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EXPERIMENTAL PAPERS

Changes in Behavioral Characteristics and Tyrosine Hydroxylase Levels in the Nucleus Accumbens of the Brain of DAT-HET Rats during Free Alcoholization

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Abstract—DAT-HET rats with their underlying hyperdopaminergia are a promising model for the investigation of neuropsychiatric diseases, which are based on impaired dopamine neurotransmission, including alcoholism. The aim of the work was to evaluate the effect of free alcoholization on drinking, locomotor, exploratory behavior, anxiety, and Tyrosine hydroxylase (TH) levels in rats with impaired functioning of the DA system (DAT-HET). The study was carried out on adult male rats of the DAT-HET (n = 15) and Wistar (n = 13), which were divided into 4 groups: “DAT-HET ethanol” (n = 10) and “Wistar ethanol” (n = 9), who were in the mode of free alcoholization for 112 days of the experiment. The DAT-HET water (n = 5) and Wistar water (n = 4) groups did not have access to an ethanol solution and did not participate in behavioral tests. Ethanol preference and consumption was assessed in the “Two-bottle choice test”. The amount of ethanol consumed in the cells was recorded weekly. Behavior was assessed using the Open Field and Elevated Plus Maze tests. After alcoholization, to assess the level of TH, an immunohistochemical (IHC). It was found that during free alcoholization, DAT-HET rats do not form preferences for ethanol. Under the low ethanol consumption, the initial hyperactivity in DAT-HET rats is leveled. The DAT-HET model leads to an increase in TH levels in nAcc. In addition, the free alcoholization reduces the level of TH in nAcc with the development of a pathological increase in TH, observed in the DAT-HET model, but has no effect on healthy animals.

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Keywords: DAT-HET rat, dopamine, ethanol, nucleus accumbens, tyrosine hydroxylase, free alcoholization, alcohol dependence, behavior

INTRODUCTION

The initial preference for alcohol that leads to alcohol dependence is related to a malfunctioning reward system. The mesolimbic part of the brain's dopamine system is the center of reward and reinforcement and is directly involved in the mechanism of alcohol dependence [1]. The mesolimbic

pathway of the reward and reinforcement system is mainly composed of dopaminergic neurons localized in the ventral tegmental area (VTA), whose projections come to the nucleus accumbens (nAcc) area [2]. Tyrosine hydroxylase (TH), the main enzyme limiting the rate of catecholamine synthesis, is contained in all dopamine neurons. TH first converts the amino acid L-tyrosine into 3,4-dihydroxyphenylalanine and then into dopamine [3]. One of the mechanisms of regulation of TH activity is a feedback mechanism, in which catecholamine breakdown products inhibit the activity of this enzyme [4]. TH is a recognized marker for studying the localization, differentiation, and development of catecholaminergic neurons.

DAT-HET rats are heterozygotes of the DAT-KO line in which the number of restriction sites for the dopamine active transporter protein (DAT) on only one allele of the gene was reduced using the “zinc-finger nucleases” genome-editing technique. As a result, the amount of extracellular dopamine was increased by 50% in DAT-HET rats compared to wild-type animals. In this regard, this lineage has a number of behavioral characteristics, such as increased excitability, locomotor activity, and unstable anxiety levels [5]. The DAT-HET model is suitable for studying diseases such as attention deficit hyperactivity disorder (ADHD), schizophrenia and addictive disorders [6].

