

CHRONIC ALCOHOLISM-MEDIATED METABOLIC VIOLATIONS IN ALBINO RATS

BRAIN

ABSTRACT

Objectives: Despite of intensive investigation of alcoholism consequences for nervous system, the full complex of such metabolic alterations in brain remains unknown. The aim of present work was to study the influence of chronic ethanol consumption on rats' brain contents of free amino acids, rates of CYP2E1 and CYP3A2 expression, NO-synthase activities, and pro- and antioxidant system parameters. **Materials and Methods:** Wistar albino male rats (body weight (BW), 160–200 g) were divided into two groups: I – alcohol, II – normal animals. The content of amino acids in rat brain was determined using an amino acid analyzer. Brain CYP2E1 and CYP3A2 were evaluated with RT-PCR method, pro- and antioxidant system parameters –by standard methods. **Results:** Changes of brain free amino acids contents with alcoholism were demonstrated for 14 amino acids and their total sum. Chronic alcohol consumption caused 1.77 times increasing of CYP2E1 mRNA expression in rat's brain, 2.34 times – of CYP3A2 mRNA expression, 2.8 times - of cNOS activity, 42% - of TBARS formation rates. Activities of SOD and catalase were decreased 19% and 25% respectively. **Conclusions:** Thus investigation of alcoholism effects on brain levels of free amino acids permits to obtain complex estimation of this pathology influence on metabolic processes in brain, especially on amino acids, proteins, ATP and NADPH metabolisms. Alterations on the level of genome in our experiments were accompanied with complex alterations on the level of protein, amino acid and nitrous metabolisms.

Key words: chronic alcoholism, brain, rats

29 **Objectives**

30 At present alcohol has become an important socio-medical problem due to its massive
 31 uncontrollable consumption in the world. Chronic alcohol consumption causes serious
 32 consequences for entire nervous system functioning and, particularly, the brain [1], associated with
 33 metabolic processes complex violations [2]. In adult human chronic alcoholics, brain damage is
 34 characterized by cerebral and cerebellar atrophy, and impaired neuronal function within the
 35 hippocampus and frontal cortex. Besides specific alcohol-related disorders, such as Wernicke–
 36 Korsakoff syndrome, hepatic encephalopathy and pellagra, heavy alcohol consumers exhibit
 37 cognitive and motor impairments, cholinergic deficits and dementia. It is estimated that 50–75% of
 38 long-term alcoholics show cognitive impairment and structural damage to the brain, making chronic
 39 alcoholism the second leading cause of dementia behind Alzheimer’s disease [3].
 40 Neuropathological analyses have provided evidence for loss of neurons in certain regions of the
 41 brain of alcoholics [4].

42 Among several proposed mechanisms of brain damage at chronic alcoholism, should be
 43 mentioned accumulation of DNA damages in the absence of repair processes, resulting in genomic
 44 instability and death of neurons [3]. The processes of alcohol metabolism are also connected with
 45 generation of reactive oxygen species (ROS) and nitric oxide (NO) via induction of
 46 NADPH/xanthine oxidase, nitric oxide synthase (NOS), and CYP2E1 [5, 6]. Additionally, ethanol
 47 activates and recruits Toll-like receptor (TLR)4 receptors within the lipid rafts of glial cells,
 48 triggering the production of inflammatory mediators and causing neuroinflammation [7].

49 It is very important to note that synaptic changes promoted by ethanol are mediated
 50 directly and indirectly by acetylcholine, dopamine, serotonin, glutamate, GABA and other
 51 neurotransmitters [8].

52 However, despite of intensive investigation of alcoholism consequences for nervous system,
 53 the full complex of such metabolic alterations remains unknown, as soon as limiting and regulating
 54 factors of alcohol metabolism in brain [9]. Especially the contribution of chronic alcohol intake to
 55 changes of brain amino acids metabolism is not known, as soon as its influence on any particular
 56 amino acid contents and functions in nervous cells. Role of individual amino acids changes and
 57 their combined effects at alcoholism on brain proteins and nucleic acids structure and/or
 58 metabolism remains unclear.

59 Based on these facts and considering that alcoholism is chronic disease highly prevalent in
 60 the world population, the present work reports the influence of chronic ethanol consumption on rats’
 61 brain contents of free amino acids, rates of CYP2E1 and CYP3A2 expression, NO-synthase
 62 activities, and pro- and antioxidant system parameters.

