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Purmorphamine Alters Anxiety-Like Behavior and Expression of Hedgehog Cascade Components in Rat Brain after Alcohol Withdrawal

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Abstract—Disturbances in the Hedgehog (Hh) signaling play an important role in dysmorphogenesis of bone tissue and central nervous system during prenatal alcohol exposure, which underlies development of fetal alcohol syndrome. The involvement of Hh proteins in the mechanisms of alcohol intake in adults remains obscure. We investigated the role of the Hh cascade in voluntary ethanol drinking and development of anxiety-like behavior (ALB) during early abstinence and assessed changes in the expression of Hh pathway components in different brain regions of male Wistar rats in a model of voluntary alcohol drinking using the intermittent access to 20% ethanol in a two-bottle choice procedure. Purmorphamine (Hh cascade activator and Smoothened receptor agonist) was administered intraperitoneally at a dose of 5 mg/kg body weight prior to 16-20 sessions of alcohol access. Purmorphamine had no effect on the ethanol preference; however, rats exposed to ethanol and receiving purmorphamine demonstrated changes in the ALB during the early abstinence period. Alcohol drinking affected the content of the Sonic hedgehog (Shh) and Patched mRNAs only in the amygdala. In rats exposed to ethanol and receiving purmorphamine, the level of Shh mRNA in the amygdala correlated negatively with the time spent in the open arms of the elevated plus maze. Therefore, we demonstrated for the first time that alterations in the Hh cascade induced by administration of purmorphamine did not affect alcohol preference in voluntary alcohol drinking. It was suggested that Hh cascade is involved in the development of anxiety after alcohol withdrawal through specific changes in the Hh cascade components in the amygdala.

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Keywords: purmorphamine, alcohol, voluntary drinking, anxiety-like behavior, rats

INTRODUCTION

Secreted hedgehog (Hh) ligands play a key role in embryogenesis and functioning of stem cells, as well as in the maintenance of tissue homeostasis due to their involvement in the injury-induced regeneration [1]. Mammals have three proteins of this family: Sonic hedgehog (Shh), Desert hedgehog (Dhh), and Indian hedgehog (Ihh). The most studied of them is Shh. The Hh signaling cascade is a multicomponent network of molecular events that depend on many factors [2]. According to the simplified model of the Hh pathway activation, (i) Patched transmembrane protein (Ptch) in its inactive state blocks the Smoothened

Abbreviations: ALB, anxiety-like behavior; EPM, elevated plus maze; Gli, transcription factor; Hh, Hedgehog; Ptch, Patched transmembrane protein; Shh, Sonic hedgehog protein; Smo, Smoothened G protein-coupled receptor.

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(Smo) G protein-associated receptor (GPCR); (ii) Hh ligand binds to Ptch thus abolishing its inhibitory effect on Smo and leading to cascade activation; (iii) Smo triggers an intracellular cascade resulting in the activation of transcription factor Gli and expression of target genes [2].

Because the Hh cascade plays a pivotal role in cell proliferation and differentiation, its dysregulation might lead to oncogenesis, which makes this system a target in the development of pharmacological agents for the treatment of malignant tumors [3]. Thus, drugs inhibiting the Hh pathway have been developed and approved for the treatment of certain cancers. However, the Hh cascade is also essential for the functioning of the central nervous system (CNS). In addition to being classical morphogens mediating CNS development in embryogenesis, Hh proteins directly or indirectly regulate multiple processes, such as neurogenesis, axonal growth, and neuroplasticity, in mature brain [4]. Hh proteins are also involved in the formation of response to the oxidative stress and inflammation in the nervous tissue [5]. Impaired functioning of the Hh signaling cascade is associated with diseases of the nervous system, such as autism, depression, dementia, stroke, epilepsy, as well as neurodegenerative and demyelinating diseases [6]. This makes the search and design of agents capable of influencing this system a very promising direction in the development of medications aimed at pharmacological correction of CNS disorders [6].

Purmorphamine, a derivative of purine, deserves special attention among compounds affecting the Hh pathway. By acting as a Smo receptor agonist, it triggers canonical Hh cascade accompanied by activation of the Gli transcription factor [7]. Systemic administration of purmorphamine is effective in a number of experimental models of CNS diseases. Purmorphamine alleviated brain tissue damage and reduced neurological deficit [8] and memory impairment in the mouse models of brain ischemia [9]. Purmorphamine decreased the neurotoxic effects of propionic acid and ethidium bromide in rats, including neurodegeneration, impairments in the motor activity and learning in the Morris water maze test, and depressive-like behavior [10, 11].

Alcohol consumption is accompanied by a complex of molecular and cellular adaptations that might underlie its transformation from a controlled process to abuse and development of alcohol dependence characterized by the withdrawal syndrome [12]. Mesocorticolimbic structures, primarily, frontal cortex, amygdala, hippocampus, and striatum, are the main anatomical structures regulating various aspects of alcohol drinking and formation of alcohol dependence [13, 14]. Studying molecular mechanisms of excessive alcohol consumption and alcohol dependence, which are accompanied by neuroplastic processes [15, 16], remains one of the priorities in neuroscience.