Several animal studies have shown how alcohol consumption in different modes of alcoholization affects the dopaminergic system and causes behavioral changes. Thus, ethanol reduced the dopamine level in the ventral striatum in Wistar rats consuming a moderate amount of ethyl alcohol solution in the regime of free alcoholization, and also had an anxiolytic effect, which was expressed in a decrease in defecation acts in rats in the “Open Field” test [7]. Chronic alcohol consumption for 365 days led to a decrease in TH enzyme and dopamine content, as well as to an increase in DAT in the ventral striatum in Sprague–Dawley rats [8]. Intermittent semi-induced coping in Wistar rats with high ethanol intake decreased the level of dopamine in the nAcc [9]. Depressive behavior and anhedonia were observed in Long–Evans rats 48 h after termination of long-term intermittent alcoholization with 20% ethanol [10]. At the same time, aggressive behavior correlated with an increase in dopamine levels in the nAcc was also observed if rats received 10% ethanol before social tests [11]. The DAT transporter is also involved in the formation of alcohol dependence. In the free-choice mode, male DAT-HET mice consumed more ethanol when the ethanol solution concentration was increased to 32% compared to male DAT-KO mice [12]. In Wistar Kyoto rats consuming large amounts of alcohol at free choice, the number of DAT binding sites in neurons of many brain regions, including the nAcc, increased [13]. Alcohol also extends its effect on TH activity. During acute exposure to orally administered 20% ethanol in Sprague–Dawley rats, an increase in TH levels was observed in many brain regions, including the substantia nigra, which is part of the mesolimbic system [14]. In the same rat line, an increase in TH mRNA expression in the ventral striatum was detected during chronic consumption of a 5% ethanol solution under free-choice conditions [15]. In Wistar rats, after continuous consumption of ethanol solution for 20 days, a decrease in immunoreactivity to TH in dendritic spines in the area of the nAcc was observed [16].

Despite a large number of studies investigating the relationship between alcohol consumption, its effect on the dopaminergic system and behavior, the mechanisms of ethanol preference in hyperdopaminergia are not fully understood. Using DAT-HET rats as a model of dopaminergic system dysfunction provides an opportunity to evaluate behavioral changes under the influence of ethanol and obtain new data to study the effects of ethanol on TH levels, which is an important link in dopamine synthesis. Intermittent and semi-instrumental alcoholization methods usually focus on the motivation of alcohol preference and include an additional stress factor, which can have a strong effect on DAT-HET rats. Previously, in a pilot study, we showed that under semi-coercive alcoholization, DAT-HET rats form ethanol preference faster than Wistar rats, and their anxiety level is reduced [17].

The regime of free alcoholization includes free access to any liquid in the home cage without additional stress exposure, so it is most suitable for

studying alcohol preference and related primary changes in animal behavior in this model. Thus, the aim of the work was to evaluate the effect of free alcoholization on drinking, locomotor, exploratory behavior, anxiety and TH levels in rats with dopaminergic system impairment (DAT-HET).

MATERIALS AND METHODS

Subject of the study. The study was conducted on sexually mature male DAT-HET rats ($n = 15$) and Wistar rats ($n = 13$) aged two months, weighing at least 180 g at the beginning of the experiment. The DAT-HET rats were obtained and verified in the vivarium of St. Petersburg State University. Verification of the presence of the knockout gene was performed by genotyping using the classical real-time polymerase chain reaction (PCR) method. Wistar rats were obtained from the vivarium of the Sechenov Institute of Evolutionary Physiology and Biochemistry, Russian Academy of Sciences.

All animals were divided into 4 groups. The experimental groups "DAT-HET ethanol" ($n = 10$) and "Wistar ethanol" ($n = 9$) were under free alcoholization for 112 days of the experiment, where they had constant access to a drinker with a 10% ethanol solution and to a drinker with water, participated in all behavioral tests. Thirty days after the end of alcoholization (day 142 of the experiment), 5 rats were randomly selected from these groups for immunohistochemical examination. Control groups: "DAT-HET water" ($n = 5$) and "Wistar water" ($n = 4$) had constant access to water and food but no access to ethanol solution, did not participate in behavioral tests, and after 142 days of the experiment were taken for immunohistochemical brain examination.

Animal confinement. All rats were kept on a standard chow of three (two) individuals each in a 570 × 350 × 250 mm cage with free access to food and drinkers with water and ethanol under conditions of 12 h light, 12 h darkness, air temperature 20 ± 3°C and humidity 50–55%. As food the animals received a balanced full-fed feed of LBC "Tosnensky Feed Mill" in pelleted form, energy value 11.39 MJ/kg.

Two bottle choice test. To assess alcohol preference, the rat was placed in a cage similar to a home cage with two identical drinkers. One of the drinkers contained water and the other contained a 10% solution of ethyl alcohol. The location of the drinkers was changed at each subsequent test. Before testing, the rats were subjected to drinking deprivation for 24 h. The proportion (%) of ethanol solution drunk in relation to the total amount of fluid drunk during the 10-min observation period was estimated.

