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ORIGINAL ARTICLE

Effects of mood stabilizer lithium on noradrenergic turnover in the prefrontal cortex of chronically stressed rats

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Abstract

OBJECTIVE: Data about the dynamics of noradrenaline (NA) transmission, storage and degradation may be very important for understanding the reduction of functional deficiency of NA and maintaining the stability of NA levels in animals with depressive-like behavior treated with lithium. This study aimed to investigate the effects of mood stabilizer lithium on concentrations of NA in the prefrontal cortex (PFC), as well as behavior rats exposed to chronic restraint stress (CRS). In addition, this study examined the effects of lithium on protein levels of noradrenaline transporter (NET), vesicular monoamine transporter 2 (VMAT2) and catechol-O-methyltransferase (COMT), as well as the enzyme activity of monoamine oxidase A (MOA) in the PFC of chronically stressed rats.
METHODS: The investigated parameters were quantified by Western blot analysis, CAT Research ELISA kits, and an assay of enzyme activity. Also, the forced swim test (FST) was used to assess the behavior of animals.
RESULTS: We found that lithium treatment decreased high protein levels of NET and VMAT2, as well as the enzyme activity of MOA in chronically stressed rats to the levels found in unstressed animals. In addition, lithium treatment decreased the concentration of NA (24%) and immobility in animals exposed to CRS.
CONCLUSION: Our data confirm that lithium-induced modulation of prefrontal noradrenergic turnover and stabilized the behavior of chronically stressed rats.

Abbreviation:

BD	- bipolar disorder
COMT	- catechol-O-methyltransferase
CRS	- chronic restraint stress
FST	- forced swimming test
MO	- monoamine oxidase
MOA	- monoamine oxidase A
NA	- noradrenaline
NET	- noradrenaline transporter
PFC	- prefrontal cortex
VMAT2	- vesicular monoamine transporter 2

INTRODUCTION

The literature data have shown that in the pathophysiology of mood disorders lithium is known as an effective drug in the long-term stabilization of moods (Alda, 2015). Numerous studies have confirmed that bipolar disorder (BD) is associated with stress-induced neuronal remodeling (Wood *et al.* 2004), as well as that chronic

restraint stress (CRS) is a good animal model which induces neuronal remodeling in the prefrontal cortex (PFC) (Wellman, 2001). Also, it is known that the stability of noradrenaline (NA) levels is very important for BD amelioration (Kurita, 2016). For this reason, data about noradrenergic turnover in the PFC may be very important for understanding the functional deficiency of NA in pathological conditions.

It is known that the dynamics of NA transmission are potently regulated by reuptake through noradrenaline transporter (NET) (Blakely and Bauman, 2000). CRS treatment significantly increased protein levels of NET and levels of NA, which probably confirms the uptake of neurotransmitter NA via NET in the PFC (Popović *et al.* 2017 a). Higher protein levels of NET suggest that NET can be upregulated in response to heightened demand for uptake of NA in conditions provoked by CRS (Popović *et al.* 2017 a). This finding indicated a functional deficiency of NA in animals with depressive-like behavior (Popović *et al.* 2017 a). However, the inhibition of the activity of NET can prolong the time during which this neurotransmitter is available in the synaptic cleft (Montoya *et al.* 2016). In addition, vesicular monoamine transporter 2 (VMAT2), which is responsible for the uptake of NA from the cytoplasm into intracellular storage vesicles, is expressed in neurons that are implicated also in the regulation of mood and behavior (Schwartz *et al.* 2003). For example, a significant elevation in VMAT2 density was observed in the platelets of untreated major depressed patients (Zucker *et al.* 2002). In addition, changes in enzyme activity of monoamine oxidase (MO) and catechol-O-methyltransferase (COMT), the enzymes which catalyze the oxidative deamination of NA are also accompanied by mood disorders. For example, the activity of mitochondrial MO is elevated in the brain during the major depression (Hu *et al.* 2011). The inhibition of the activity of MO by antidepressants can prolong the time during which neurotransmitters are available in the synaptic cleft (Montoya *et al.* 2016). It is known that antidepressants may be effective only for short-term treatment of bipolar depression, because these drugs may increase the risk of switching to mania in BD (Kurita, 2016). However, very little is known about prefrontal noradrenergic turnover in animals exposed to CRS treated with mood stabilizer lithium.

