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Seven-day ethanol administration influence on the rat brain histaminergic neurons

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ABSTRACT

The purpose of the study is to clarify the effect of 7 days of ethanol administration upon brain histaminergic neurons in rats. Male Wistar rats were injected intraperitoneally (i.p.) with 20% ethanol/saline (0.85% NaCl) daily, over 7 days, whereas control rats were given saline. The animals were decapitated 24 h after the 7th injection and samples of hypothalamus were prepared for light and electron microscopy, accompanied by morphometry to examine the histaminergic neurons. It was found that ethanol administration gradually decreased the duration of alcohol-induced sleep and decreased the total amount of histaminergic neurons and the amount of histologically normal neurons, but increased the amount of hypochromic neurons and shadow cells. The histaminergic neuron bodies and nuclei decreased in size. The ultrastructural changes in histaminergic neurons demonstrated activation of their nuclear apparatus, both destruction or hypertrophy and hyperplasia of organelles, especially lysosomes. The histochemical examination revealed the activation of lactate dehydrogenase and acid phosphatase, and inhibition of NADH–, NADPhH, and succinate dehydrogenases. Following 7 days of ethanol administration, histaminergic neurons exhibit the structural signs of hyperactivity, which can be related to neuronal adaptation to the actions of ethanol, and increased behavioral tolerance to ethanol.

Introduction

The histaminergic system is one of the most important and well-established neurotransmitter systems of the brain. The bodies of histaminergic neurons are situated in the posterior hypothalamus only, forming five cluster groups, E1–E5 (Brown, Stevens, & Haas, 2001; Zimatkin, Kuznetsova, & Strik, 2006). Their processes reach all brain regions, and regulate the activity of other neurotransmitter systems and brain functions. The brain histaminergic system participates in neuroendocrine and cardiovascular regulation, brain blood flow, sleep and wakefulness, hibernation, feeding and drinking behavior, memory, cognition, and learning. It is involved in some pathological conditions and diseases, including addiction (reviews of Blandina, Munari, Provensi, & Passani, 2012; Brown et al., 2001; Haas & Panula, 2003; Haas, Sergeeva, & Selbach, 2008; Zimatkin, 2007).

There are data suggesting interactions between brain histamine and ethanol, and participation of the brain histamine system in alcohol-related behavior and alcoholism pathogenesis (reviewed in Panula & Nuutinen, 2011; Zimatkin, 2007; Zimatkin & Anichtchik, 1999). The histamine and ethanol metabolic pathways in the brain both utilize the enzyme aldehyde dehydrogenase. Therefore, the highly active ethanol metabolite, acetaldehyde, can interfere with histamine degradation by competition with N–tele–methylimidazole acetaldehyde for this enzyme (Ambroziak & Pietruszko, 1987). This may be the metabolic basis for the alcohol–histamine interactions in the brain (Zimatkin & Anichtchik, 1999). Our previous investigations demonstrated that histamine H1–receptor antagonists that pass the blood–brain barrier increase ethanol metabolism in rats, but decrease tolerance to the hypnotic effects of ethanol, because they increase the sensitivity of the brain to ethanol (Zimatkin, Liopo, & Zakharov, 1997).

High sensitivity of histaminergic neurons to alcohol can be theoretically predicted because of high activity of the ethanol-oxidizing enzyme catalase and low activity of aldehyde dehydrogenase, providing the conditions for toxic acetaldehyde accumulation in brain aminergic neurons (Zimatkin & Lindros, 1996). The influence of alcohol on the brain histamine level and metabolism has been studied biochemically in brain homogenates (Fogel, Andrzejewski, & Maslinski, 1991; Nowak & Maśliński, 1984; Prell & Mazurkiewicz-Kwilecki, 1981; Subramanian, Schinzel, Mitznegg, & Estler, 1980). The effects of acute ethanol administration on histamine levels in the

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brain strongly depend on the dose of alcohol, the species of experimental animals, and the brain structure studied. The activity of the histamine-synthesizing enzyme histidine decarboxylase has been reported to be increased after the administration of ethanol in the hypothalamus, midbrain, and brain cortex of adult rats (Prell, Bielkiewicz, & Mazurkiewicz-Kwilecki, 1982), but decreased in the brain cortex and thalamus (Subramanian et al., 1980). Almost all authors consider that following alcohol administration, the activity of the histamine-degrading enzyme histamine N–methyltransferase failed to change in any brain region (Prell et al., 1982; Prell & Mazurkiewicz-Kwilecki, 1981; Subramanian et al., 1980). Ethanol increases the steady-state N–tele-methylhistamine levels in the mouse hypothalamus, probably by inhibiting the elimination of this metabolite in the brain (Itoh, Nishibori, Oishi, & Saeki, 1985). Thus, the biochemical data indicate that alcohol can influence histamine content and metabolism in the brain.