Consumption of the weight of the ethanol solution drunk per week was estimated. Each week the weight of the ethanol solution drunk from the drinker was determined. For each cell the weight of ethanol drunk in g per kg of animal body weight in terms of 100% ethanol was determined, and then the average for one rat for one day of the week was calculated.

Open Field test. The test was conducted in an installation, which is an arena of size 600 × 600 mm, equipped with five "mink" holes and illuminated by an incandescent lamp of 720 lux, at a distance of 1.5 m from the floor. The following parameters were recorded for 5 min: length of the walk, rears (number), peeks into the "minks" (number), grooming (duration, s), boluses (number), urinations (number).

Elevated Plus Maze test. The test was conducted on an installation with two closed and two open arms, at the intersection of which is an open area. The labyrinth was set at a height of 1 m from the floor. The size of each arm was 50 × 10 cm, and the size of the maze center was 10 × 10 cm. The rat was placed in the center of the setup with its nose toward the open arm and the time spent in the open and closed arms, time spent in the center of the maze, as well as rears, hangings from the maze, acts of defecation, and urination were recorded for 5 min.

Immunohistochemical study. All selected rats were anesthetized intraperitoneally with chloral hydrate (400 mg/kg) and decapitated. The brains were fixed by immersion in 4% paraformaldehyde solution for 6 days (at 4°C). After cryoprotection in a 30% sucrose solution diluted in 0.9% NaCl phosphate-salt buffer (pH 7.4), brains were frozen with isopentane (Sigma, USA) at –42°C and stored at –80°C. Using a cryostat (Leica CM-1520, Germany), alternating series of frontal brain slices

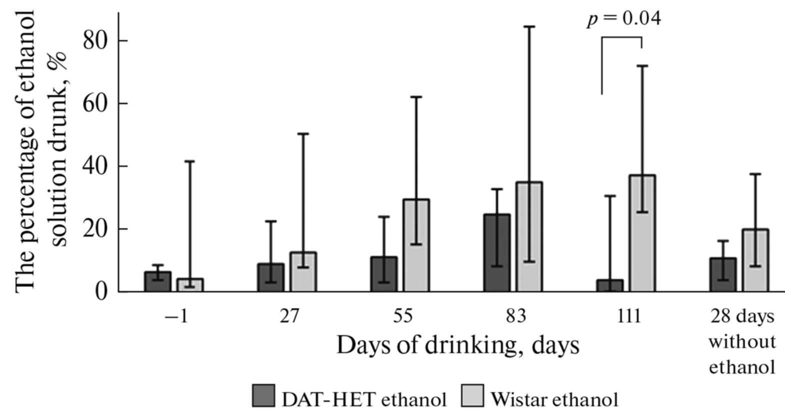

Fig. 1. Ethanol preference in the "Two bottle choice test". *Abscissa*: days of alcoholization, *ordinate*: percentage (%) of alcohol drank. Hereinafter the significance of differences ($p < 0.05$) according to Mann–Whitney *U*-test is indicated.

(20 μm thickness) from the nAcc were made according to the rat brain atlas [18]. Each sixth slice was mounted on Super Frost/plus glasses (Menzel, Germany), dried at room temperature, and stored at –20°C. The protocol described earlier was used for the study [19]. For preliminary antigen demasking, the slides were boiled in citrate buffer (pH 6.0) for 5 min. The reaction was performed using primary mouse anti-TH antibodies (TH, Sigma, USA) diluted 1:1500, secondary goat anti-Mouse IgG antibodies conjugated with biotin (VectorLabs, UK) diluted 1:600, and a streptovidin peroxidase solution (BioLegend, USA) diluted 1:700. The glasses were subjected to standard histological treatment and were placed in transparent Bio-Mount medium (Bio-Optica, Italy). Specificity of the reaction was checked using a negative control (reaction without primary antibodies). Images from the nAcc region were acquired in transmitted light using a Carl Zeiss Imager A1 microscope (Germany) with a built-in Axiocam 712 video camera, Zen 3.4 (blue edition) image capture software. Using Image J software (NIH, USA) we estimated the optical density of TH in immunopositive sprouts. The results are presented in arbitrary unit (au).

Study protocol: the weight of the ethanol solution drunk in home cages in rats was assessed weekly. Two glass test was performed on (–1st), 27-, 55-, 83- and 111th days and 28 days after the end of alcoholization. The Elevated Plus Maze test was performed on (–2nd), 54- and 110th days of alcoholization, the Open Field test was performed on (–4th), 52- and 108th days. Rats were weighed every week of the experiment.