63 **Material and methods**

Wistar albino male rats, body weight (b.w.) of 150 g to 170 g, were used in the study. They were kept under a controlled temperature (from 22 °C to 24 °C), relative humidity of 40 % to 70 %, lighting (12 h light-dark cycle), and on a standard pellet feed diet ("Phoenix" Ltd., Ukraine). The study was performed in accordance with the recommendations of the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes and approved by the Institutional Animal Care and Use Committee. For the experimental (chronic alcoholism) model, reproducing male rats were selected according to the method for measuring voluntary alcohol self-administration in rats, which provides a continuous choice between an alcohol solution and water (two bottle preference test) [10]. The six selected rats were used for chronic alcoholism modeling by replacing water with a 15 % ethanol solution during 150 days. Six intact male rats (of the same age and weight) were used as a control.

Six intact male rats (of the same age and weight) were used as controls. From the beginning of the experiment, they were kept in the same conditions as experimental animals but were given only water *ad libitum*.

After 150 days, both the experimental and control rats were sacrificed under a mild ether anaesthesia by decapitation. Their brains were used for investigation.

To brain homogenates samples (1ml) equal volumes of 3 % sulfa salicylic acid were added and obtained mixtures were left for 10 minutes in refrigerator at t+4⁰C. The formed sediments were removed by centrifugation (5000g, 10 min, 4 °C) [11]. Supernatants contained free amino acids from brains. Contents of free amino acids were determined on the amino acid analyzer AAA-881 (Czech Republic). The expression of CYP2E1 and CYP3A2 (ortholog of CYP3A4) mRNA in the brain was determined by a reversed transcriptase polymerase chain reaction (RT-PCR). Brain samples (50 mg) were collected, quickly frozen in liquid nitrogen, and stored at -80 °C before RNA extraction. The isolation of total mRNA was carried out with a TRI-Reagent (Sigma, USA). The integrity and concentration of RNA was analyzed in a 2 % agarose gel. First-strand complementary DNA (cDNA) was synthesized using a First-Strand cDNA Synthesis Kit (Fermentas, Germany). The reaction mixture contents for PCR, amplification protocol, and specific primers for the CYP2E1 gene were chosen according to SM Lankford et al. [12]. The primer sequences were: sense, 5'-CTTCGGGCCAGTGTTCAC-3' and anti-sense, 5'-CCCATATCTCAGAGTTGTGC-3'. The reaction mixture contents for PCR, amplification protocol, and specific primers for the CYP3A2 gene were chosen according to W Jäger et al. [13]. The primer sequences were: sense, 5'-TACTACAAGGGCTTAGGGAG-3' and anti-sense, 5'-CTTGCCTGTCTCCGCCTCTT-3'. RT-PCR with primers of β - actin sense, 5' -GCTCGTCGTCGACAACGGCTC - 3' and antisense 5' - CAACATGAT CTGGGTCATCTTCT -3') was carried out for internal control. All of the primers were synthesized by "Metabion" (Germany). The MyCycler thermocycler (BioRaD, USA)

99 was used for amplification. PCR products (CYP2E1-744 bp and β -actin-353 bp) were separated in
100 a 2 % agarose gel, stained with ethidium bromide, and visualized under a UV transilluminator
101 (BIORAD, USA). Data analysis was carried out with Quantity One Software (USA) and presented
102 in relative units as the ratio of CYP2E1 mRNA contents and β -actin mRNA.

103 iNOS and cNOS activities measure in brains were performed by spectrophotometric
104 method [14]. Concentration of protein in brain fractions was determined by the Bradford protein
105 assay using bovine serum albumin as a standard.

106 Lipid peroxidation was investigated as the rate of ascorbate-induced thiobarbituric acid
107 reactive substances (TBARS) formation [15], superoxide dismutase activity (SOD) was determined
108 in accordance to Chevary [16], catalase activity was detected using the colorimetric ammonium
109 molybdate method [17].

110 The obtained data were calculated by one-way analysis of variance (ANOVA) and
111 expressed as the mean $\pm$ standard error of the mean (M $\pm$ S.E.M.). Data were compared using Tukey
112 test. Differences were considered to be statistically significant at $p < 0.05$.

113 **Results**

114 Investigation of chronic alcoholism effects on rat brain contents of free amino acids (table
115 1) showed that statistically significant changes as compared with control were registered out for 14
116 amino acids and total sum of free amino acids.

