.

Material and Methods

Animals and stress model

Wistar 11-week-old male rats (300–340 g) were maintained under standard laboratory conditions with water and food *ad libitum* and kept three to four per cage (Gavrilovic et al., 2010). In accordance with our previous protocol (Popović et al., 2017), animals were divided into two groups. The control group (N = 10) was not

Received March 6, 2019. Accepted March 5, 2020.

This work was supported by the Ministry of Education, Science and Technological Development of the Republic of Serbia, Contracts Nos. III 41027, OI 173041 and III41022.

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Abbreviations: BDNF – brain-derived neurotrophic factor, CRS – chronic restraint stress, DBH – dopamine-β-hydroxylase, NA – noradrenaline, NET – noradrenaline transporter, VMAT2 – vesicular monoamine transporter 2.

exposed to stress. In the CRS group (N = 10), the animals were exposed to chronic restraint stress. Restraint stress was achieved by placing each animal in a 25 × 7 cm plastic bottle, as described previously by Gamaro et al. (1999). The animals in these groups were exposed to 2 h of restraint stress every day at random times during the light period of the light/dark cycle to avoid habituation during the experimental procedure of 14 days (Kim and Han, 2006). To reduce variance in the physiological parameters due to daily rhythms, the remaining animals were sacrificed at the same time point in the circadian cycle, between 9:00 and 11:00 am, i.e., one day after the last treatments. Animals were sacrificed under no-stress conditions by rapid decapitation. The hippocampi were rapidly dissected, frozen in liquid nitrogen and stored at –70 °C until analysed.

Western blot analysis

The protein concentration was determined using the BCA method (Thermo Scientific Pierce, Waltham, MA), described by Stich (1990). DBH, NET, VMAT2 and BDNF proteins were assayed by Western blot analysis as described previously by Gavrilović et al. (2013). Antibodies used for quantification of specific proteins were as follows: for DBH, ab63939 (Abcam, Cambridge, UK), for NET, ab41559 (Abcam), for VMAT2, ab70808 (Abcam), for BDNF, ab6201 (Abcam) and for β-actin, ab8227 (Abcam). After washing, the membranes were incubated in secondary anti-rabbit (Amersham ECL™ Western Blotting Analysis System, Little Chalpont, UK) antibodies conjugated to horseradish peroxidase. The secondary antibody was then visualized by the Western blotting enhanced chemiluminescent detection system (ECL, Amersham Biosciences). The membranes were exposed to ECL film (Amersham Biosciences). The result was expressed in arbitrary units normalized in relation to β-actin, which is in accordance with our previous protocol (Gavrilović et al., 2013).

Noradrenaline measurement

Noradrenaline concentration in the hippocampus fractions was determined using 3-CAT Research ELISA kits (Labor Diagnostica Nord, Nordhorn, Germany) according to the manufacturer's protocol, as described previously by Gavrilović et al. (2018). Absorbance was determined at 450 nm using a microplate reader (Stat Fax 2100, Ramsey, MN). Values were expressed as ng of NA per g of tissues, which is in accordance with our previous protocol (Gavrilović et al., 2018).

Data analysis

The data are presented as means ± S.E.M. Differences of gene expression of *DBH*, *NET*, *VMAT2* and *BDNF*, as well as of NA concentration between control and CRS animals in the hippocampus, were analysed by *t*-test. Statistical significance was accepted at $P < 0.05$. Correlations of the protein levels (DBH, VMAT2 and BDNF) and NA levels were analysed by the Pearson test, using the Sigma Plot v10.0 (with SigmaStat integration, San Jose, CA).

Results

Changes of DBH, NET, VMAT2 and BDNF protein levels in the hippocampus

The animals exposed to CRS showed increased levels of DBH protein by 30 % ($P < 0.05$, *t*-test, Fig. 1a),

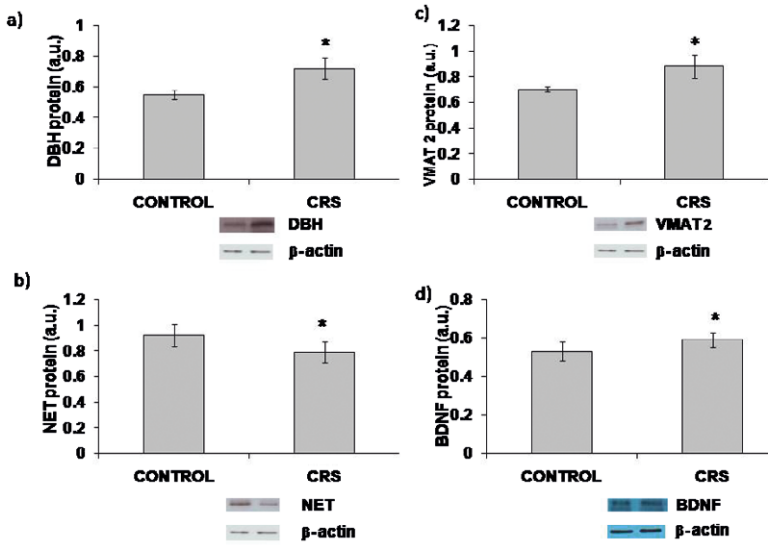

Fig. 1. Effects of chronic restraint stress (CRS) on dopamine-ß-hydroxylase (DBH) [a], noradrenaline transporter (NET) [b], vesicular monoamine transporter 2 (VMAT2) [c], and brain-derived neurotrophic factor (BDNF) [d] protein levels in the hippocampus