Statistical processing of the results. The results of immunohistochemical study were evaluated using Kruskal–Wallis H-criterion followed by a posteriori analysis of intergroup differences by Mann–Whitney *U*-criterion with Holm–Bonferroni correction. Results are presented as boxplots with a median (M) of 50% with interquartile ranges. Significance of differences in behavioral tests was assessed using Mann–Whitney *U*-test. The results of the behavioral tests were presented as graphs with median and interquartile ranges.

RESULTS

When evaluating the preference for ethanol in the Two bottle choice test it was found that all animals did not prefer ethanol to water during the experiment under free-choice conditions in the home cage (the proportion of ethanol drunk in the tests did not exceed 50% of the total amount of fluid drunk). "DAT-HET ethanol" rats significantly less preferred ethanol compared to "Wistar ethanol" rats at day 111 of alcoholization. At 28 days after the end of alcoholization, the proportion of ethanol drunk did not exceed 30% for both groups (Fig. 1).

Analysis of weekly measurements of the weight of the ethanol solution drunk in home cages showed that the rats of the "DAT-HET ethanol" group consumed significantly less alcohol in the middle of drinking [mean (7th–11th) week] compared to the "Wistar ethanol" rats and did not dif-

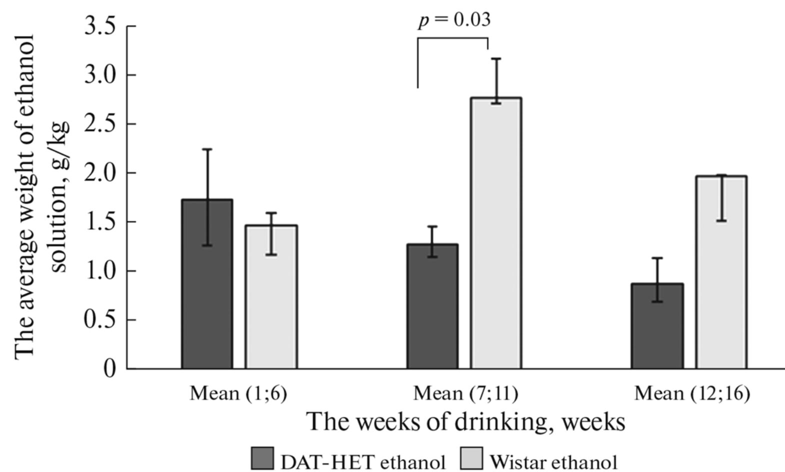

Fig. 2. Average weight of ethyl alcohol solution g/kg drunk by one rat from the group in one day (converted to pure ethanol) in home cages. *Abscissa*: weeks of alcoholization, *ordinate*: the average weight of ethanol solution drunk by one rat from the group during one day, in g/kg of the animal's body weight.

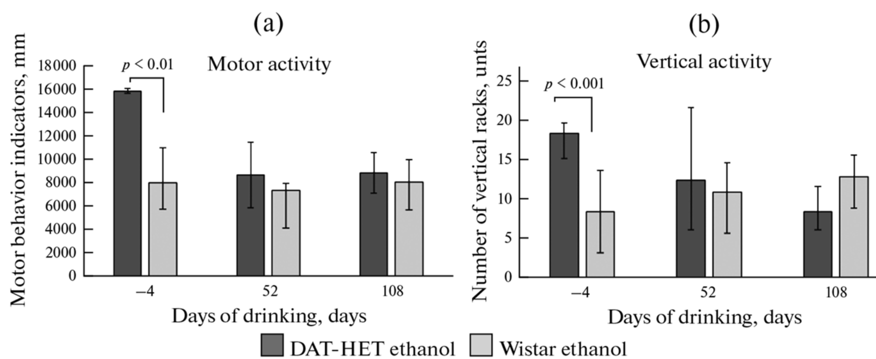

Fig. 3. Motor and vertical activity in rats during alcoholization. *Abscissa*: days of alcoholization; *ordinate*: length of walking distance, in mm (a), rears, number (b).

fer from them in the first [mean (1st–6th) week] and last [mean (12th–16th) week] weeks of alcoholization (Fig. 2).