Our previous investigation for the first time demonstrated the effect of single alcohol administration on the morphology and metabolism of brain histaminergic neurons (Zimatkin, Fedina, & Kuznetsova, 2013). The aim of the present study is to estimate the effect of subacute (7 days) ethanol administration on alcohol tolerance (sensitivity of rats to hypnotic effect of ethanol) and the histology, histochemistry, and ultrastructure of brain histaminergic neurons.

Materials and methods

Animals, chemicals, and experimental design

Eighteen male Wistar rats were obtained from the breeding colony of the Grodno State Medical University. Their weight was 175 ± 23 g. All experimental procedures complied with European Community Council Directive (86/609/EEC) for care and use of laboratory animals. Protocols were reviewed and approved by the Ethical Committee of the Grodno State Medical University (protocol #1, 20.01.2010). All efforts were made to minimize animal suffering. Rats were housed in cages with free access to food and water and kept under controlled environmental conditions. A solution of 20% ethanol/saline (0.85% NaCl) was injected i.p. into nine rats at a dose of 4 g/kg daily, between 9:00 and 11:00 AM for 7 days. After every ethanol injection, the duration of alcohol-induced sleep was measured by the interval between loss and recovery of the righting reflex as the method to measure sensitivity to the hypnotic effect of ethanol. Nine control animals were injected with saline, with volumes corresponding to the injections received by the ethanol-injected rats. All rats were decapitated 24 h following the last injection.

All the chemicals were obtained from Sigma–Aldrich (USA).

Histology and histochemistry

Six rats from each group were anesthetized, and fresh brain was removed following decapitation. Pieces of hypothalamus were then obtained, frozen, and stored in liquid nitrogen for further analysis. For light microscopy, 10–μm serial sagittal sections of the frozen hypothalamus were prepared using a cryostat (Leica CM 1840, Germany). They were stained with a 0.1% solution of toluidine blue (Nissl method) to assess general cytology of neurons. To determine the activity of the marker enzyme of histaminergic neurons, monoamine oxidase type B (MAO B, EC 1.4.3.4), was detected using the method published by us earlier (Zimatkin & Tsydik, 1996). It has been confirmed that MAO B is a good histochemical marker for histaminergic neurons and their grouping in rat hypothalamus (Zimatkin et al., 2006). To assess the activity of the oxidizing enzymes (such as succinate dehydrogenase [SDH, EC 1.3.99.1], lactate dehydrogenase [LDH, EC 1.1.1.27], glucose-6–phosphate dehydrogenase [G–6–PDH, EC 1.1.1.49], NADH dehydrogenase [NADHDH, EC 1.1.1.49] and NADPhH dehydrogenase [NADPhDH, EC 1.6.1.1]) and to estimate the activity of lysosomal enzyme acid phosphatase (AP, EC 1.4.3.4), we used methods described in Pearse, 1960. Briefly, to assess enzyme histochemistry, the cryostat sections were placed into the corresponding incubation medium consisting of buffer, substrate, co-factor (if necessary), and chromogen for times ranging from 30 min to 5 h, to visualize the location of enzymatic activity. Sections were then washed and embedded in a suitable plastic medium.

Microphotography and morphometric analysis of histaminergic neurons

For the identification of histaminergic neurons in brain sections, the stereotaxic atlas and corresponding topographic schemes were used (Paxinos & Watson, 2007; Zimatkin et al., 2006). The hypothalamus sections for histology and histochemistry were made in parallel (as serial-sections ribbon), and the location of histaminergic neurons was compared with preparations stained for MAO B. The location of E2 histaminergic neurons is shown in Fig. 1.

The microphotography and morphometry of histological preparations were performed using an Axioskop 2 plus microscope (Zeiss, Germany), equipped with a digital camera (Leica DFC 320, Germany). Image analysis was done using Image Warp software (Bit Flow, USA). In Nissl-stained preparations, all visible histaminergic neurons were estimated according to their type of chromatophilia (the intensity of staining of neurons cytoplasm) and divided into normochromic (normal, medium staining), hyperchromic (intensive staining), hypochromic (pale staining), and shadow cells (very pale remnants of dyed neurons with no visible nucleus).

To estimate the size and shape of neuronal bodies and nuclei in preparations stained by the Nissl technique, the images of up to 30 histaminergic neuron bodies and their nuclei were outlined in every preparation on the computer monitor and the mean values were used for further statistics. Maximal and minimal diameter (D), perimeter (P), area (A), and volume, as well as form factor ($4\pi A/P^2$ – parameter of sphericity and folding) and factor of elongation (maximal D/minimal D – parameter of sphericity) were quantified in Nissl-stained neuron bodies (perikaryons).

The enzyme activities were determined in the cytoplasm of neurons by the optical density of chromogen obtained in the course of histochemical reactions.