Data are shown as means ± S.E.M. of 10 rats. Statistical significance: *$P < 0.05$, CRS animals compared to control animals (*t*-test)

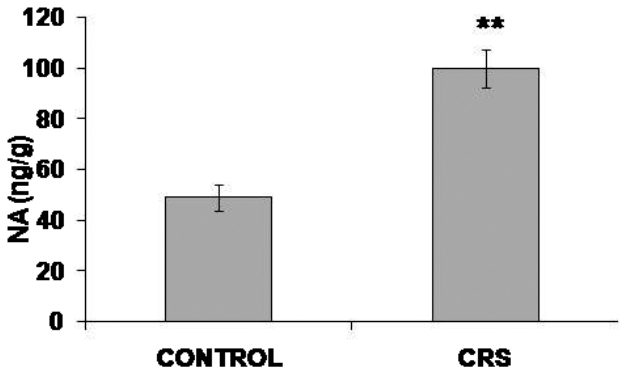

Fig. 2. Effects of chronic restraint stress (CRS) on the concentrations of noradrenaline (NA) in the hippocampus
Data are shown as means ± S.E.M. of 10 rats. Statistical significance: **P < 0.01, CRS animals compared to control animals (*t*-test)

VMAT2 protein by 11 % (P < 0.05, *t*-test, Fig. 1c) and BDNF protein by 11 % (P < 0.05, *t*-test, Fig. 1d) in the hippocampus, compared with the control animals. However, CRS significantly decreased the levels of NET protein by 16 % (P < 0.05, *t*-test, Fig. 1b) compared with the control animals.

Changes of the NA concentration in the hippocampus

We found that in the hippocampus, CRS significantly increased NA concentration by 104 % (P < 0.01, *t*-test, Fig. 2), compared with the control animals. In the control animals, we did not record any significant correlation between the levels of VMAT2 protein and NA concentration in the hippocampus. However, a significant positive correlation was found between the levels of VMAT2 protein and NA concentration (Pearson, r = 0.793; P < 0.05) in the hippocampus of the animals exposed to CRS. In addition, we found a significant positive correlation between the levels of DBH protein and NA concentration (Pearson, r = 0.865; P < 0.01), as well as significant positive correlation between the levels of BDNF protein and NA concentration (Pearson r = 0.704; P < 0.05) in the hippocampus of animals exposed to CRS.

Discussion

In the present study, we found that CRS increased the hippocampal NA levels. In addition, we noted that the significant increase of hippocampal NA concentration coincided with the increased levels of DBH enzyme. This finding suggests the possible increase of conversion of neurotransmitter dopamine to NA in the hippocampus of the animals exposed to CRS. In addition, data about NA transmission are very important for understanding changes of hippocampal noradrenergic capacity in the stress condition. According to the literature data, the monoamine hypothesis of mood disorders states that depression is caused by insufficient signalling by monoamines (Carlson, 1988). It is known that the dynamics of NA transmission is regulated by reuptake through NET (Blakely and Bauman, 2000). In this study, we found that CRS significantly decreased the protein levels of NET. It is possible that the reduced functional deficiency of NA is an adaptation to the prolonged exposure to stress. In addition, an important result in this work is that CRS increased the protein levels of VMAT2. We recorded a significant positive correlation between the levels of VMAT2 and NA in the hippocampus of chronically stressed rats. It is known that VMAT play a critical role not only in sorting, storage and release of monoamines, but also in the fine-tuning of neuronal informational output (Eiden, 2000). Repeated stress increased expression of VMAT2 levels in many NA-synthesizing cells, indicating an adaptation to the prolonged exposure to a strong stressor, which could provide a mechanism for facilitating utilization of the enhanced noradrenergic capacity (Tillinger et al., 2010).

In this study we also found that CRS significantly increased the protein levels of BDNF. The increased protein levels of BDNF could restore neurohormone stocks highly solicited during chronic stress, thus playing a "protective" role during the stress (Nawa et al., 1993; Givalois et al., 2004a,b; Naert, 2006). We recorded that the increased concentrations of NA positively correlate with the protein levels of BDNF in the hippocampus of animals exposed to CRS. Our results confirm that the increased level of BDNF may play an important role in the adaptive physiological processes required by neurons to maintain the noradrenergic capacity in the conditions provoked by chronic stress. The molecular mechanisms by which CRS increases the hippocampal NA level represent an important adaptive phenomenon of the noradrenergic system in the stress condition. Our results may be important in the research of numerous psychiatric diseases caused by chronic stress.

Conflict of interest

The authors report no conflict of interest.

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