117 Contents of lysine, histidine, arginine, ornithine, serine, glutamic acid, glycine, valine,
118 isoleucine, leucine, tyrosine, glutamine and total sum of free amino acids were decreased while
119 contents of methionine and phenylalanine – increased. The most profound changes were registered
120 for contents of serine (– 80%), lysine (-47,5%), ornithine (-55%), arginine (-45%), valine (-50%)
121 and tyrosine (-54%).

122 To evaluate the effect of 150 days ethanol exposure on transcriptional activation of
123 CYP2E1 and CYP3A2 in brain RT-PCR was performed.

124 According to data in figure 1 chronic alcohol consumption caused 1.77 times increasing of
125 CYP2E1 mRNA expression in rat's brain as compared with group of control animals. At the same
126 times CYP3A2 mRNA expression in ethanol-treated rat's brain increased 2.34 times (figure 2).

127 Results on brain nitric oxide synthases activities following 150 days alcohol administration
128 are demonstrated in figure 3. The level of iNOS in ethanol-treated rat's brain was within the normal
129 limits. At the same times cNOS activity was raised 2.8 times as compared with control.

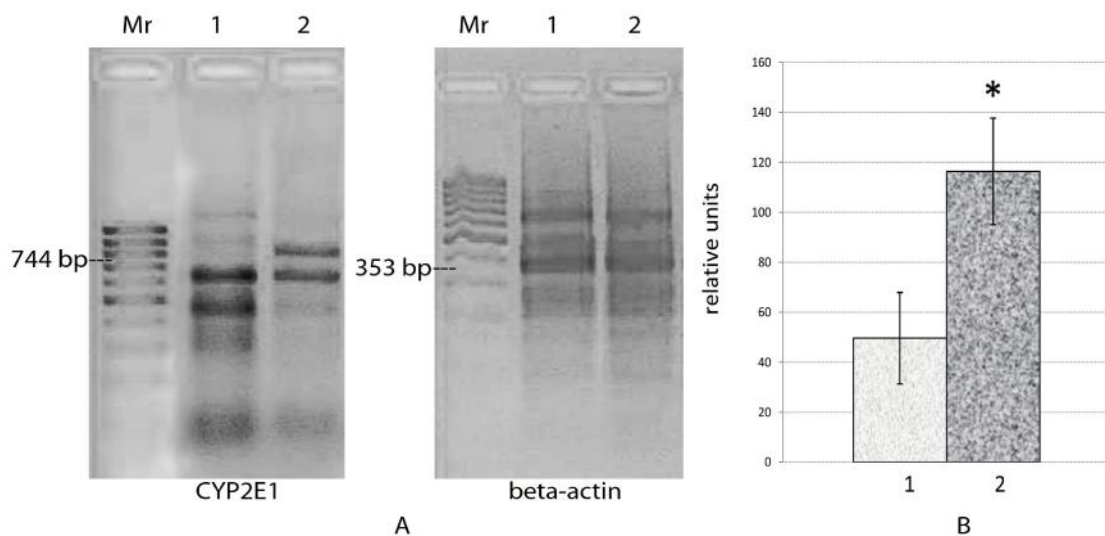
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131 Table 1 Rat brain contents of free amino acids in norm and with alcoholism. (M $\pm$ m, n=6, mg/ 100 g
132 of moist tissue)

Amino acid	Norm	Alcoholism
Lysine	2.00±0.06	1.05±0.04*
Histidine	0.80±0.02	0.50±0.02*
Arginine	2.00±0.04	1.10±0.01*
Ornithine	0.20±0.04	0.09±0.02*
Aspartic acid	18.70±2.50	12.80±3.07
Threonine	4.20±1.30	4.17±0.10
Serine	6.70±2.00	1.30±0.08*
Glutamic acid	133.2±4.80	116.70±1.70*
Proline	0.40±0.10	0.32±0.10
Glycine	4.10±0.10	3.00±0.10*
Alanine	6.00±1.09	5.00±0.10
Cysteine	1.70±0.50	1.20±0.12
Valine	0.80±0.10	0.40±0.05*
Methionine	0.40±0.05	0.60±0.05*
Isoleucine	0.40±0.10	0.28±0.07*
Leucine	0.90±0.20	0.60±0.16*
Tyrosine	1.30±0.10	0.60±0.05*
Phenylalanine	1.30±0.04	1.80±0.08*
Glutamine	13.50±1.20	10.00±0.80*
Total sum	221.10±18.40	173.11±10.77*