It is well known that the Hh cascade is involved in the developmental disorders of the bone tissue and CNS in prenatal alcohol exposure and is one of the molecular links in the development of fetal alcohol syndrome [17, 18]. However, the role of Hh in the regulation of alcohol consumption and accompanying behavioral disorders in adults remains poorly studied despite the relevance and prospects of this research. There are indirect indications that the Hh cascade participates in the mechanism regulating alcohol drinking and concomitant changes in the CNS activity. In particular, it was demonstrated that expression of Ptch in the rat hippocampus during early abstinence period in the model of voluntary drinking with the intermittent access to 20% ethanol depended on the pattern of alcohol consumption [19]. In this model, rats demonstrated the ability to increase alcohol consumption up to 10 g/kg body weight per day, which corresponds to the excessive alcohol drinking in humans [20]. It should be noted that the intermittent access to ethanol allowed rats to consume more alcohol than protocols using continuous access to ethanol [20]. The abstinence period (up to 72 h, but not later) was characterized by memory impairment, anxiety, and depression-like symptoms accompanied by changes in the CNS at the molecular level [20]. In the same model, during the early abstinence period, there was a significant increase in the Shh level in the hippocampus and striatum of rats that had an access to alcohol and received 7,8-dihydroxyflavone (brain-derived neurotrophic factor mimetic) vs. animals that only had access to alcohol [21].

As mentioned above, Hh participates in the formation of various types of behavior. In regards of alcohol consumption and accompanying impairments it is important that activation of the Hh cascade attenuated obsessive-compulsive disorders [22] and had an anxiolytic effect [23, 24]. Abstinence after chronic alcohol consumption can lead to the development of anxiety-like disorders [25, 26], but the relationship between the Hh cascade and anxiety-like behavior (ALB) during alcohol withdrawal has not been investigated before.

Based on the published reports and our own data, we suggested that activation of the Hh signaling may alter alcohol consumption and/or behavior during the early abstinence period. The main goal of this work was to study the role of purmorphamine in alcohol consumption in a model of voluntary alcohol drinking with the intermittent access to 20% ethanol and in the development of ALB in rats after alcohol withdrawal. We also evaluated changes in the levels of Shh, Ptch, Smo, and Gli mRNAs in the key mesocorticolimbic

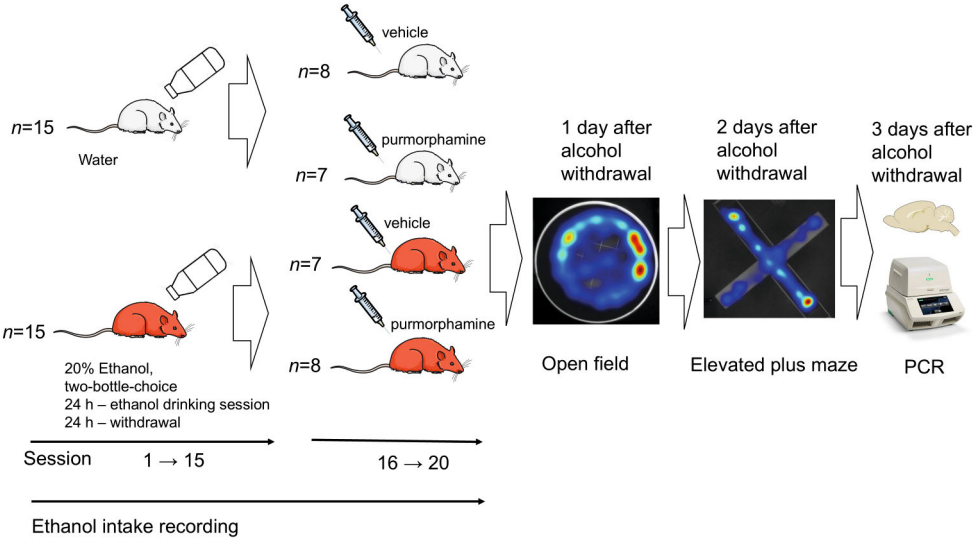

Fig. 1. Scheme of the experiment. Voluntary alcohol drinking was modeled using the intermittent access to 20% ethanol in a two-bottle choice procedure; alcohol was presented together with water for a period of 24 h, followed by alcohol replacement with water for another 24 h. Purmorphamine was administered intraperitoneally at a dose of 5 mg/kg body weight before sessions 16-20 of alcohol access. Alcohol intake was recorded during 20 sessions, after which the rats were accessed water only. ALB was assessed in the open field and elevated plus maze tests one day and two days after alcohol withdrawal, respectively. On the third day, the levels of mRNA for Shh, Ptch, Smo, and Gli (signaling cascade components) were determined in the frontal cortex, amygdala, hippocampus, and striatum.

brain structures, since an increase in the expression of Hh signaling pathway components is considered to be a molecular marker of cascade activation in many cell types [3].

MATERIALS AND METHODS

Animals. Male Wistar rats were obtained from the Stolbovaya Branch of the Scientific Center of Biomedical Technologies of the Federal Medical Biological Agency (Moscow Region, Russia). The animals were 12 weeks old with the average body weight of 288 ± 4 g. The adaptation period (from the moment the rats were housed at the animal facility to their inclusion into the experiment) was at least 7 days. The rats were kept in individual cages under standard conditions with an artificial 12/12-h light/dark illumination cycle at constant temperature (21-23°C) with ad libitum access to water and granulated chow.

Design of the experiment. The scheme of the experiment and the number of animals in the experimental and control groups are shown in Fig. 1.