Electron microscopy

For electron microscopy, the samples of posterior hypothalamus from three controls and three alcohol-injected rats were fixed in 2.5% glutaraldehyde in Millonig buffer (pH 7.4) for 4 h at 4 °C. Then the lateral parts of the posterior hypothalamus, where the histaminergic neurons of the largest group, E2, are situated, were fixed in 1% osmium tetroxide in Millonig buffer (pH 7.4) for 1 h at room temperature (Millonig, 1961), dehydrated in an increasing concentration of ethanol and acetone, and embedded in epoxide gum. The sections were obtained with ultramicrotome MT–7000 (RMC, USA), contrasted by uranyl acetate and lead citrate (Reynolds, 1963), and examined with transmission electron microscope JEM–1011 (JEOL, Japan). Ultraphotographs were acquired by digital camera (Olympus MegaView III, Germany). The morphometry of ultrastructures was carried out with image analysis iTEM software (Olympus Soft Imaging Solutions, Germany). Mitochondrial and lysosomal profiles were outlined by a cursor on the computer monitor to estimate their number, and their individual and relative areas in the cytoplasm of histaminergic neurons.

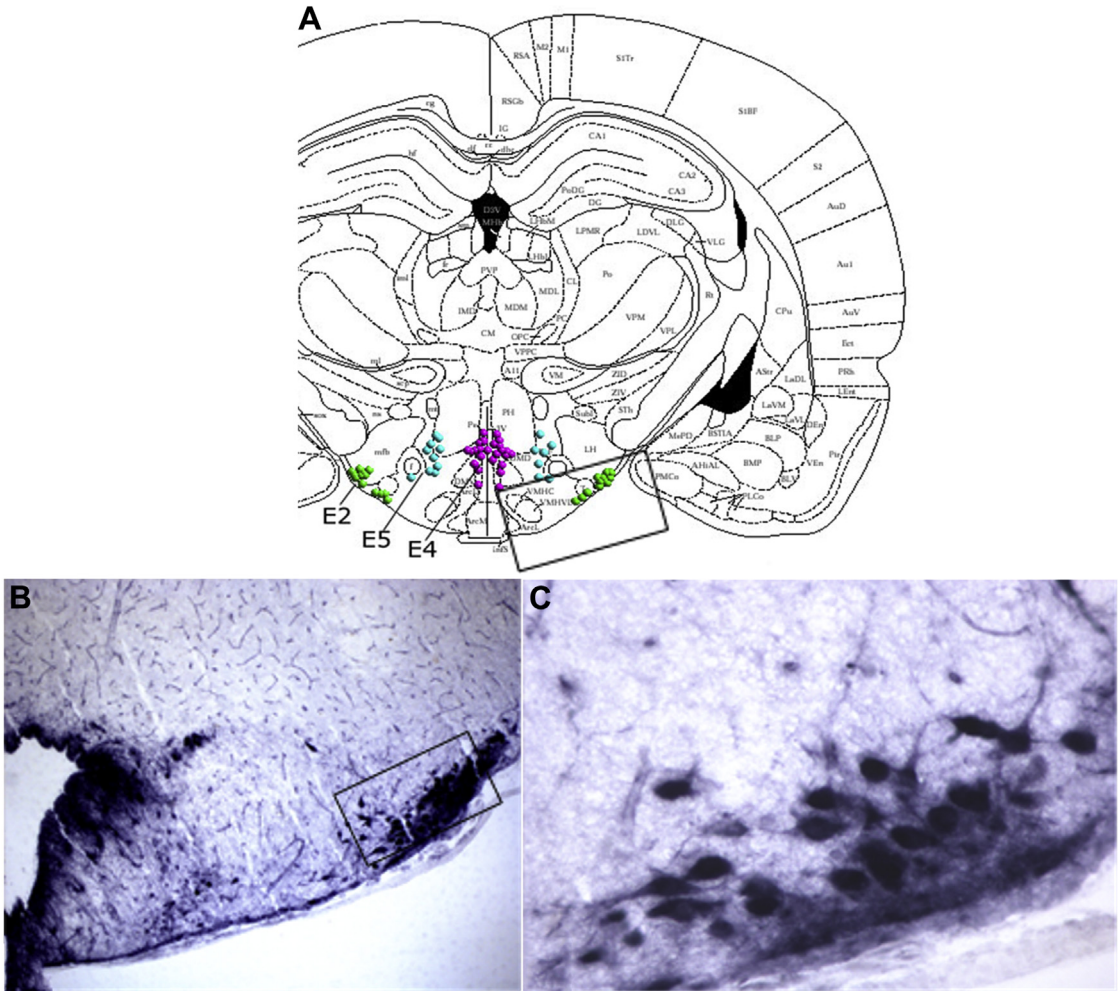

Fig. 1. Location of E2 histaminergic neurons in rat hypothalamus. (A) Histaminergic neurons of E2 nucleus on a diagram from a stereotaxic atlas. (B) Histochemical staining of histaminergic neurons for MAO B (detail of rectangular box in A). Magnification: ×40. (C) Histochemical staining of histaminergic neurons for MAO B (detail of rectangular box in B). Magnification: ×200.

