PROCEEDINGS OF THE YEREVAN STATE UNIVERSITY

Chemistry and Biology 2016, № 3, p. 40–47

Biology

EFFECTS OF *d*-AMPHETAMINE ON REGIONAL ACTIVITY OF ARGINASE ISOFORMS IN RAT CORTICOLIMBIC BRAIN

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In the present work cytoplasmic and mitochondrial arginase isoforms (ARG1 and ARG2 respectively) are studied in the rat corticolimbic brain regions in amphetamine induced bipolar disorder (BD). Escalating non-neurotoxic doses of *d*-amphetamine sulfate (AMPH) injected to rats over 24 days induce changes in behavior mimicking BD accompanied by alterations in resident microbiota with manifestation of pathogenic bacteria. Both ARG1 and ARG2 are stimulated in the brain corticolimbic regions involved in the formation of emotions, learning and memory. Simultaneously, NO stable metabolite levels are diminished or not changed in the cytoplasm and mitochondria of the brain regions studied in AMPH-treated rats. It is suggested that arginase isoforms may contribute the NO-synthase inhibition and prevention of detrimental effects of the reactive nitrogen species overproduced in BD.

***Keywords*:** arginase, bipolar disorder, corticolimbic brain, cytoplasm, nitric oxide, *d*-amphetamine, mitochondria.

Introduction. Bipolar disorder (BD) is a complex neuropsychiatric disorder characterized by intermittent episodes of mania and depression [1]. According to the WHO reports bipolar disorder affects over 60 million people worldwide [2]. The pathophysiology of BD is complex, and cycling between states of depression and mania disorder remains unclear [3]. Plenty of evidences suggest that arginase might be involved in the BD pathophysiology. Arginase hydrolyzes L-arginine to L-ornithine and urea and exists in 2 isoforms, cytoplasmic arginase (ARG1) and mitochondrial arginase (ARG2) synthesized as a pre-protein, imported to mitochondria, and processed to the mature form in the mitochondrial matrix [4]. Differential expression of arginase isoforms could provide a means to preferentially direct ornithine either to proline or excitatory amino acid, glutamate synthesis via ornithine aminotransferase in cytoplasm or to polyamine synthesis via ornithine decarboxylase in mitochondria [5]. Ornithine is decarboxylated to putrescine, involved in the synthesis of

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polyamines, spermidine and spermine, implicated in pathogenesis of BD and schizophrenia [6]. In the present work arginase isoforms are studied in the rat corticolimbic brain regions in amphetamine induced BD.

Materials and Methods. All procedures involving animals are in accordance with Directive 86/609/EEC and approved by the respective Institutional Animal Care and Use Committees of the NAS of the Republic of Armenia and the local committee of biomedical ethics (H. Bunyatyan Institute of Biochemistry, RA). Two-month-old male Wistar rats weighing 150–200 *g* obtained from our breeding colony were used.

Experimental Part. Escalating non-neurotoxic doses of *d*-amphetamine sulfate (AMPH) ("Sigma", St. Louis, MO) 2–6 *mg*/*kg* of the weight to mimic the pattern of drug use in addicts [7]. Intra-peritoneal injections of AMPH (0.2 *mL* once a day) were performed each weekday omitting weekends over 24 days (18 injection days). Animals were divided into two groups: I – AMPH-treated animals; II – control with age and sex matched intact rats. At the end of AMPH administration rats were tested in open field an elevated plus-maze and sacrificed by decapitation immediately after behavioral testing.

Open Field (OF) Test. The rats were placed singly into an OF (diameter 1 *m*, divided by 2 concentric circles into 16 equal sections on the floor of the arena) and observed in 3 *min* to assess locomotor activity (the number of sectors crossed with all paws (crossing or crossovers), 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 behavior) and defecation boluses (anxiety) counted manually/visually [8].

Elevated Plus-Maze (EPM) Test. Immediately after the OF test the rats were placed singly into a common central platform (10×10 (*cm*)) of elevated plus-maze comprised of two open and two closed arms (45×10×10 (*cm*)) and elevated to a height of 80 *cm* above the floor. During 3 *min* observation period, the following parameters were assessed: number of open arm entries and closed arm entries. Percentage of the number of entries into the open arm of the total number of entries into all arms was calculated. Exploration (grooming and rearing) and risk assessment (number of hanging over the open arm) were also examined. 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 [9].