Alcohol consumption in the model of intermittent access to 20% ethanol. The model of voluntary drinking with the intermittent access to 20% ethanol in a two-bottle choice procedure was implemented according to the protocol proposed by Simms et al. [27]. In the experimental groups, 20% ethanol solution and water were presented to rats simultaneously in two bottles for 24 h. Rats chose the bottle and the amount of liquid to drink. A total of 20 sessions of alcohol access were performed; the positions of the bottle in the cage were alternated. The period between the sessions was 24 h, during which the rats were presented with two bottles of water. The control group had a continuous access to two bottles of water. The ethanol access sessions began during the dark phase of the light/dark illumination cycle. Animals and bottles were weighed each time before and after the drinking session. The level of alcohol preference was estimated as the ratio of the volume of alcohol intake per session (daily) to the total volume of liquid consumed and expressed in percent.

Purmorphamine administration. The animals were injected intraperitoneally with a suspension of purmorphamine (ab120933; Abcam, USA) in 10% dimethyl sulfoxide, 40% PEG-400, and 50% isotonic sodium chloride at a dose of 5 mg/kg body weight (3 ml/kg body weight) 1 h before ethanol presentation during sessions 16 to 20. It was previously demonstrated that purmorphamine administered systemically at a dose of 5 mg/kg affected various types of rat behavior [10, 11, 22]. Animals that did not receive purmorphamine were injected with the vehicle (3 ml/kg body weight). Prior to the first purmorphamine administration, animals exposed to ethanol were randomly distributed according to the ethanol preference over the last 5 sessions. The following four groups were formed: 1) Control group with a constant access to water only; 2) Control + purmorphamine group (control animals receiving purmorphamine injections); 3) Ethanol

group that was presented with alcohol and water (according to the model described above); and 4) Ethanol + purmorphamine group (rats injected with purmorphamine prior to the ethanol access).

Open field test was used for the assessment of motor activity and ALB. The animals were tested in a Circular Open Field for Rats (TS0501-R; Open Science LLC, Russia) with a white round open arena (diameter, 97 cm; wall height, 42 cm). The arena was divided into three equal concentric zones (center, middle, and periphery) and illuminated at 500 lux. Rats were placed in the central zone and allowed to explore the arena for 5 min. After each test, the arena was purified with 20% ethanol. Animal behavior was recorded with a DMK 23GV024 GigE digital monochrome video camera (Imaging Source Europe GmbH, Germany) and analyzed with the Ethovision XT11 software (Noldus, Netherlands). The assessed parameters included the distance traveled, movement speed, time spent in the arena center, and number of entries into the center.

Elevated plus maze (EPM) test was used to evaluate ALB. EPM was a cross-shaped platform (TS0502-R3; Open Science LLC, Russia) consisting of the central area (14×14 cm) and four open-top crossing arms (50×14 cm): two opposing open arms with a wall height of 1 cm and two opposing closed arms with a wall height of 30 cm. The maze was placed 50 cm up from the floor and installed so that the open arms were illuminated in the same way as the entire experimental room (500 lux), while the closed arms were darkened. The animal was placed into the central area with its nose toward the open arm, and its behavior was recorded with a DMK 23GV024 GigE digital monochrome video camera for 5 min. After each test, the maze was cleaned with 20% ethanol. Animal behavior was analyzed with Ethovision XT11 software. The assessed parameters were the distance traveled, movement speed, number of entries into the open and closed arms, time spent in the open and closed arms, and the number of head dips and stretch-attend postures.

Polymerase chain reaction (PCR). The animals were sacrificed by decapitation, the brains were removed and washed in ice-cold isotonic sodium chloride solution. Frontal cortex, amygdala, hippocampus, and striatum were isolated on ice and stored at –80°C. Total RNA was extracted with ExtractRNA reagent (#BC032; Evrogen, Russia) and treated with 2.5 U of DNase I (#M0303; NEB, USA) to remove genomic DNA impurities. Reverse transcription was performed with 100 U Protoscript II reverse transcriptase (#M0368; NEB) in the reaction mixture containing 0.5 μg of total RNA, 1 μM Oligo(dT)$_{15}$ (#SB001, Evrogen), and 1 μM random decanucleotide primers (#SB002, Evrogen). PCR was performed in a CFX96 thermal cycler (Bio-Rad, USA) in two parallel repeats using 2 pmol of synthetic oligonucleotide primers and qPCRmix-HS SYBR PCR premix (#PK147S; Evrogen). The sequences of primers used to amplify cDNAs for Shh [28], Ptch, and Smo [29], Gli [30], and rpS18 [31] were described previously. Relative mRNA levels were assessed as suggested in [32].

Statistical analysis. The results were analyzed and statistically processed with the STATISTICA 8.0 software package (StatSoft Inc., USA) and Prism 8.0 (GraphPad Software Inc., USA); the data were analyzed for the normality of distribution using the Shapiro–Wilk test and presented as mean ± standard error of mean (SEM) or as median and interquartile range. The differences between the levels of alcohol preference during the first and each following drinking session were calculated using the mixed-effects model (restricted maximum likelihood model) and Dunnett's method for multiple comparisons. The significance of differences between the dependent samples was evaluated with the repeated measures analysis of variance (ANOVA) and Tukey's test for multiple comparison of means. Comparison of several independent samples was carried out with the two-factor ANOVA and Tukey's test or the Kruskal–Wallis test followed by the *post hoc* analysis of multiple comparisons and the Dunn's test. Correlation analysis was performed by estimating the Spearman correlation coefficient. The differences were considered significant at $p < 0.05$.