Because of the critical role of NA in the switch of bipolar depression to mania, monitoring of the changes of noradrenergic turnover in the PFC in animals with depressive-like behavior treated with lithium may be very important in research of lithium role in reduction of functional deficiency of NA in pathological conditions and for maintaining normal NA levels. Therefore, in this study we examined: levels of enzymes involved in NA reuptake (noradrenaline transporter-NET), storage (vesicular monoamine transporters-VMAT2), and degradation (monoamine oxidase A - MOA and catechol-O-methyltransferase - COMT), as well as concentrations of NA in the PFC of chronically stressed rats treated with lithium. An additional aim of the study was to test the behavior of chronically stressed rats treated with lithium.

MATERIALS AND METHODS

Animals and stress models

Wistar11-week-old male rats (300-340g) were maintained under standard laboratory conditions with water and food *ad libitum* and kept three to four per cage (Gavrilović *et al.* 2018). In accordance with our previous protocol (Popović *et al.* 2019) animals were divided into three groups: **CONTROL group** (n=20) was not exposed to any treatment, **CRS group** (n=20) consisted of animals exposed to chronic restraint stress treatment, and **CRS+Li group** (n=20) consisted of animals exposed to chronic restraint stress treatment with Li given each day immediately prior to daily restraint. Restraint stress was performed by placing each animal in a 25 x 7 cm plastic bottle as described previously by Gamaro *et al.* (1999). The animals in these groups were exposed to 2h of restraint stress every day for 14 days within random times during the light period of the light/dark cycle, to avoid habituation (Kim and Han, 2006). Lithium was administered intraperitoneally to the animals, once a day for 14 days as described previously (Popović *et al.* 2019). The initial lithium dose was 1.5 mEq/kg for 2 days, and was then increased to 2.3 mEq/kg for 7 days, followed by 3 mEq/kg for 5 days. This lithium administration protocol maintained the plasma lithium concentration above the minimum therapeutic concentration (*i.e.* 0.4 mM) for the treatment of bipolar disorder throughout the treatment period. Depression-like behaviors were assessed by elevated forced swimming test (FST). Ten animals from each group were tested on the FST. Animals that were used to test the behavior were not used for further analysis. To reduce variance in the physiological parameters due to daily rhythms, remaining animals (n=10 from each group) 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 prefrontal cortex was rapidly dissected, frozen in liquid nitrogen and stored at -70 °C until analyzed.

Noradrenaline measurement

Noradrenaline concentration in the prefrontal cortex fractions was determined using 3-CAT Research ELISA kits (Labor Diagnostica Nord, Nordhorn, Germany) according to the manufacturer's protocol. Absorbance was determined at 450 nm using a microplate reader (Stat Fax 2100). Values were expressed as ng of NA per g of tissues following our previous protocol (Gavrilović *et al.* 2018).

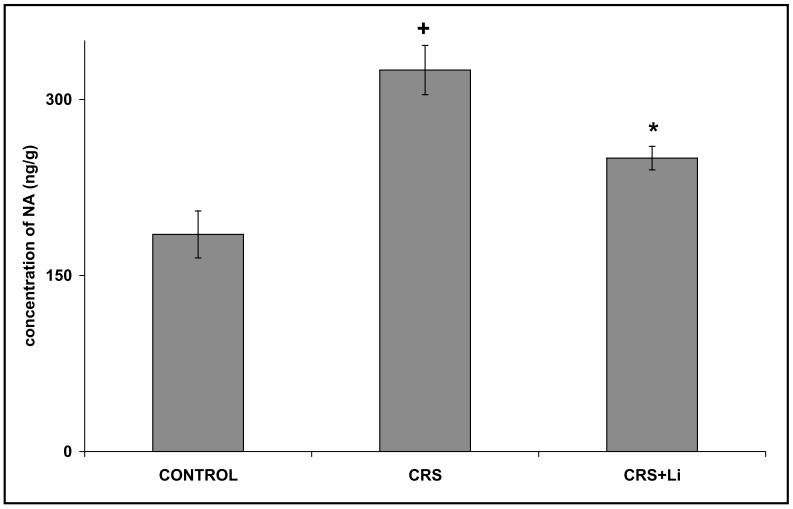

Fig. 1. Effects of lithium on the concentration of noradrenaline (NA) in the prefrontal cortex of animals exposed to CRS. The values are means ± S.E.M. of 10 rats. Statistical significance: +$p<0.05$ animals exposed to CRS vs. CONTROL animals (t-test); *$p<0.05$ animals exposed to CRS+Li *vs.* CRS animals (t-test). The concentration of NA was expressed as ng per gram of tissue (ng/g).