133

134 *- P<0.05 statistically significant in comparison with norm



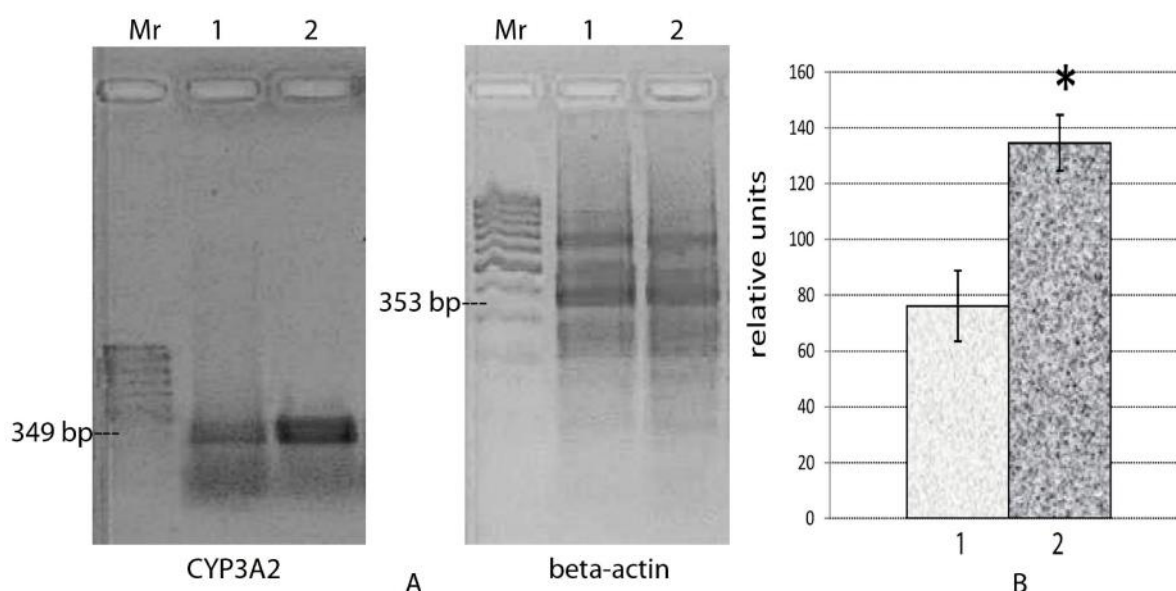
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136 Figure 1. CYP2E1 mRNA in rats' brain after 150 days 15% ethanol consumption:

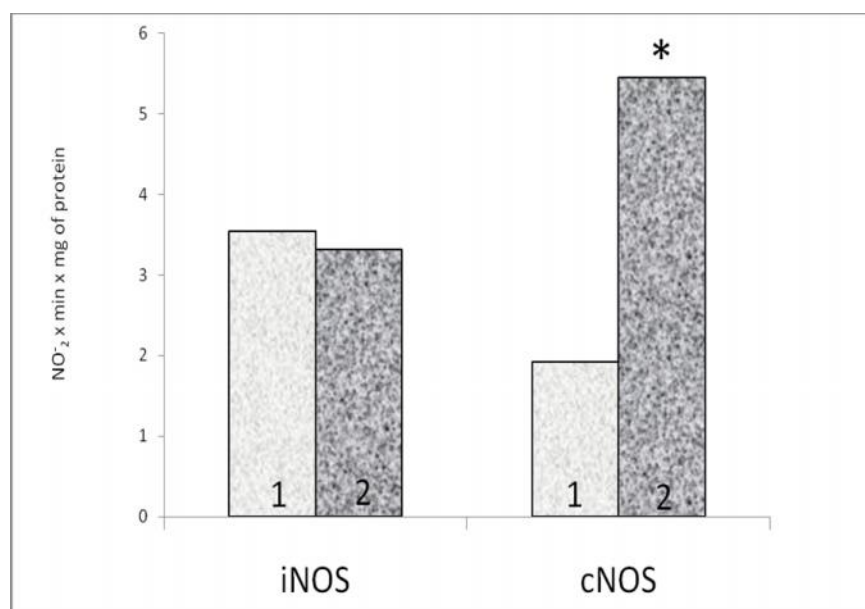
137 A – electrophoregram of CYP2E1 (744 b.p) and reference-gene β -actin (353 b.p.) RT-PCR products
 138 (arrows indicate appropriate DNA fragments): Mr – DNA marker, 1-control, 2- chronic alcoholism.