RESULTS

Ethanol preference. According to the mixed-effects model, the level of alcohol preference varied significantly between the sessions ($F = 2.453$; $p = 0.034$). Based on the Dunnett's test, the preference for alcohol increased and was higher after sessions 7-9 and 12-13 compared to the alcohol preference after the first session (Fig. 2).

The data on the effect of purmorphamine on the alcohol preference are shown in Fig. 3. According to repeated measures ANOVA, ethanol drinking session had a significant effect on the alcohol preference ($F_{(5,60)} = 5.269$; $p < 0.001$). Purmorphamine administration did not influence alcohol preference ($F_{(1,12)} = 0.110$; $p = 0.745$). The interaction of "ethanol drinking session" and "purmorphamine administration" factors had not significant effect on the preference for ethanol ($F_{(5,60)} = 0.237$; $p = 0.945$).

Open field test was used to evaluate the ALB parameters one day after alcohol withdrawal (Fig. 4). The time spent in the center of the arena (Fig. 4a; $H_{(3,29)} = 0.728$; $p = 0.867$), number of entries to the center (Fig. 4b; $H_{(3,29)} = 0.605$; $p = 0.895$), traveled distance (data not shown), and movement speed (data not shown) did not differ between the groups.

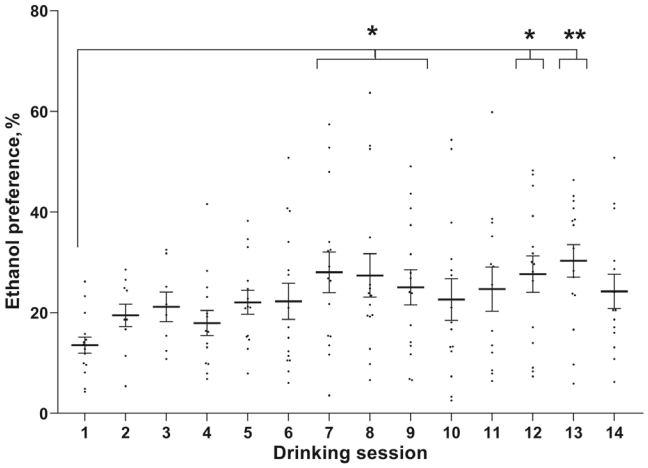

Fig. 2. Changes in ethanol preference in the model of voluntary drinking with the intermittent access to 20% ethanol in a two-bottle choice procedure. The data are presented as mean ± SEM; * $p < 0.05$; ** $p < 0.005$, significant differences with the first session (mixed-effects model and Dunnett's test for multiple comparison).

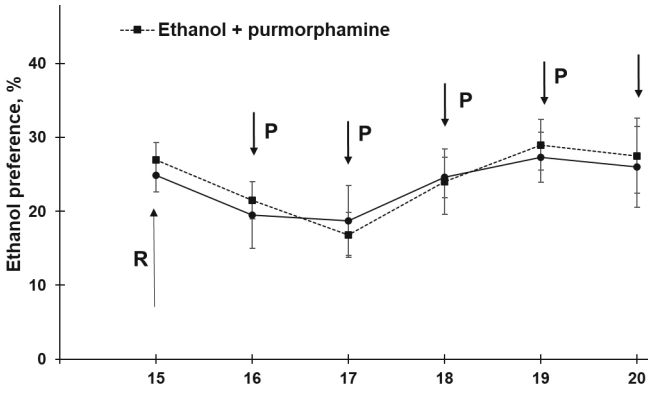

Fig. 3. Effect of purmorphamine on ethanol preference in the model of voluntary drinking with the intermittent access to 20% ethanol in a two-bottle choice procedure. R, randomization by the preference for alcohol over 5 sessions before the start of purmorphamine administration; P, intraperitoneal administration of purmorphamine at a dose of 5 mg/kg body weight 1 h before alcohol access (sessions 16-20). The data are shown as mean ± SEM.

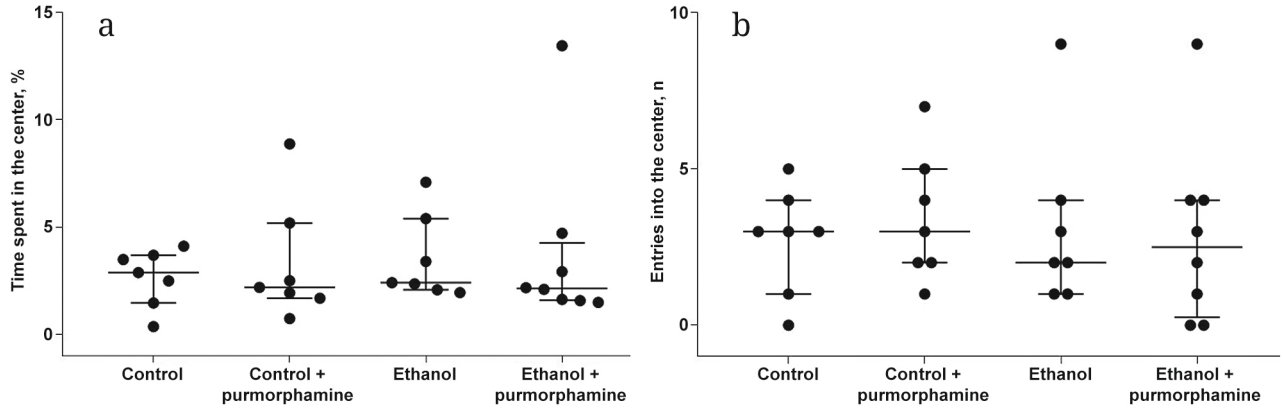

Fig. 4. Effect of purmorphamine on the ALB in the open field test after implementation of the voluntary drinking model with the intermittent access to 20% ethanol in a two-bottle choice procedure. a) Time spent in the center; b) number of entries to the center. The data are presented as median and interquartile range. Purmorphamine was administered intraperitoneally at a dose of 5 mg/kg body weight one hour before ethanol drinking sessions 16-20. The test was performed one day after alcohol withdrawal.