Statistics

The primary data obtained were processed with nonparametric statistics (because of the small number of animals in the groups) using software STATISTICA 6.0 (StatSoft, Inc., USA). In descriptive statistics, the values of median (Me) and interquartile range (IQR) were determined. The differences were considered significant at $p < 0.05$ (Mann–Whitney U test).

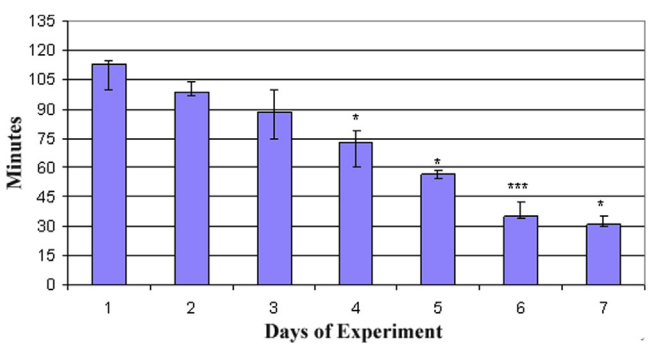

Fig. 2. Duration of alcohol-induced sleep in rats during 7-day i.p. administration of ethanol at a dose of 4 g/kg/day. Data are presented as median ± interquartile range; $^{*}p < 0.05$, $^{***}p < 0.001$, as compared to controls.

Results

The 7-day i.p. ethanol administration at a dose of 4 g/kg gradually decreased the mean duration of alcohol-induced sleep (4–fold, from 115 to 30 min). The differences became significant, as compared to controls, starting from the 4th day (Fig. 2).

Morphometric and histochemical changes in histaminergic neurons following ethanol administration

Histological examination of hypothalamus histaminergic neurons in rats within 24 h following 7-day ethanol administration has revealed a decline in the amount of normal, normochromic

Table 1
Types of chromatophilia (in %) of histaminergic neurons in rat hypothalamus after 7 days of ethanol administration at 4 g/kg.

Types of neurons	Control ($n = 6$)	Alcohol ($n = 6$)
Normochromic neuron	90.65 ± 7.99	78.54 ± 9.45
Hypochromic neuron	3.70 ± 2.54	11.33 ± 4.43*
Hyperchromic neuron	3.15 ± 3.00	4.67 ± 6.34
Shadow cell	2.79 ± 1.65	6.80 ± 4.33*

Data are presented as median ± interquartile range; $^{*}p < 0.05$, as compared to controls.

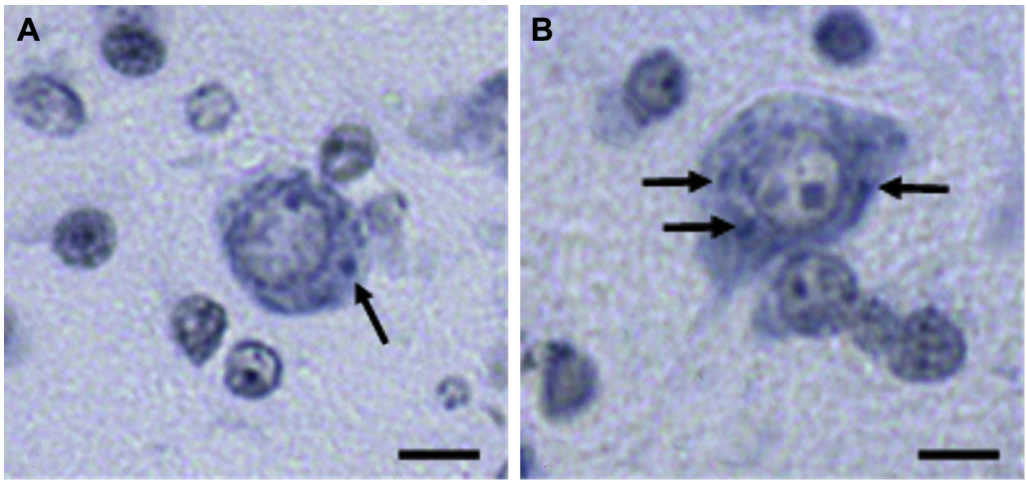

Fig. 3. Histaminergic neurons of hypothalamus in rats 24 h after the 7th alcohol administration. (A and B) Arrows indicate nucleolus-like bodies in cytoplasm of neurons. Stained by Einarson. Scale bars = 10 μm.

neurons, but a significant increase in the amount of hypochromic neurons and shadow cells (Table 1). The amount of histaminergic neurons per area of section decreased by 4% ($p = 0.016$). In some histaminergic neurons stained for nucleic acid determination, the increased size of nucleoli and their shift to the nuclear border were observed, as well as the appearance in cytoplasm of the round or oval-shape bodies resembling the nucleoli. They may be called "nucleolus-like bodies" (Fig. 3).