139 B) Average rate of CYP2E1 mRNA expression in rats' brain:1-control, 2- chronic alcoholism.

140 *- P<0.05 statistically significant in comparison with norm



141 Figure 2. CYP3A2 mRNA in rats' brain after 150 days 15% ethanol consumption:
 142 A – electrophoregram of CYP3A2 (349 b.p) and reference-gene β -actin (353 b.p.) RT-PCR products
 143 (arrows indicate appropriate DNA fragments): Mr – DNA marker, 1-control, 2- chronic alcoholism.
 144 B) Average rate of CYP3A2 mRNA expression in rats' brain: 1-control, 2- chronic alcoholism.
 145 *- P<0.05 statistically significant in comparison with norm
 146



148 Figure 3. Levels of iNOS and cNOS activities in rat' brain after 150 days 15% ethanol
 149 consumption (1 — control; 2 — chronic alcoholism).
 150 *- P<0.05 statistically significant in comparison with norm
 151

153 Data on brain pro- and antioxidant system state are shown in table 2.

154

155 Table 2 Rat brain pro- and antioxidant system state after 150 days 15% ethanol
156 consumption (M±S.E.M., n = 6).

Indices	Animal groups	
	Control	Chronic alcoholism
TBARS content, nmoles×mg of protein ⁻¹ ×min ⁻¹	86.80±3.29	123.02±2.95*
SOD activity, relative units×min ⁻¹ ×mg of protein ⁻¹	1.73±0.07	1.40±0.14*
Catalase activity, nmol H ₂ O ₂ min ⁻¹ ×mg of protein ⁻¹	1.30±0.10	0.97±0.03*

157 *- P<0.05 in comparison with control

158

159

160 Compared with the unaffected animals, ethanol-treated rats showed brain pro- and antioxidant
161 system alteration. The chronic 15 % ethanol administration led to increase of TBARS rate formation
162 (42 %). Under this condition the activities of antioxidant enzymes SOD and catalase were decreased
163 19% and 25% respectively.

164 Discussions

165 First of all character of amino acids changes at alcoholism could be evidence of
166 considerable general disturbances in metabolism of brain proteins [18]. Amino acid availability is a
167 rate-limiting factor in the regulation of protein synthesis [19]. Our results are in good accordance
168 with other authors' data [20], which demonstrated impaired protein synthesis with ethanol
169 consumption.

170 Changes of glutamic acid contents are of special interest. Glutamate, the major excitatory
171 transmitter in the vertebrate brain, is involved in neuronal development and synaptic plasticity.
172 Glutamatergic stimulation leads to differential gene expression patterns in neuronal and glial cells.
173 A glutamate-dependent transcriptional control has been established for several genes [21].
174 Glutamate is emerging as a major factor stimulating energy production in central nervous system.
175 Brain mitochondria can utilize this neurotransmitter as respiratory substrate and specific
176 transporters are required to mediate the glutamate entry into the mitochondrial matrix. Glutamate
177 transporters of the Excitatory Amino Acid Transporters (EAATs) family have been previously well
178 characterized on the cell surface of neuronal and glial cells, representing the primary players for
179 glutamate uptake in mammalian brain [22]. Thus observed in our experiments decreasing of free
180 glutamic acid contents in brain pools could cause profound changes in brains cells metabolism and

181 functioning with alcoholism.

182 Among this with alcoholism could be damaged amino acids exchange mechanism. The
183 system x(C)- (Sx(C)-) transporter functions as mediator of the exchange of extracellular cystine (L-
184 Cys(2)) and intracellular glutamate (L-Glu)[23, 24].

185 Observed changes of glutamine and ornithine contents could be an evidence of
186 disturbances in processes of amino acids excess catabolism in the urea cycle. Among this glutamine
187 is the major transport form of ammonia from all organs to the blood plasma [25, 26, 27]. In case of
188 its concentration decreasing this function could be partially substituted by free alanine, serine,
189 glycine and aspartic acid [25]. But with alcoholism contents of not only glutamine, but also alanine,
190 serine, glycine and aspartic acid are decreased in comparison with norm. It could affect the course
191 of nitrogen metabolism processes in brain and in the body as a whole.