Elevated plus maze (EPM) test. Two days after alcohol withdrawal, rat behavior was assessed in the EPM test (Fig. 5). According to the Kruskal–Wallis test, experimental groups differed in the number of head dips (Fig. 5a; $H_{(3,30)} = 11.800$; $p = 0.008$), time spent in the open arms (Fig. 5c; $H_{(3,30)} = 9.440$; $p = 0.024$), and number of entries to the closed arms (Fig. 5d; $H_{(3,30)} = 8.121$; $p = 0.044$), but not in the number of entries to the open arms (data not shown; $H_{(3,30)} = 1.985$; $p = 0.576$) and number of stretch-attend postures (Fig. 5b; $H_{(3,30)} = 1.604$; $p = 0.659$). *Post hoc* analysis of multiple comparisons using the Dunn's test showed that the number of head dips in the Ethanol + purmorphamine group was significantly lower compared to the Control group (Fig. 5a). The Ethanol + purmorphamine group also showed a trend ($p < 0.1$) toward an increase in the time spent in the open arms compared to the Control + purmorphamine ($p = 0.084$) and Ethanol ($p = 0.053$; Fig. 5c) groups, as well as a decrease in the number of entries to the closed arms compared to the Control ($p = 0.088$) and Control + purmorphamine ($p = 0.095$; Fig. 5d) groups. The distance traveled and the speed of movement did not differ between the groups (data not shown).

Expression of Shh, Ptch, Smo, and Gli mRNAs in brain structures. Three days after alcohol withdrawal, the relative content of mRNAs for Shh, Ptch, Smo, and Gli in the frontal cortex, amygdala, hippocampus, and striatum was evaluated (Table 1). According to ANOVA, the factor "ethanol" had a considerable effect