Image analysis following ethanol administration revealed the decrease in minimal and maximal diameter, perimeter, area, and volume of histaminergic neurons. Form factor increased by 8.6%, as compared to controls (Table 2). Similar changes were found in neuronal nuclear size and shape. The nuclear/cytoplasmic ratio was not altered (not shown).

Histochemical investigation demonstrated that 24 h after the 7-day ethanol administration, the MAO B activity in the cytoplasm of histaminergic neurons had not changed. The activity of SDH had decreased by 13.7%, NADHDH activity had decreased by 7%, and NADPhHDH activity had decreased by 16%, but the activity of LDH had increased by 23% and the activity of acid phosphatase had increased by 31% (Figs. 4 and 5).

Ultrastructural alterations of histaminergic neurons following ethanol administration

Twenty-four hours after the 7-day ethanol administration, folding of the nuclear envelope was found to have increased, as well as the extension of perinuclear space (Fig. 6B and E), as compared to controls (Fig. 6A). Some nuclei contained unusual vacuoles of polygonal shape surrounded by a single biological membrane (Fig. 6C). There was an increase in nucleoli and translocation of nucleoli to the nuclear envelope, and an aggregation of ribosomal subunits between nucleoli and the nuclear envelope (Fig. 6D), which are signs of their increased transport to the cytoplasm through the nuclear pores (not shown).

Alcohol induced various changes to the organelles in the cytoplasm of histaminergic neurons. There was hypertrophy of the Golgi complex, and extension of the canals and cisterns of the endoplasmic reticulum (Fig. 6C–E). The amount of binding ribosomes noticeably decreased, but the amount of free polyribosomes in cytoplasm increased (Fig. 6D–F). In some neurons, the Golgi cisterns were located concentrically, forming unusual circular, lamellar bodies (Fig. 6B).

The most prominent ultrastructural feature caused by ethanol's action on histaminergic neurons was the appearance of round or oval-shape bodies about 1–2 μm in size, consisting of osmeophilic granular aggregations. They closely resembled the nucleoli and may be called "nucleolus-like bodies" (Fig. 6E).

After 7 days ethanol administration, most mitochondria in histaminergic neurons contained the densely packaged crista and dark osmeophilic matrix (Fig. 6E and F). Both the amount and comparative area of mitochondria per area of neuronal cytoplasm and the form factor increased significantly (Fig. 6F; Table 3).

The amount and size of lysosomes in the cytoplasm of histaminergic neurons following 7-day ethanol administration visibly increased (Fig. 6B and D). The morphometric analyses have also revealed a significant increase in their amount, area, and relative area in cytoplasm; the factor of elongation of lysosomes decreased (Table 4).

Table 2
Morphometric parameters of histaminergic neuron perikaryons in rat hypothalamus after 7-day ethanol administration at 4 g/kg.

Parameters	Control ($n = 6$)	Alcohol ($n = 6$)
Minimal diameter (μm)	12.27 ± 0.70	11.55 ± 0.38*
Maximal diameter (μm)	19.12 ± 0.52	17.56 ± 1.02*
Perimeter (μm)	54.96 ± 2.64	49.36 ± 4.32**
Area (μm^2)	182.57 ± 22.73	161.46 ± 32.52*
Volume (μm^3)	1856.36 ± 343.25	1544.32 ± 468.14*
Form factor	0.75 ± 0.06	0.82 ± 0.06**
Factor of elongation	1.53 ± 0.12	1.46 ± 0.21

Data are presented as median ± interquartile range; *$p < 0.05$, **$p < 0.01$, as compared to controls.

Discussion

The duration of alcohol-induced sleep gradually decreased over the course of the 7 days of alcohol administration, at a dose of 4 g/kg/day. This suggests the formation of a behavioral tolerance to the hypnotic effects of ethanol in animals. Since one of the main functions of brain histaminergic neurons is the regulation of sleep and wakefulness, their involvement in alcohol-induced sleep and awakening have been suggested (Haas et al., 2008).

Twenty-four hours after the 7th ethanol administration at a dose of 4 g/kg, the histaminergic neuron bodies became smaller and more spherical. The possible reason for this might be disturbances