Isolation of Cytoplasm and Mitochondria from Rat Brain. Brains were rapidly removed from the skulls, placed on cold plate, and prefrontal cortex (PFC), 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 and centrifuged at 15000 *rpm* for 20 *min*, cytoplasm was separated in the supernatant and mitochondria were separated in the pellets. Mitochondria were washed twice with the above mentioned buffer, re-suspended and homogenized.

Arginase Assay. The samples were incubated at 37°*C* for 60 *min* in the reaction mixture containing 20 *mM* HEPES buffer pH 7.4, 0.05 *M* $MnCl_2 \cdot 4H_2O$, 0.1 *M* L-arginine·HCl. The reaction was stopped by adding of 10% TCA, and protein-free samples were obtained by centrifugation at 15000 *rpm* for 3 *min*, incubated with 4.5% ninhydrin at 90°*C* for 30 *min*. Cooled samples were read at an absorbance of 505 *nm* using a spectrophotometer Specoll-211 (Germany) to measure L-ornithine produced by arginase [10]. Arginase activity is expressed as *μmoles* of L-ornithine $\cdot mg^{-1}$protein$\cdot h^{-1}$.

Measurement of the NO Stable Metabolites. Protein-free samples (0.5 *N* NaOH and 10% $ZnSO_4$ were used for deproteinization) were analyzed for stable intermediates of NO using colorimetric technique based on diazotization using Griess-Ilosvay reagent (1:1 mixture of 0.17% sulfanilic acid and 0.05% α-naphthylamine in 12.5% acetic acid), and the absorbance was measured spectrophotometrically at 546 *nm* [11].

Protein content is measured according to Lowry, using crystalline bovine serum albumin as standard [12].

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

Results and Discussion. Effects of AMPH long-term treatment on the behavioral characteristics of rats were studied in OF and EPM. The behavior in rats was scored at 3 *min* intervals during the period between 30 and 60 *min* after AMPH administration, and the crossings and rearing were markedly increased in OF test of 6.2 and 3.4 times, respectively as compared with control, and began to decline at around 90 *min*, gradually decreased, and disappeared at around 180 *min* after AMPH administration (Fig. 1).

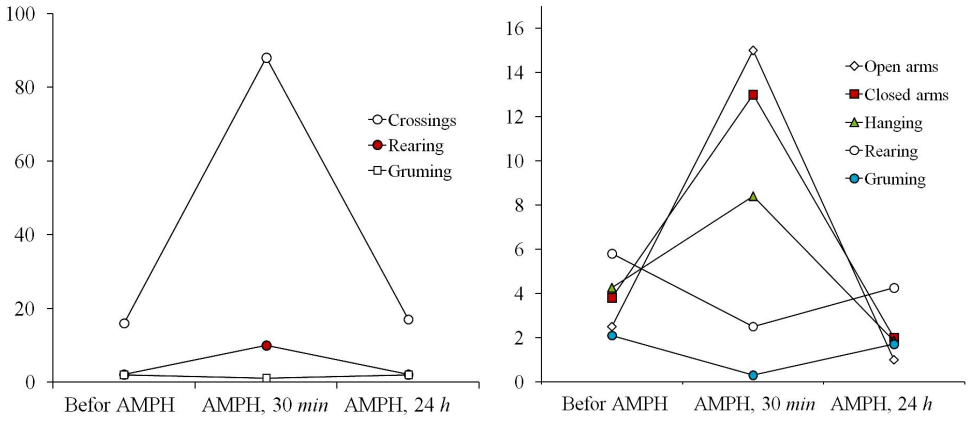

Fig. 1. AMPH-induced behavioral changes in the open field and elevated plus-maze tests. Data are expressed as M ± S.E.M., *n*=12, statistical comparisons were made by one way ANOVA followed by the post-hoc test of Holm-Sidak. Differences are considered significant if $p < 0.05$. # $p > 0.05$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Notably, behavioral pattern in OF was also normal 24 *h* post-AMPH withdrawal. In EPM, the number of entries into the open and closed arms and

hanging over the open arms were increased of 6, 3.6 and 2 times 30 *min* after AMPH administration to rats and dropped of 2.8, 1.8 and 2.3 times 24 *h* post-AMPH withdrawal compared respectively to control values. The exploratory activity of rats was at some extent suppressed by AMPH, as it was confirmed by a 1.8 and 4.3-fold decrease in the number of grooming in OF and EPM respectively, and a 2-fold drop in rearing in EPM in AMPH/ 30 *min* rats.