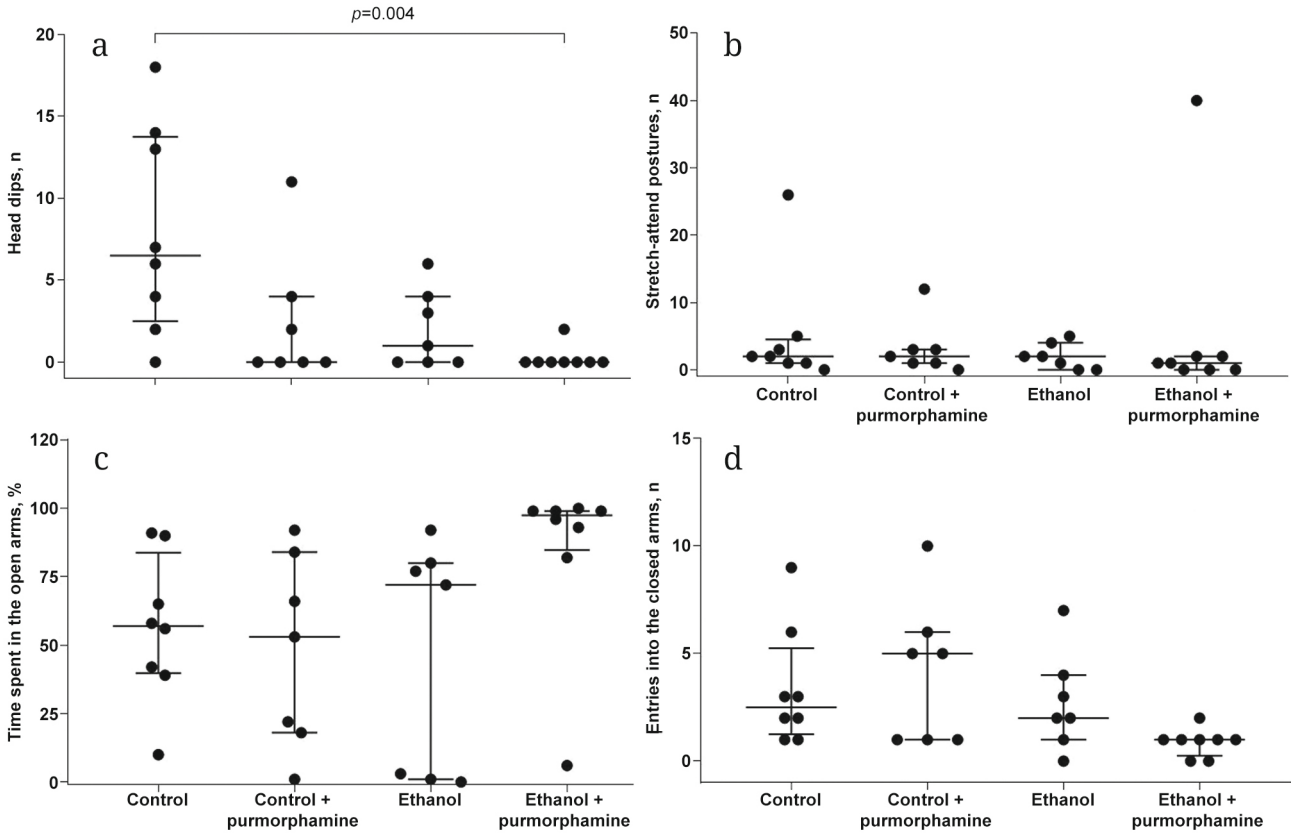

Fig. 5. Effect of purmorphamine on rat behavior in the EPM test after implementation of the voluntary drinking model with the intermittent access to 20% ethanol in a two-bottle choice procedure. a) Number of head dips; b) number of stretch-attend postures; c) time spent in the open arms; d) number of entries to the closed arms (Kruskal–Wallis test followed by *post hoc* analysis of multiple comparisons with the Dunn's test). The data are presented as median and interquartile range. Purmorphamine was administered intraperitoneally at a dose of 5 mg/kg body weight one hour before ethanol drinking sessions 16-20. The test was carried out two days after alcohol withdrawal.

on the relative level of Shh mRNA in the amygdala, while the effects of the factor "purmorphamine" and interaction of "ethanol" and "purmorphamine" factors did not reach the level of significance (Table 1). The factor "ethanol" (but not "purmorphamine" or interaction of "ethanol" and "purmorphamine" factors) had a considerable effect on the relative Ptch mRNA level in the amygdala (Table 1). Subsequent multiple comparison of means revealed no significant differences between the groups. Nevertheless, it should be noted that the Ethanol + purmorphamine group showed a trend ($p < 0.1$) toward a decrease in the Shh mRNA level in the amygdala compared to the Control ($p = 0.068$) and Control + purmorphamine ($p = 0.062$) groups (Fig. 6). At the same time, in the Ethanol + purmorphamine group, the level of Shh mRNA in the amygdala exhibited a significant inverse correlation with the time spent in the open arms ($r = -0.786$; $p = 0.036$), whereas in the Control ($r = 0.167$; $p = 0.693$), Control + purmorphamine ($r = -0.071$; $p = 0.879$) and Ethanol ($r = 0.357$; $p = 0.432$) groups, this correlation did not reach the level of significance. There were no considerable changes in the content of Ptch, Smo, and Gli mRNAs in the amygdala, as well as in the content of all studied mRNAs in the frontal cortex, hippocampus, and striatum (Table 1).

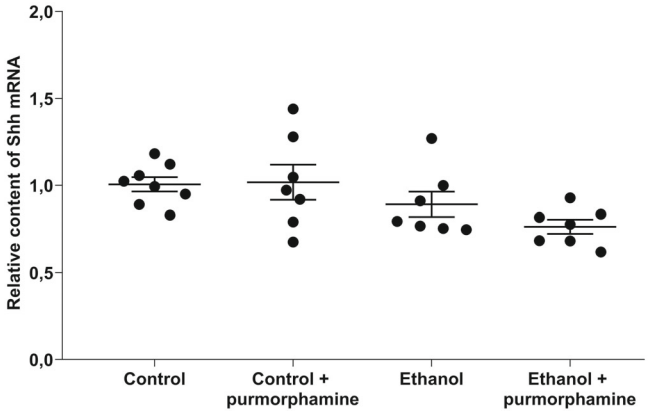

Fig. 6. Effect of purmorphamine on the relative Shh mRNA content in the amygdala after the voluntary drinking model with the intermittent access to 20% ethanol in a two-bottle choice procedure. The data are presented as mean ± SEM. Purmorphamine was administered intraperitoneally at a dose of 5 mg/kg one hour before the ethanol drinking sessions 16-20. mRNA levels were assessed by PCR three days after alcohol withdrawal.

Table 1. Relative content of mRNAs for the Shh signaling cascade components in different brain regions after implementation of the voluntary drinking model with the intermittent access to 20% ethanol in a two-bottle choice procedure