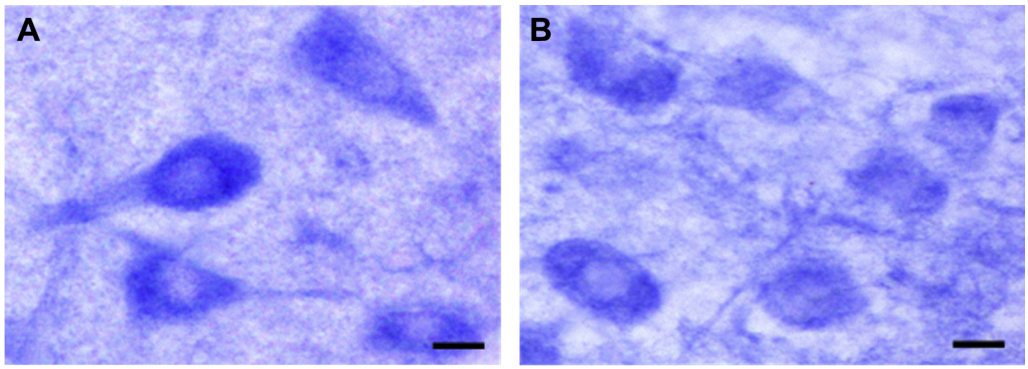

Fig. 4. NADPhH dehydrogenase activity in cytoplasm of histaminergic neurons of the E2 hypothalamic nucleus of control rats (A) and 24 h after 7-day ethanol administration at a dose of 4 g/kg/day (B). Stained by the Hess, Scarpelli, Pearse technique. Scale bars = 10 μm.

of the electrolyte balance and alteration of the cytoskeleton of neurons, induced by ethanol. It may also be the way of adaptation of neurons to the predictable alcohol-induced swelling of their bodies (Zimatkin et al., 2013).

The activity of the marker aerobic oxidative enzymes SDH, NADHDH, and NADPhHDH in the cytoplasm of brain histaminergic neurons decreased, but the activity of the marker enzyme of anaerobic glycolysis, lactate dehydrogenase, increased. This probably indicates both the disturbances of energy metabolism and metabolic adaptation of neurons to alcohol, taking into consideration the alcohol-induced hypoxia.

On the ultrastructural level, histaminergic neurons of animals at 24 h following the 7-day ethanol administration displayed some compensatory, adaptive changes, intended to increase the viability of these neurons during the next possible exposure to ethanol. The signs of the activation of their nuclei were observed: increased size of nucleoli, the shift of nucleoli to the nuclear border, an aggregation of ribosomal subunits between nucleoli and nuclear envelope, the signs of their increased transport to the cytoplasm through the nuclear pores, broadening of the perinuclear space, and increased folding of the nuclear envelope. In addition to hypertrophy and hyperplasia of the endoplasmic reticulum and Golgi complex it may reflect an activation of the synthetic processes in those neurons. In particular, the increase in the comparative amount of free ribosomes may indicate the switching of the protein biosynthesis for the cell's own needs to compensation for the damaged or lost structures following the alcohol exposure. It appears that brain histaminergic neurons have prepared for the next possible alcohol exposure.

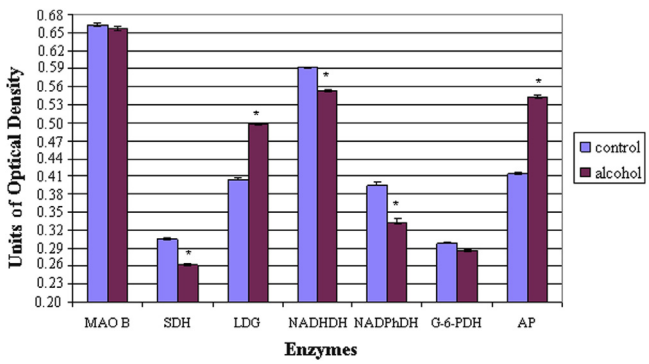

Fig. 5. Changes of enzyme activities in cytoplasm of brain histaminergic neurons 24 h after 7 days of ethanol administration at a dose of 4 g/kg/day. The enzymes studied (as described in the Materials and methods section) are represented on the horizontal axis, and the optical densities of chromogen obtained in the course of corresponding histochemical reactions are plotted on the vertical axis. Data are presented as median ± interquartile range; $^{*}p < 0.05$, as compared to controls.

The "nucleolus-like bodies" observed in the cytoplasm of histaminergic neurons following the 7-day ethanol administration were described earlier in neurons and glial cells of hypothalamus in other experimental conditions (Kawabata, 1965). In some publications they are called processing bodies or stress granules (Decker & Parker, 2012; Thomas, Loschi, Desbats, & Boccaccio, 2011). They contain the RNA-binding proteins and mRNA. They regulate the transcription and are involved in mRNA degradation or storage, contributing to cell survival (Decker & Parker, 2012; Thomas et al., 2011).

The amount and relative area of mitochondria in neuronal cytoplasm were significantly increased. They contained the densely packaged crista, to be used for intensive oxidation of the first, and most toxic, ethanol metabolite acetaldehyde. Some of the mitochondria were in close proximity to the nucleus, endoplasmic reticulum, and Golgi complex to provide energy for upcoming intense metabolic demands. The flat Golgi cisterns forming the unusual circles were observed in some histaminergic neurons. It is not clear whether this is a feature of the organelles' degeneration or their hyperactivity.