Monoamine oxidase enzyme activity

The enzyme activity of MOA was determined using the Amplex Red Monoamine Oxidase Assay (A12214, Molecular Probes, USA) described by Zhou and Panchuk-Voloshina (1997). This assay is based on the detection of H_2O_2 in a horseradish peroxidase-coupled reaction using N-acetyl-3, 7-dihydroxyphenoxazine (Amplex Red), a highly sensitive and stable probe for H2O2. Fluorescence was measured with a fluorimeter using excitation at 560±10 nm and fluorescence detection at 590±10 nm. Monoamine oxidase activity was expressed as U/mg of protein, following our previous protocol (Gavrilović *et al.* 2018).

Western blot analysis

The prefrontal cortex was homogenized in 0.05 M sodium phosphate buffer (pH 6.65). Subsequently, the protein concentration was determined using the bicinchoninic acid (BCA) method (Thermo Scientific Pierce, USA) described by Stich (1990). NET, VMAT2, and COMT proteins were assayed by Western blot analysis as described previously by Gavrilović *et al.* (2018). Antibodies used for quantification of specific proteins were as follows: NET (ab41559 Abcam, USA), for VMAT2 (ab70808 Abcam, USA), for COMT (ab208647 Abcam, USA) and for β-actin (ab8227 Abcam, USA). After washing, the membranes were incubated in the secondary anti-rabbit (dilution 1:5000, Amersham enhanced acridan-based chemiluminescent/chemi-fluorescent substrate (ECL™) Western Blotting Analysis System, UK) antibodies conjugated to horseradish peroxidase. A secondary antibody was then visualized by the Western blotting enhanced chemiluminescent detection system (ECL, Amersham Biosciences, UK). The membranes were exposed to ECL film (Amersham Biosciences, UK). The result was expressed in arbitrary units normalized in relation to β actin, following our previous protocol (Gavrilović *et al.* 2018).

Forced swim test (FST)

The Porsolt forced swim tank consisted of a clear, cylindrical Plexiglas tank measuring 45 cm high and 20 cm in diameter with a water (28 °C) depth of 30 cm following our previous protocol (Popović *et al.* 2017 b). These testing parameters are consistent with other protocols using FST as a measure of depressive-like behavior (Porsolt *et al.* 1978). The forced swim test was comprised of a two-day protocol. On the first day, a rat was placed into the swim tank for 15 min. Afterwards, the rat was removed and placed under a heat lamp for one hour before being returned to its home cage and transported back to the housing colony. The FST tanks were rinsed after every animal and refilled with fresh tap water (28 °C). On the second day, each rat was placed back into the swim tank for 5 min and behavior was videotaped. As was done during the previous day, each rat was warmed under a heat lamp for one hour before being returned to its home cage and transported back to the animal colony. FST behavior was scored using a time sampling technique (Detke *et al.* 1995), where every five seconds, the behavior was characterized as either swimming, climbing, or being immobile. Swimming was defined as paw movement underwater, climbing was defined as the paws breaching the surface of the water, and immobility was defined by a lack of movement. Rats spending more time immobile in the FST have been characterized as reflecting increased depressive-like behavior (Porsolt *et al.* 1978). For the purpose of this paper, we will use the "depressive-like" description.

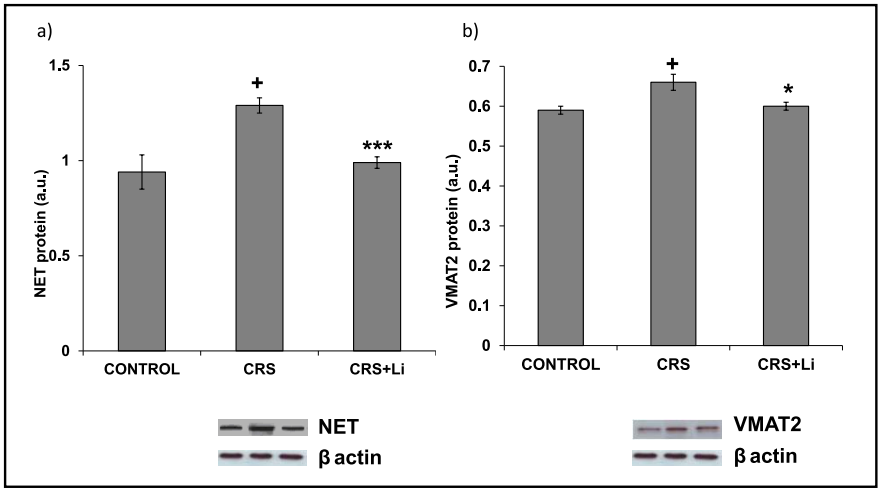

Fig. 2. Effects of lithium on noradrenaline transporter (NET) [a] and vesicular monoamine transporter 2 (VMAT2) [b] protein levels in the prefrontal cortex of animals exposed to CRS. The values are means ± S.E.M. of 10 rats. Statistical significance: +p<0.05 animals exposed to CRS *vs.* CONTROL animals (t-test); *p<0.05, ***p<0.001 animals exposed to CRS+Li *vs.* CRS animals (t-test). The result was expressed in arbitrary units normalized in relation to β actin.