On average across all fifteen measurements, the "DAT-HET ethanol" rats also drank significantly less alcohol compared to the Wistar ethanol rats of 1.3 ± 0.2 and 2.0 ± 0.2 g/kg, respectively (p = 0.03).

In the Open Field test, the "DAT-HET ethanol" rats demonstrated high locomotor and vertical activity (walking length, rears) compared to the "Wistar ethanol" rats before the beginning of alcoholization and did not differ in exploratory activity (peeking). After the beginning of alcoholization, the motor activity of the "DAT-HET ethanol" rats decreased, and the behavior of the animals of both groups did not differ (Fig. 3). The exploratory activity decreased in the middle and at the end of alcoholization in the rats of both groups; no differences were observed between the groups. In terms of autonomic acts and grooming, the rats did not differ throughout the experiment.

In the elevated plus maze test, the "DAT-HET ethanol" rats, in contrast to the "Wistar ethanol" rats, preferred open arms at the beginning and 54 days after the beginning of alcoholization. The "DAT-HET ethanol" rats spent significantly less time in the center of the cruciform maze before and at day 54 of alcoholization compared with the animals in the "Wistar ethanol" group. The "DAT-HET ethanol" rats spent significantly less time in the closed arms of the maze before alcoholization. At the end of alcoholization, no significant differences were found between the groups. The number of hangs from open sleeves was greater in the "DAT-HET ethanol" group at the

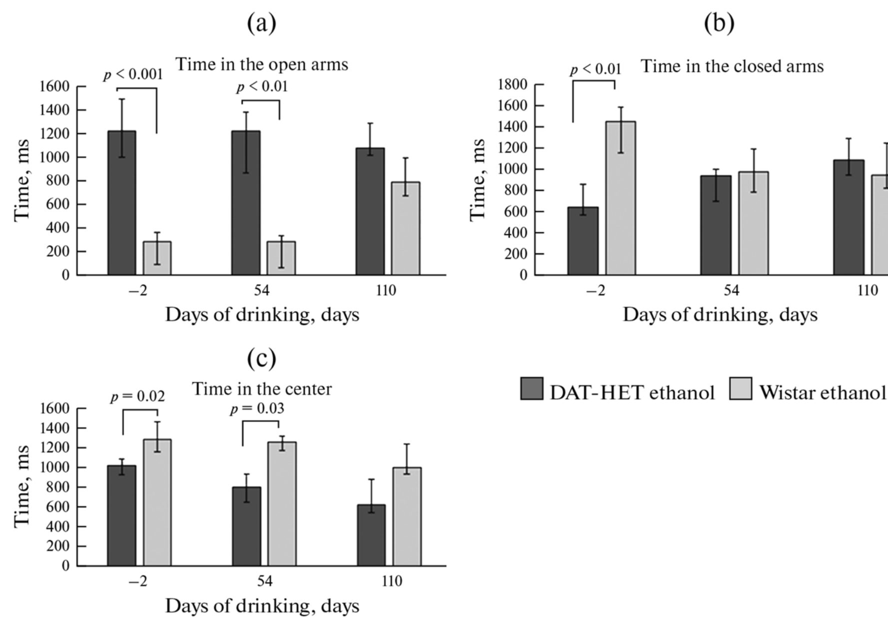

Fig. 4. Time spent in closed (a), open arms (b), and in the center (c) of the elevated plus maze. *Abscissa*: days of alcoholization; *ordinate*: time in ms.

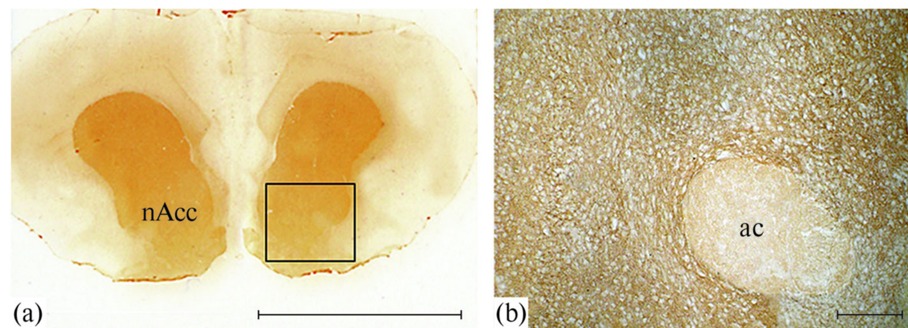

Fig. 5. Immunohistochemical reaction to tyrosine hydroxylase on the frontal section of the brain of the Wistar rat with the area of the nAcc. At low magnification, the nAcc area is squared; at higher magnification, tyrosine hydroxylase-immunopositive outgrowths are located in nAcc. Label: ac—anterior commissure, scale: 5 mm (a) and 200 μm (b).