The significant increase in the amount and size of lysosomes in histaminergic neurons accompanied by an activation of the lysosomal marker enzyme acid phosphatase may be a process of the neurons preparing for increased autophagy for the removal of alcohol-damaged microstructures.

Previous studies have found that acute administration of ethanol to experimental animals increased, did not change, or decreased the level of histamine in the brain (Itoh et al., 1985; Nowak & Maśliński, 1984; Prell et al., 1982; Rawat, 1980; Subramanian et al., 1980). It is difficult to correlate our results with the above-mentioned biochemical data, because we examined the morphology of histaminergic neurons, while the above-mentioned studies were performed in homogenates of the whole brain or its large dissected regions, which invariably includes various types of cells.

On the other hand, these dramatic disturbances in brain histaminergic neurons were observed following 7-day ethanol administration: reduction in the number of brain histaminergic neurons per section area, an increase in the amount of abnormal, hypochromic neurons, and an increase in shadow cells among the surviving neurons. These results can be the consequence of neurotoxic effects of ethanol. As we examined specifically the histaminergic neurons of hypothalamus only, it is not clear whether the effects of ethanol were restricted to the histaminergic neurons or whether all neurons in the slice showed similar effects. This may be the aim of another investigation.

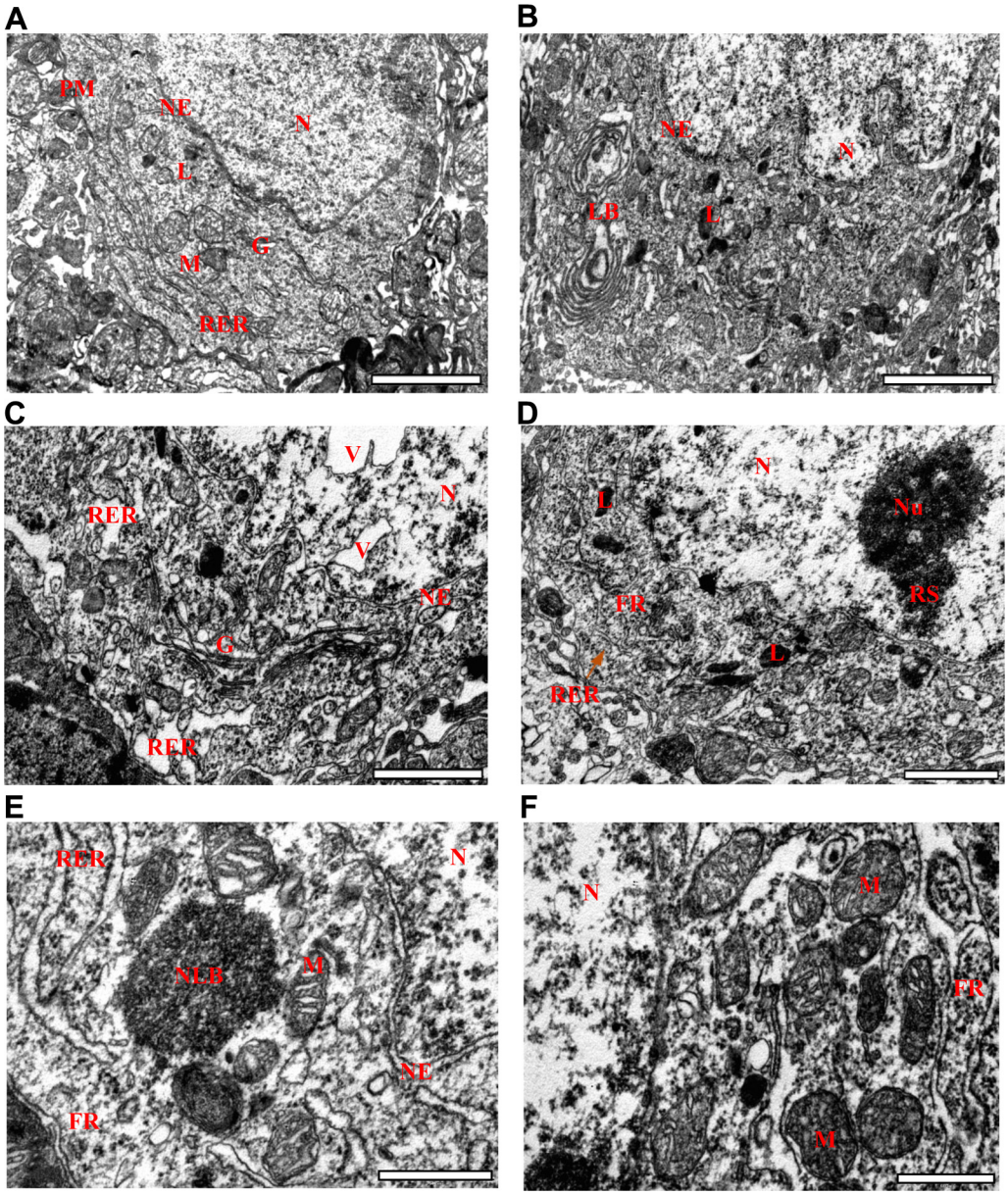

Fig. 6. Fragments of hypothalamus E2 neurons of control rat (A) and after 7 days of ethanol administration at a dose of 4 g/kg/day (B–F). Designations: FR – free ribosomes; G – Golgi complex; L – lysosome; LB – lamellar bodies; M – mitochondria; N – nucleus; NE – nuclear envelope; NLB – nucleolus-like body; Nu – nucleolus; PM – plasma membrane; RER – rough endoplasmic reticulum; RS – aggregation of ribosomal subunits; V – vacuole. Scale bars: A and B – 2 μm; C and D – 1 μm; E and F – 0.5 μm.