Data analysis

The data are presented as means ± S.E.M. Differences of protein levels of NET, VMAT2, and COMT; activity of enzymes MOA; concentration of NA, as well as animal behavior between CRS and CRS+Li animals were analyzed by t-test. Statistical significance was accepted at p<0.05. Correlations of protein levels VMAT2 and NA levels were analyzed by the Pearson test, using the Sigma Plot v10.0 (with Sigma Stat integration).

RESULTS

Lithium treatment decreased NA, NET, MOA, but not COMT, in the prefrontal cortex of CRS rats

In our previous study (Popović *et al.* 2017 a) we found that in the prefrontal cortex CRS significantly increased NA concentration by 49% (p<0.05, t-test, Figure 1), levels of NET protein by 25% (p<0.05, t-test, Figure 2a), VMAT2 protein by 12% (p<0.05, t-test, Figure 2b) and COMT protein by 59% (p<0.05, t-test, Figure 3b), as well as activity of MOA by 58% (p<0.05, t-test, Fig. 3a), compared with control animals. In this study, we found that lithium treatment in animals exposed to CRS significantly decreased the concentration of NA by 24% (p<0.05, t-test, Figure 1) compared with CRS animals. In addition, lithium treatment decreased levels of NET protein by 23% (p<0.001, t-test, Figure 2a) and decreased levels of VMAT2 protein by 10% (p<0.05, t-test, Figure 2b) in animals exposed to CRS. Also, the animals exposed to CRS treated with lithium showed a decreased activity of MOA by 34% (p<0.01, t-test, Figure 3a), while levels of COMT protein remained unchanged (Figure 3b) compared with CRS animals. A significant positive correlation was found between protein levels of VMAT2 and the concentration of NA (Pearson R=0.633; p<0.05) of animals exposed to CRS+Li.

Changes in animal behavior

In our recent research (Popović *et al.* 2017 b) we found that CRS significantly increased immobility by 20% (p<0.05, t-test, Figure 4), compared with the control animals. Using the FST model, CRS rats treated with lithium have significantly less immobility by 13 % (p<0.05, t-test, Figure 4) than untreated CRS rats.

DISCUSSION

Our earlier research showed that CRS influenced depressive-like behavior in rats (Popović *et al.* 2017 b), as well as that it induced a significant increase of NA concentration in rat PFC (Popović *et al.* 2017 a). Also, we recorded significantly increased protein levels of NET, VMAT2, and COMT, as well as enzyme activity of MOA in the PFC of chronically stressed rats (Popović *et al.* 2017 a). These results indicated a functional deficiency of NA (Popović *et al.* 2017 a). In the present study, we found that lithium treatment decreased the high concentration of NA in chronically stressed rats to the levels of unstressed animals found in our previous research (Popović *et al.* 2017 a). An important result of this study is that lithium treatment in animals exposed to CRS decreased high protein levels of prefrontal NET to the levels of unstressed animals found in our previous research (Popović *et al.* 2017 a). Decreased protein levels of NET can prolong the time during which NA is available in the synaptic cleft. Lithium treatment likely affects the reduction of functional deficiency of NA in animals with depressive-like behavior.

In addition, we observed that lithium treatment in animals exposed to CRS decreased high protein