beginning of the experiment (8.3 ± 4.6 vs. 3.0 ± 1.0, $p < 0.01$), but in subsequent tests the differences between the groups were not reliable. There were also no significant differences between the groups in terms of time spent in open arms (Fig. 4).

In this study, we measured the optical density of TH immunopositive material in the nAcc (Fig. 5) in all groups of DAT-HET and Wistar rats.

A higher initial optical density of TH-immunopositive outgrowths in the area of the adjoining nucleus was observed in the "DAT-HET water" rats: M = 113 (0.108; 0.126) compared with the "Wistar water" group: M = 108 (0.103; 0.115). After alcoholization, the "DAT-HET ethanol" rats showed a decrease in TG levels: M = 109 (0.103; 0.114) compared with the "DAT-HET water" group (Fig. 6). A decrease in the optical density of TH in the "DAT-HET ethanol" rats was also observed compared to the "Wistar ethanol" group: M = 112 (0.106; 0.117) ($p = 0.04$).

DISCUSSION

In the present experiment, DAT-HET rats showed no preference for ethanol under conditions of free alcoholization in the "Two glass test"

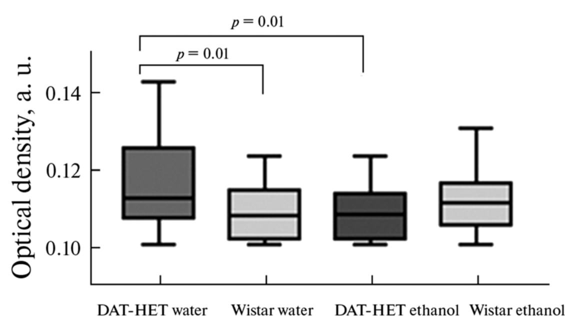

Fig. 6. Optical density of TH-immunopositive outgrowths in the area of the adjoining nucleus in the studied groups of DAT-HET and Wistar rats. Ordinate axis: optical density in a.u. Notations: the black line inside the box is the median, the limit lines are the interquartile range. Significance of differences at $p < 0.05$, by Mann–Whitney *U*-test.

and showed a low level of its consumption in the home cages during the week. It has been previously shown that DAT-HET rats increased ethanol consumption and preference under conditions of semi-induced alcoholization compared to Wistar rats [17]. The results obtained in this experiment echo earlier studies on the role of DAT in the formation of preference for ethanol exactly under conditions of free alcoholization. Thus, under similar conditions in male DAT-KO and DAT-HET mice, ethanol consumption and preference decreased [20]. It should be noted that Wistar rats consuming more ethanol in weekly samples in the middle of the experiment also did not demonstrate reliably stable preference during alcoholization and one month after it. Our data are in agreement with the results of works showing that similar dynamics of ethanol consumption is characteristic for animals which receive ethanol at free choice [21].

Locomotor activity in DAT-HET rats before alcoholization was significantly higher than in Wistar rats, which was repeatedly noted earlier [5, 6]. Subsequently, against the background of alcoholization, the locomotor activity of the DAT-HET line animals decreased by the middle of the experiment and did not differ from the parameters of alcoholized Wistar rats. No differences were found in the exploratory activity of DAT-HET rats compared to Wistar rats; it was high in the first test before alcoholization and decreased in subsequent tests. The same results are noted at the end of the experiment. Perhaps, against the background of free alcoholization, in the absence of ethanol preference formation, the decrease in locomotor and exploratory activity in DAT-HET rats is due to the sedative effect of ethanol, which reduces the hyperreactivity of this group of animals. It can also be suggested that this may be influenced by factors such as habituation to the open-field test arena and aging of the animals during the experiment.