In this study, we demonstrated that histaminergic neurons exhibit the structural signs of hyperactivity, with the increased activity probably related to their adaptation to alcohol. The nuclear apparatus of neurons showed the obvious structural features of activation, supporting the processes of biosynthesis in cytoplasm. The enlargement of endoplasmic reticulum and the Golgi complex, as well as the increased amount of free ribosomes and appearance of "nucleolus-like bodies" also confirm that. The hyperplasia of

Table 3
Morphometric parameters of mitochondria in brain histaminergic neuron cytoplasm after 7-day ethanol administration at 4 g/kg.

Parameters	Control ($n = 9$)	Alcohol ($n = 8$)
Average area (μm^2)	0.10 ± 0.02	0.12 ± 0.04
Perimeter (μm)	1.56 ± 0.36	1.56 ± 0.33
Form factor	0.66 ± 0.29	0.70 ± 0.22**
Factor of elongation	2.14 ± 0.47	1.98 ± 0.58
Relative area (in μm^2)/100 μm^2	10.00 ± 6.01	12.74 ± 6.64*
Amount/100 μm^2	100.18 ± 47.41	121.03 ± 62.12*

Data are presented as median ± interquartile range; *$p < 0.05$, **$p < 0.01$, as compared to controls.

Table 4
Morphometric parameters of lysosomes in cytoplasm of brain histaminergic neurons after 7-day ethanol administration at 4 g/kg.

Parameters	Control ($n = 9$)	Alcohol ($n = 8$)
Average area (μm^2)	0.06 ± 0.01	0.09 ± 0.04*
Perimeter (μm)	0.91 ± 0.29	0.99 ± 0.31
Form factor	0.74 ± 0.16	0.79 ± 0.19
Factor of elongation	1.64 ± 0.36	1.56 ± 0.43*
Relative area (in μm^2)/100 μm^2	1.00 ± 0.54	1.92 ± 0.66*
Amount/100 μm^2	13.14 ± 24.89	32.01 ± 29.65*

Data are presented as median ± interquartile range; *$p < 0.05$, as compared to controls.

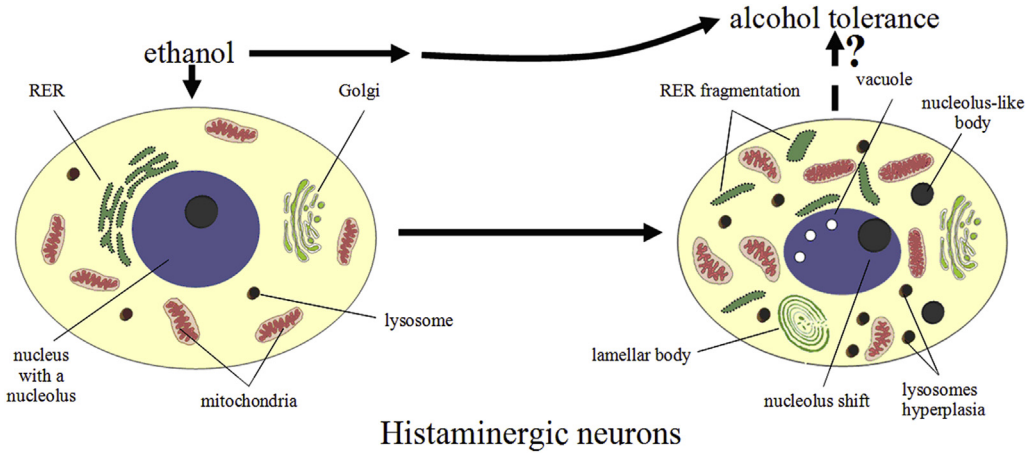

Fig. 7. Schematic drawing of the seven-day ethanol administration effects on the rat brain histaminergic neurons.

mitochondria and lysosomes demonstrate the huge potential capabilities of those neurons as well (Fig. 7). All together, these observations indicate an adaptation of the rat brain histaminergic neurons to alcohol, but the question about their role in modulating alcohol-induced sleep and wakefulness remains open.

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Conflict of interest statement: none declared.

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