A possible role for gut infection in triggering brain responses, such as anxious behavior and mood changes, in the absence of overt gut inflammation or elevation of plasma cytokines have been described in mice [13]. Analysis of microbiota showed a substantial enhancement in the number of resident *Escherichia coli* and *Candida albicans* and manifestation of *Staphylococcus aureus* and hemolytic *E. coli* accompanied by a reduced number of obligate microbes in the rats exposed to long-term treatment with escalating doses of AMPH. Of note, *S. aureus* and *C. albicans* may contribute to a decrease in 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 [14]. *C. albicans* could be involved in yeast-induced illness-mental depression, anxiety, and the abnormalities in brain function that can be cleared completely with anti-yeast therapy [15]. An anti-fungal composition is successfully used for treatment or prophylaxis of BD [16].

Host catecholamines including adrenaline and noradrenaline (NA) have been shown to stimulate the growth of non-pathogenic isolates of *E. coli* as well as the enterohaemorrhagic *E. coli* (via NA-mediated iron supply from transferring) and could induce the production of an "autoinducer" which, in turn, promotes *E. coli* growth in the absence of NA [17]. A matter of special relevance is that blood-brain barrier structure or function may be affected, as it is the case in our model of AMPH-induced BD, when single colonies of *E. coli* were detected in bloodstream and brain. Thus, AMPH treatment could provide its effects indirectly through alterations in microbiota that can unbalance the gastrointestinal immune responses and influence distal effector sites leading to CNS disease including both demyelination and affective disorders [18].

Simultaneous study of the arginase activity was performed in the corticolimbic brain regions of AMPH-treated rats. As shown in Fig. 2, a, ARG1 activity was increased by 1.6, 1.6, 2.5 and 1.5 times in the cytoplasm of PFC, striatum, hippocampus and hypothalamus, respectively. Interestingly, ARG1 could exert fungicidal activity [19]. Thereby, ARG1 may modulate AMPH-induced changes in microbiota that in turn affect the metabolic pathways in CNS. Oxidative stress might be a trigger for ARG1, as superoxide anion ($O_2^{\bullet-}$) and H_2O_2 is shown can enhance mRNA content and ARG1 activity in the rat alveolar macrophages [20].

AMPH also increased the ARG2 activity of 2, and 1.4 times in the hippocampus and hypothalamus respectively, but no changes were observed in the PFC, and 1.6 fold decrease in the ARG2 activity was detected in the striatum (Fig. 2, b). It is of interest that ARG2 promotes macrophage inflammatory responses through mitochondrial reactive oxygen species ($O_2^{\bullet-}$ and H_2O_2) stimulating their production NOS/NO-independently [21]. Therefore, ARG2 may exert antibacterial activity, as

well as involved in the stimulation of ARG1 by releasing of reactive oxygen species in the brain regions studied, except striatum.

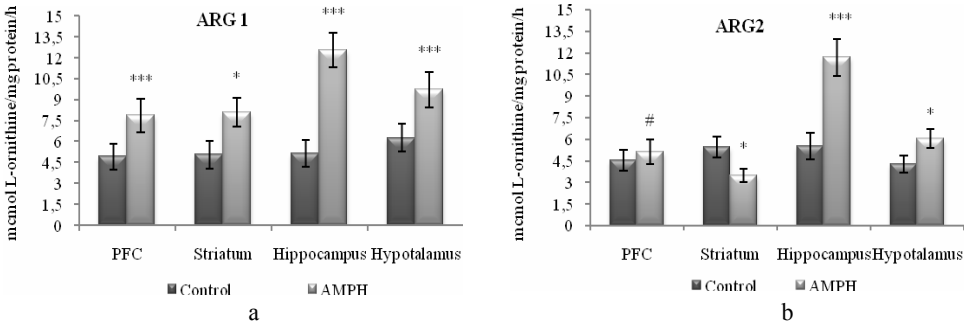

Fig. 2. Cytoplasmic arginase ARG1 (a) and mitochondrial arginase ARG2 (b) activities in the corticolimbic brain regions following long-term treatment with AMPH. Data are expressed as M ± S.E.M., n = 12, statistical comparisons were made by one way ANOVA followed by the post-hoc test of Holm-Sidak. Differences are considered significant if $p < 0.05$. # $p > 0.05$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Both ARG1 and ARG2 are stimulated in hippocampus following AMPH injections. It should be noted that hippocampal lesions produced moderate and reliable memory impairment in the novel object recognition task suggesting that hippocampus is important for object recognition memory [22]. Thus, we have demonstrated for the first time AMPH-induced activation of the arginase isoforms in the corticolimbic brain regions involved in the formation of emotions and memory consolidation.