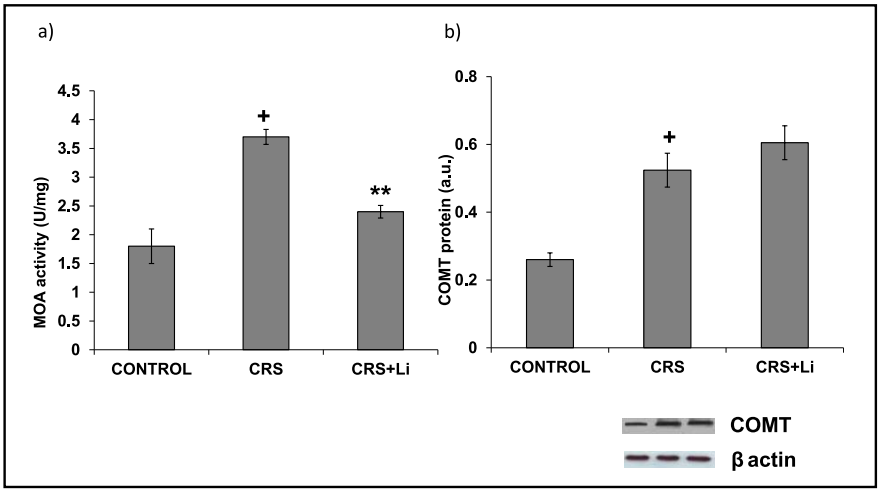

Fig. 3. Effects of lithium on the enzyme activity of monoamine oxidase A (MOA) [a] and catechol-O-methyltransferase (COMT) protein levels [b] in the prefrontal cortex of animals exposed to CRS. The values are means ± S.E.M. of 10 rats. Statistical significance: +p<0.05 animals exposed to CRS *vs.* CONTROL animals (t-test); **p<0.01 animals exposed to CRS+Li *vs.* CRS animals (t-test). The level of MOA was expressed as units per milligram of protein (U/mg) and protein levels of COMT were expressed in arbitrary units normalized in relation to β actin.

levels of VMAT2 to the levels of unstressed animals found in our previous research (Popović *et al.* 2017 a). This finding confirms that treatment with lithium is involved in maintaining a constant level of NA characteristic of unstressed animals. A significant positive correlation between the levels of VMAT2 and NA in the PFC of chronically stressed rats after lithium treatment is indicative of such a statement.

It is known that the inhibition of MO is an important therapeutic strategy for several neuropsychiatric disorders (Cesura and Pletscher, 1992). For example, Cesura and Pletscher (1992) and Knoll (2000) found that the increase of monoamine levels was caused by MO inhibitors. The literature data confirm that lithium is a very weak inhibitor of MO (Fisar *et al.* 2010). However, Sastre *et al.* (2005) found that levels of NA metabolites are reduced by lithium. In the present study, we found that lithium treatment in animals exposed to CRS decreased prefrontal MOA activity to the levels of unstressed animals found in our previous research (Popović *et al.* 2017 a), while levels of COMT protein remained unchanged. Finally, our results indicate that the treatment of CRS animals with lithium is an important factor for maintaining NA levels as in unstressed animals.

In addition, chronic treatment with mood-stabilizing drug lithium in animals exposed to CRS significantly decreased immobility to the level of unstressed animals found in our previous research (Popović *et al.* 2017 b). Significantly decreased immobility in chronically

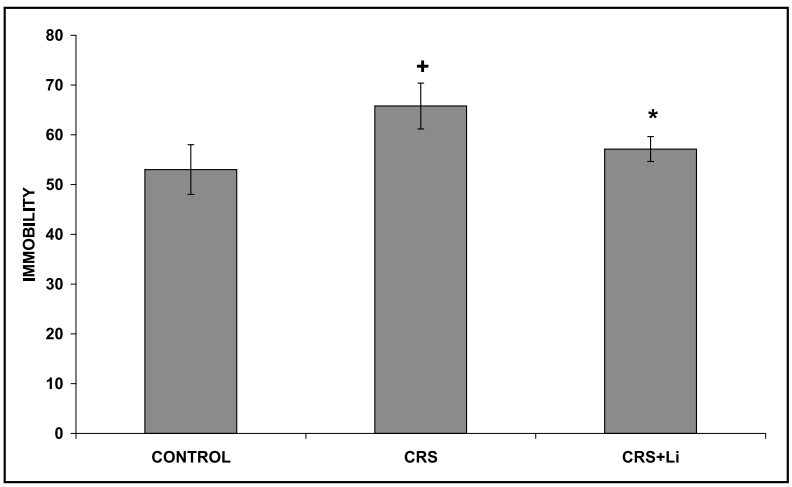

Fig. 4. Effects of lithium on immobility (I) in animals exposed to CRS. The values are means ± S.E.M. of 10 rats. Statistical significance: +p<0.05 animals exposed to CRS *vs.* CONTROL animals (t-test); *p<0.05 animals exposed to CRS+Li *vs.* CRS animals (t-test).

stressed rats treated with lithium makes the animals more ready to "fight or flight" under the conditions of stress that may occur later. This finding confirms that lithium stabilizes behavior in animals with depressive-like behavior and makes them more prepared for a new challenge.

In conclusion, modulation of noradrenergic turnover in the prefrontal cortex of chronically stressed rats by lithium reduced functional deficiency of NA, maintained normal NA levels, and stabilized behavior. This finding may be very important for understanding the role of lithium in maintaining the stability of the prefrontal noradrenergic system in the condition of bipolar depression.

ACKNOWLEDGEMENTS

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CONFLICT OF INTEREST

The authors report no conflict of interest.

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