Brain area	mRNA	Control, n = 8	Control + Purmorphamine, n = 7	Ethanol, n = 7	Ethanol + Purmorphamine, n = 8	ANOVA: F; p		
						Ethanol	Purmorphamine	Ethanol × Purmorphamine
Frontal cortex	Shh	1.03 ± 0.11	1.17 ± 0.09	1.21 ± 0.14	1.03 ± 0.16	0.02; 0.89	0.02; 0.89	1.46; 0.24
	Ptch	1.03 ± 0.10	1.13 ± 0.08	1.08 ± 0.10	1.00 ± 0.16	0.11; 0.74	0.01; 0.91	0.69; 0.42
	Smo	1.05 ± 0.13	1.10 ± 0.08	1.00 ± 0.12	1.03 ± 0.10	0.34; 0.57	0.17; 0.68	0.01; 0.92
	Gli	1.09 ± 0.18	1.20 ± 0.22	1.54 ± 0.36	1.06 ± 0.26	0.32; 0.58	0.48; 0.49	1.24; 0.28
Amygdala	Shh	1.01 ± 0.04	1.02 ± 0.10	0.89 ± 0.07	0.76 ± 0.04	**7.58**; **0.01**	0.76; 0.39	1.10; 0.30
	Ptch	1.02 ± 0.07	1.05 ± 0.06	0.93 ± 0.07	0.81 ± 0.11	**4.28**; **0.05**	0.30; 0.59	0.90; 0.35
	Smo	1.02 ± 0.07	1.10 ± 0.07	0.96 ± 0.06	0.94 ± 0.12	1.55; 0.22	0.10; 0.75	0.33; 0.57
	Gli	1.22 ± 0.29	1.21 ± 0.14	1.04 ± 0.11	1.05 ± 0.27	0.60; 0.45	0.00; 0.99	0.00; 0.98
Hippocampus	Shh	1.02 ± 0.08	0.86 ± 0.13	0.98 ± 0.07	0.85 ± 0.06	0.09; 0.77	2.82; 0.11	0.05; 0.83
	Ptch	1.03 ± 0.09	0.91 ± 0.13	0.97 ± 0.10	0.73 ± 0.08	1.29; 0.27	3.21; 0.08	0.34; 0.56
	Smo	1.03 ± 0.09	0.99 ± 0.17	1.10 ± 0.13	0.82 ± 0.09	0.18; 0.68	1.82; 0.19	1.07; 0.31
	Gli	1.05 ± 0.11	0.84 ± 0.23	0.92 ± 0.07	0.75 ± 0.07	0.69; 0.41	2.02; 0.17	0.02; 0.88
Striatum	Shh	1.09 ± 0.16	1.43 ± 0.17	1.09 ± 0.17	1.28 ± 0.14	0.20; 0.66	2.76; 0.11	0.22; 0.64
	Ptch	1.05 ± 0.10	1.07 ± 0.09	1.12 ± 0.18	1.09 ± 0.07	0.17; 0.68	0.00; 0.98	0.04; 0.85
	Smo	1.04 ± 0.10	0.95 ± 0.10	1.17 ± 0.19	1.03 ± 0.11	0.70; 0.41	0.78; 0.39	0.05; 0.83
	Gli	1.17 ± 0.25	1.08 ± 0.11	1.13 ± 0.27	1.03 ± 0.12	0.05; 0.83	0.21; 0.65	0.00; 0.99

Note. Statistically significant differences (two-factor ANOVA) are shown in bold.

DISCUSSION

Hh cascade and alcohol drinking. The role of Hh proteins in the mechanisms of alcohol consumption in adults and development of disorders associated with chronic intoxication is still poorly understood. Our work is the first one to assess the effect of purmorphamine on the voluntary alcohol drinking in adult rats. According to the obtained results, purmorphamine had no significant effect on the preference for ethanol. However, we should mention the earlier reported data on the effectiveness of purmorphamine in the model of obsessive-compulsive disorder, which might also be involved in the formation of pathological forms of alcohol drinking. In particular, it was demonstrated that systemic administration of purmorphamine attenuated obsessive-compulsive behavior caused by the injection of serotonin 1A receptor agonist into the raphe nuclei [22]. Furthermore, the authors of this work observed a decrease in the Shh content in the cerebrospinal fluid, blood plasma, and brain tissue, i.e., purmorphamine stabilized the level of Shh without affecting its content [22].

Hh cascade and ALB. Anxiety is a typical affective symptom associated with the alcohol withdrawal syndrome [25, 26]. Alcohol intake in voluntary drinking models, including the model of intermittent access to 20% ethanol in a two-bottle choice procedure, has been accompanied by changes in the ALB. It was shown that alcohol withdrawal after the intermittent access to 20% ethanol led to the pronounced manifestations of ALB in the open field and EPM tests in mice [33] and rats [34]. However, some researchers failed to demonstrate the effect of alcohol intake on

ALB in the voluntary drinking models. Thus, providing free access to 10% alcohol for 4 h during the dark phase of the day/night cycle for 12 days did not affect the number of entries to the center in the open field test 8 days after alcohol withdrawal [35]. No behavior changes in the EPM test were reported one day [36] or one week [37] after stopping the intermittent access to 20% ethanol. Similarly, continuous access to alcohol in the voluntary drinking model had no effect on animal behavior in the EPM test [38]. Hence, the indications that voluntary alcohol drinking in rodents can modify their behavior in the anxiety tests are rather contradictory. Here, we found no behavioral changes in the open field and EPM tests after alcohol withdrawal.

The Hh cascade is known to participate in the molecular mechanisms of ALB. Direct involvement of the Hh family proteins in the regulation of ALB was demonstrated in Dhh-deficient mice [39]. The authors investigated different forms of behavior, including motor activity, learning, and memory formation, as well as anxious- and depressive-like behavior, and found an increase in the depressive-like behavior (in the forced swim test) and ALB (in the Vogel conflict test) in Dhh-deficient male mice compared to the wild-type controls [39]. In the model of fetal alcohol syndrome in *Danio rerio*, upregulation of Shh expression in fish exposed to ethanol in early embryogenesis prevented CNS dysmorphogenesis and ALB at the late development stages [40]. Conditional Smo knockout in neural stem cells disrupted neurogenesis in the mature hippocampus, which was accompanied by exacerbation of anxiety- and depressive-like behavior, without affecting motor activity and learning [23].

Here, we demonstrated for the first time that alterations in the animal behavior in the EPM test might be caused by the interaction between voluntary alcohol drinking and systemic administration of purmorphamine leading to the activation of the Shh cascade. These results are consistent with the concept that stimulation of the Hh cascade produces the anxiolytic effect upon access to alcohol, as evidenced by the trend toward an increase in the time spent in the open arms and decrease in the number of entries to the closed arms in the EPM test.