An updated meta-analysis of oxidative stress markers in BD shows that lipid peroxidation, NO and DNA/RNA damage, are significantly increased in BD patients [23]. However, our regimen and dosage of AMPH treatment were mainly accompanied by a decrease in the intracellular content of NO stable metabolites in the rat corticolimbic brain regions (Fig. 3, a and b).

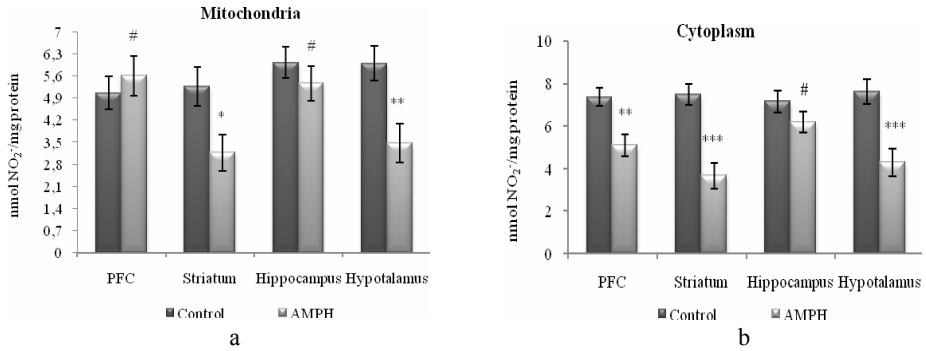

Fig. 3. Cytoplasmic (a) and mitochondrial (b) NO stable metabolite levels in the corticolimbic brain regions, following long-term treatment with AMPH. Data are expressed as M ± S.E.M., n =12, statistical comparisons were made by one way ANOVA followed by the post-hoc test of Holm-Sidak. Differences are considered significant, if $p < 0.05$. # $p > 0.05$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

NO stable metabolite levels were decreased of 1.5, 2.0, 1.2 (non-significant) and 1.8 times in the cytoplasm of the PFC, striatum, hippocampus and hypothalamus compared respectively to control. No changes in the content of NO metabolite were detected in the mitochondria isolated from PFC, and hippocampus, whereas of about 1.7-fold decrease was observed in those from the striatum, and hypothalamus (Fig. 3, b). It is not excluded, that under conditions of AMPH-induced oxidative stress a reduction of NO stable metabolite level occurred because of the rapid reaction NO with superoxide by formation of a potent oxidant, detrimental compound peroxynitite [24].

Previously, we demonstrated role of gut microbiota in the persistent activation of the mitochondrial iNOS in the corticolimbic brain that was involved in the mitochondrial dysfunction and other pathological processes following chronic stress-induced depression [25]. Importantly, *S. aureus* has been shown to elicit immune functions in both *microglia* and *astrocytes* on the level of responses to Toll-like receptors-2 that should trigger the innate immune effector molecules including iNOS [26]. LPS from bacterial translocation is responsible for TLR-4 activation in the brain and upregulation of the iNOS and associated depression-like behaviour of rats [27]. Presumably, AMPH-developed conditions will inevitably lead to the stimulation of iNOS/NO overproduction. We can speculate that concomitant stimulation of arginase isoforms could partially interfere with above mentioned processes preventing iNOS/NO overproduction and protect against detrimental effects of the reactive nitrogen species generated during oxidative stress in AMPH-induced BD. Arginase depletion of L-arginine reducing its bioavailability has proved that it is a rate limiting factor in NO synthesis, particularly via iNOS, a high-output form strongly dependent on the presence of arginine [28]. In addition, arginase produces urea, which inhibits a conversion of iNOS monomers to its active dymers [29]. Of interest, putrescine may also inhibit iNOS translation and NO overproduction [30].

Conclusion. Escalating non-neurotoxic doses of *d*-amphetamine sulfate injected to rats over 24 days induce changes in behavior mimicking BD accompanied by alterations in resident microbiota with manifestation of pathogenic bacteria. Both cytoplasmic and mitochondrial arginase isoforms are stimulated in the brain corticolimbic regions involved in the formation of emotions and memory consolidation. Simultaneously, NO stable metabolite levels are diminished or not changed in the cytoplasm and mitochondria of the brain regions studied in AMPH-treated rats. It is suggested that arginase isoforms may be contributed to the NO-synthase inhibition and prevention of detrimental effects of the reactive nitrogen species in BD.

Received 01.09.2016

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