192 Serine, arginine, and tyrosine can act as a NO depot, which is not only responsible for the
193 blood vessels relaxation, but also reacts with the iron atoms (in heme and free state), with
194 superoxide anions, oxygen molecules, hydroperoxide, organic peroxides and peroxide radicals [28].
195 Thus, changes of serine, arginine, and tyrosine contents with alcoholism in our experiments could
196 adversely affect at the level of NO, peroxidation rates and vascular system functioning both in brain
197 and whole organism [29].

198 Among this arginine could act as an antioxidant and powerful immunomodulator [30], as
199 soon as precursor in biosynthesis of polyamines, which regulate cells proliferation processes in
200 organism.

201 Serine is a key compound in biosynthesis of ethanolamine and phosphoethanolamine -
202 metabolites associated with the membranes structure and metabolism [30, 31, 32]: serine is
203 transformed into ethanolamine with further phosphorylation to phosphoethanolamine, followed by
204 methylation to phosphatidylcholine [25, 31]. In our experiments decreasing of serine contents at
205 alcoholism was accompanying by the opposite tendency of methionine content changes (required
206 for methylation processes). Possibly it could be a result of disturbances in membrane lipids
207 biosynthesis processes with alcoholism.

208 Chronic ethanol consumption also caused profound changes in contents of free amino acids
209 involved into processes of purines and pyrimidines biosynthesis (lysine, histidine, glutamic acid,
210 aspartic acid, glycine, glutamine, histidine, serine) [25], which may affect the biosynthesis of
211 nucleotides, nucleic acids and the cell chromosomal apparatus state.

212 According to some authors, the earliest manifestations of DNA repair processes violations
213 at the level of methylation may be free cysteine and methionine contents changes [33], such as we
214 demonstrated in our present experiments.

215 Special attentions deserve changes of methionine in brain pools. This amino acid could act

as antioxidant and immunomodulator [30, 34]. Among this, methionine is a precursor of taurine – antioxidant and membrane stabilizer. Therefore, increasing of its concentrations with alcoholism in our experiments can be regarded as a protective mechanism [30]. Methionine could be also precursor of polyamines – stimulators and regulators of proliferation processes [30, 35]. At alcoholism biosynthesis S-adenosylhomocysteine and homocysteine could be also broken [36]. On such possibility indicate changes in contents of methionine, glycine and serine – amino acids involved into synthesis of these compounds [35, 36]. Consequence of the homocysteine contents increasing is the accumulation of hydroperoxy radicals capable to damage cells [33, 35].

Changes of lysine contents could cause additional loss of leucine [33].

Essential for the brain cells normal functioning is their provision by energy resources (especially ATP). These cells actively catabolise branched chain amino acids with simultaneous energy storage in special molecules (mainly ATP) [25]. Therefore, in alcoholism observed changes of the amino acids involved in energy metabolism (valine, leucine, isoleucine) should be of special attention.

Detected changes of brain free amino acids contents at alcoholism may also indicate on disturbances in metabolism of ATP and NADPH both at the stage of glycolysis (changes of glycine, serine) and in the Krebs cycle (changes of glutamic acid, orn, glutamine, arginine, valine and methionine in serum) [37].

Among this decreasing of alanine contents could be evidence of NH_4^+ - transport disturbances [25]. Decrease of both valine and alanine concentrations in brain pools may cause disturbances in proteins catabolism and glucoso-alanine cycle [25, 38].

Changes of phenylalanine contents at alcoholism could be an evidence of great disturbances in phenylalanine hydroxylase functioning at this pathology [33].

Changes of such amino acids as glycine in brain pool are of special importance because of its involvement (with other amino acids) into glutathione biosynthesis [33, 35]. These data are in correspondence with our results on GSH contents changes at alcoholism. On the other hand as glutathione realize trans-membrane transport of amino acids [33, 39, 40], its contents changes could cause changes in free amino acids contents, especially essential [33, 39, 40].