B**iochemical markers of the Hh signaling activation in the brain and ALB after alcohol withdrawal.** According to the ANOVA, alcohol drinking affected the content of only Shh and Ptch mRNAs and only in the amygdala, i.e., in the brain structure responsible for the formation of emotions associated with anxiety. Since activation of genes coding for the Hh signaling components (primarily Ptch and Gli) is considered to be a marker of the Hh cascade activation in many cell types [3], including glia [41] and neurons [42, 43], we expected to demonstrate upregulation of the corresponding mRNAs in the brain of animals treated with purmorphamine. Contrary to our expectations, we did not detect it. Instead, there was an obvious trend towards a decrease in the content of Shh mRNA in the animals that had an access to alcohol and received purmorphamine.

In experimental models, expression of the Hh signaling cascade components in the brain may reflect the neuroprotective properties of certain compounds or their impact on behavior, including ALB. In this case, the direct pharmacological activity of these substances might not necessarily be associated with the Hh cascade.

Thus, in the rat model of chronic unpredictable stress, the flavanone naringenin prevented the development of depressive-like behavior in the forced swim test and cognitive impairment in the Morris water maze test, as well as normalized the content of Shh and Gli mRNAs previously reduced by the stress in the hippocampus [44]. In the same model, exacerbation of anxiety was accompanied by a decrease in the content of Shh and Gli mRNAs in the hippocampus, while nicotine was able to abolish behavioral impairments and changes in gene expression [45].

Intoxication of rats with propionic acid or ethidium bromide was accompanied by a reduction in the Shh content in the brain, while systemic administration of purmorphamine had a neuroprotective effect in both these models, which was accompanied by normalization of the Shh levels [10, 11]. Chronic systemic administration of the Smo agonist SAG (Smoothened agonist), a derivative of benzothiophene, alleviated anxiety in the open field and EPM tests in the high-fat diet-fed mice [24]. Moreover, SAG normalized the reduced content of Shh in these animals without affecting the amount of Shh in the neocortex [24]. Systemic administration of purmorphamine in mice subjected to the middle cerebral artery occlusion reduced the brain infarction area and decreased the development of neurological deficits, which was accompanied by a decrease in the apoptotic death of neurons [8]. Interestingly, occlusion of the middle cerebral artery upregulated expression of mRNAs for the Hh cascade components (Shh, Ptch, Smo, and Gli) in the neocortex. However, despite its physiological activity, purmorphamine did not affect expression of these proteins [8]. Therefore, purmorphamine activity does not necessarily reflected by changes in the expression of the Hh signaling components.

We found that in the Ethanol + purmorphamine group. behavior in the EPM test and the Shh content in the amygdala showed changes in opposite directions. This pattern was confirmed by the existence of the inverse correlation between the time spent in the open arms and relative content of the Shh mRNA in the amygdala only in this group of animals.

The amygdaloid complex plays a key role in the formation of stress response and anxiety [46-48]. The activity of this brain region was found to change during withdrawal after chronic intermittent intoxication with alcohol vapors and might be associated with manifestations of the withdrawal [49, 50]. The data on the Hh functioning in the amygdala are scarce, with the exception of few works showing Hh involvement in memory formation. The manifestation of the conditional fear response to a combination of sound signal and electric shock in mice was accompanied by the activation of cell proliferation and increase in the levels of Shh, Ptch, and Gli in the amygdala [51]. At the same time, suppression of Shh in mitotic neurons of the basolateral amygdala by injection of a retrovirus with an interfering RNA attenuated formation of conditional fear response and neurogenesis [51]. Moreover, by regulating neurogenesis in the amygdala, Shh normalized attenuation of the conditional fear response over time. Shh overexpression in the amygdala stimulated neurogenesis and attenuated fear response, while interfering RNAs had the opposite effect [52].

CONCLUSION

This work is the first one to investigate the activity of purmorphamine in the voluntary ethanol drinking in rats and to study their behavior in the open field and EPM tests after alcohol withdrawal. Although purmorphamine did not modify alcohol preference, it altered rat behavior in the EPM test after alcohol withdrawal. Rat behavior in the EPM test, as well as the Shh mRNA content in the amygdala demonstrated changes in opposite directions and correlated inversely only if the access to alcohol was combined with purmorphamine administration. The data obtained suggest the involvement of the Shh signaling in the formation of mechanisms of alcohol withdrawal-induced emotional disturbances in the amygdala. Further studies are needed to reveal specific details of this involvement.

Contributions. D.I.P., M.Yu.S., and N.V.G. developed the study concept; D.I.P. and N.V.G., supervised the study; D.I.P., N.I.S., and A.A.K. conducted the experiments; D.I.P., analyzed the results and wrote the text of the article; M.Yu.S. edited the manuscript; N.V.G. prepared the final version of the manuscript.

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Ethics declarations. All applicable institutional guidelines for the care and use of animals (Guide for the Care and Use of Laboratory Animals, Eighth Edition, 2010), the requirements of the European Convention for the Protection of Vertebrate Animals Used for Experiments or for Other Scientific Purposes (Strasbourg, 1986 with Appendix dated 15.06.2006), principles of Good Laboratory Practice (Order of the Ministry of Health of the Russian Federation no. 199n dated 01.04.2016, GOST R 53434-2009), as well as "Guidelines for Working with Animals" approved by the Bioethical Committee of the Institute of Higher Nervous Activity and Neurophysiology were followed. The authors of this work declare that they have no conflicts of interest.

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