DAT-HET rats also show hyperreactivity in the Elevated plus maze test, which is also consistent with the behavioral characteristics of the line [6]. Before alcoholization, they statistically significantly spend more time in the open arms and less time in the center of the maze compared to Wistar. Such a ratio of spending time in the open arms and in the center, perhaps, indicates a high reactivity of the rats of this line. Against the background of alcoholization, this tendency persists until the middle of the experiment, but at the end of the experiment the differences with Wistar rats are leveled.

Projections of dopaminergic neurons localized in the VTA come to the adjoining nucleus region and constitute the mesolimbic pathway controlling the reward and reinforcement system [2, 22]. Therefore, TH levels were assessed in immunopositive outgrowths of dopaminergic neurons in the adjoining nucleus region. Despite the low consumption of ethanol solution in the DAT-HET ethanol rats, the level of TH in the projections of dopaminergic neurons in the adjoining nucleus area was lower in the DAT-HET water rats, but did not differ from the Wistar ethanol rats. There was also a tendency for the level of TH to decrease in the DAT-HET rats compared to the alcoholized Wistar rats, but in statistical processing after the Holm–Bonferroni correction the differences are leveled, probably due to the small sample.

DAT is known to regulate signal transduction at all pre- and postsynaptic dopamine receptors by removing the neurotransmitter from the extracellular space, by reuptake [23], its density is highest in the striatum and adjoining nucleus [24]. In all DAT-knockout rats, impaired reuptake leads to an imbalance of dopamine synthesis, in particular, DAT-KO has decreased mRNA TH levels in the midbrain and TH protein levels in the striatum, while DAT-HET has increased extracellular dopamine levels in the striatum [5]. For this rea-

son, in DAT-HET rats the initially increased amount of TH in the projections of dopaminergic neurons in the adjoining nucleus region may be related to the high content of extracellular dopamine in the striatum. It can be assumed that even small doses of ethanol can affect dopaminergic neurons, causing a decrease in the total TH level in their projections in the adjoining nucleus, and probably reduce the level of extracellular dopamine in the ventral striatum and other structures of the mesolimbic system. At the same time, DAT-HET rats have no preference for ethanol because of the compensatory mechanisms at work in the dopaminergic system. As a consequence, this leads to a decrease in dopamine content and a change in the characteristic behavior for this animal model, primarily their hyperactivity.

Thus, we can conclude that the DAT-HET line rats show an increase in the TH level in the nAcc. In addition, the regime of free alcoholization decreases the TH level in the nAcc during the development of the pathological increase in the TH content observed in the DAT-HET line animals, but has no effect on the animals in the control groups. This conclusion is in good agreement with the obtained behavioral data, in which excessive locomotor activity is reduced in DAT-HET rats under conditions of alcoholization. However, the fact that the DAT-HET rats did not form an ethanol preference under conditions of free alcoholization and consumed less alcohol than the animals in the control group should be taken into account. Thus, the compared groups were exposed to different amounts of ethanol, which causes difficulties when interpreting the data from the position of neurochemical and pharmacological approaches. However, these difficulties can be overcome in the following experiments, for example, using forced exposure to the same low volumes of ethanol.

Limitations of the study. Only the index of total TH enzyme in the fibers of the adjoining nucleus was investigated. Different number of animals in the groups. Individual catecholamine levels were not assessed.

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AUTHORS’ CONTRIBUTION

Idea of work and planning of the experiment discussion of the results (E.V.F., A.Yu.E., E.O.K., I.V.A.), data collection (A.E.V., E.O.K, I.V.A.), genotyping of the knockout animal line, discussion of the results (Z.V.A.), statistical processing of results, preparation of illustrative material (I.V.A., I.Yu.M., E.O.K.), immunohistochemical examination (I.Yu.M., I.V.A., A.E.V.), writing and editing the article (I.V.A., A.Yu.E., E.O.K., E.V.F.).

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COMPLIANCE WITH ETHICAL STANDARDS

The experiments were performed according to the study design approved by the Ethical Committee of the Sechenov Institute of Evolutionary Physiology and Biochemistry of the Russian Academy of Sciences, European Communities Council Directive 1986 (2010/63/EEC) and according to the rules set forth in the Guide for the Care and Use of Laboratory Animals.

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

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