Brain P450s are regulated differentially from those in liver [41]. The availability of the prosthetic heme group appears to be essential for correct membrane insertion and enzymatic functionality of brain P450s. Furthermore, although not contributing to body's overall drug metabolism, brains P450s fulfill particular functions within specific cell types of the brain. In astrocytes of brain's border lines P450 isoforms are expressed at very high level. They form a metabolic barrier regulating drugs' influx, modulate blood-flow regulation, and act as signaling enzymes in inflammation. In neurons, however, P450s apparently have different function. In

specified brain regions such as hypothalamus, hippocampus and striatum they provide signalling molecules like steroids and fatty acids necessary for neuronal outgrowth and maintenance. Induction of these P450s by neuroactive compounds can alter steroid hormone signalling directly in target cells, which may cause reproductive disorders and sexual or mental dysfunction [41].

In our experiments violation in brain free amino acids homeostasis were accompanied with increased expression of cytochromes P450 2E1 and P4503A2 (ortholog of human 3A4) in brain tissues. Our data was in good correspondence with other investigations [42, 43]. The expression of cytochrome P4502E1 (CYP2E1) in the brain with alcoholism has been demonstrated in several regions. This enzyme catalyzes first two reactions of acetone transformation in the lactic acid, ethanol oxidation, metabolism of fatty acids and their hydroperoxides [44]. It has long been suggested that some of the neuropharmacological, neurochemical and behavioural effects of ethanol are mediated by its first metabolite, acetaldehyde. Acetaldehyde, formed in the brain tissues through the peroxidatic activity of catalase and by oxidation via cytochrome P-4502E1, may mediate some of the central nervous system actions of ethanol including tolerance and dependence[45] Genetic investigations demonstrated that sequence changes in or near CYP2E1 affect the level of response to alcohol providing a predictor of risk of alcoholism [46]. Implicating CYP2E1 in the level of response to alcohol allows inferences to be made about how the brain perceives alcohol. Also CYP2E1 appears to play a key role in 3-NT formation, protein degradation, and brain damage, which is independent of NOS [47].

Cytochrome P450 3A4 (CYP3A4) metabolizes approximately 50% of the drugs in organism [43]. Although CYP3A4 induction by ethanol is known, CYP3A4-changes interrelations with other alterations during chronic alcohol consumption are unclear. Also little is known about the expression of CYP3A proteins in the brain with alcoholism [48]. Both CYP3A4 and CYP3A5 immunoreactivity were found to varying extents in the microsomal fractions of cortex, hippocampus, basal ganglia, amygdala, and cerebellum. However, only CYP3A4 expression was observed in the mitochondrial fractions of these brain regions. Remains unclear how ethanol consumption influences on expression of CYP3A subfamily in different brain regions.

Our data along with other authors' results demonstrate that comparative expression studies are an efficient approach to discover interacting gene and metabolic networks that underlie the development of organism's final reaction to alcohol chronic consumption [49].

Increased cellular oxidative stress and altered antioxidant pool have been implicated at alcoholism. Our data on state of brain's pro- and antioxidant system at alcoholism coincide with data of other authors. Ethanol enhances ROS production in brain through a number of pathways including increased generation of hydroxyl ethyl radicals, induction of CYP2E1, alteration of the cytokine signaling pathways for induction of iNOS and sPLA(2), and production of prostanoids

286 through the PLA(2)/COX pathways. Excess of free oxygen radicals could cause brain tissue
287 damage not only via lipid peroxydation, but also proteins denaturation, enzymes inactivation,
288 nucleic acids damage, Ca^{2+} release and cytoskeleton destruction. Influence of free radicals mediates
289 blood-brain barrier functions violations, endothelial dysfunctions with constant vasodilatation [50].

290 Our results on cNOS activity rates at alcoholism are in good correspondence with other
291 authors data [51]. According to their results glutamatergic system is directly involved into
292 mediating acute and chronic alcohol effects. Its receptors are included into NO signal way, as
293 NDMA glutamatergic receptors stimulation caused Ca^{2+} exit from the depot with further calmodulin
294 binding and nNOS activation. nNOS gene is of key importance for regulation behavioural effects of
295 alcohol [51].

296 **Conclusions**

297 Thus investigation of alcoholism effects on brain levels of free amino acids permits to obtain
298 complex estimation of this pathology influence on metabolic processes in brain, especially on
299 amino acids, proteins, ATP and NADPH metabolisms. Our results may possibly indicate on serious
300 changes in brain amino acids, other nitrogenous compounds, ATP and NADPH metabolisms during
301 chronic ethanol consumption. Alterations on the level of genome in our experiments were
302 accompanied with complex alterations on the level of protein, amino acid and nitrous metabolisms.

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