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Behavioral Features and Blood Enzyme Activity in Offspring of Rats Conceived from an Alcohol-Intoxicated Father

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Abstract—Quite often, conception of a child occurs after consuming small doses of alcohol. However, effect of this factor on offspring has not been studied at all. The aim of this study was to examine the level of motor activity, anxiety-like and depressive-like behavior, sensitivity to analgesic effect of ethanol, as well as activity of the enzymes DPP-IV, PEP, and ADG in the blood of rats whose fathers received ethanol immediately before mating. As a result of the conducted experiments, it was found that the males conceived by the intoxicated fathers have significant differences in behavior compared to control animals. Thus, motor activity in the rats conceived by males under the influence of alcohol was 2-2.5 times less intense; they exhibited decreased severity of the anxiety-like and depressive-like behavior. In such animals, activity of DPP-IV and ADG was increased and activity of PEP in the blood was reduced. In the rats conceived by the fathers under the influence of alcohol, analgesic effect of ethanol was decreased, and there was also reduction in response of the activities of ADG, DPP-IV, and PEP enzymes to ethanol administration. It is assumed that a single use of ethanol by male rats immediately before mating leads to the decrease in methylation of the paternal inherited genes in offspring. As a result, activity of a number of enzymes could change, which leads to the change in the balance of neuropeptides involved in mediation of animal behavior.

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INTRODUCTION

It has been shown that alcohol consumption during pregnancy negatively affects behavior of the offsprings [1, 2]. There are also data that significant and persistent overconsumption of ethanol by the father prior to conception could affect behavior of the offsprings. In particular, offsprings of the alcoholised mice exhibited attention deficit and hyperactivity [3], hypoactivity [4], learning disabilities [5], decrease of the anxiety level [6], decrease of sensitivity to ethanol [7], and decrease of alcohol craving [8]. According to unofficial data no less than 20% of all conceptions in the world, and in Russia especially, occurs after consumption of moderated doses of ethanol. However, effects of this factor on behavior of the future offsprings have been not investigated sufficiently. We have found only two studies devoted to the effects of consumption of a single moderate dose of ethanol by the male before mating. It has been reported that the offsprings demonstrated developmental problems [9], as well as that the offsprings demonstrated much less of the risk-assessment behavior and were more aggressive that the 'normal' animals [10].

Abbreviations: ADG, alcohol dehydrogenase; DPP-IV, dipeptidyl peptidase-IV; PEP, prolyl endopeptidase.

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Behavior of humans and animals is determined mainly by the processes mediated by neuromediators and neuromodulators in the central nervous system. Neuropeptides play an important role in these processes with their levels depending significantly on activity of the enzymes of their degradation. In particular, dipeptidyl peptidase-IV (DPP-IV) participates in degradation of such peptides as growth factors, chemokines, incretins, neuropeptides, and vasoactive peptides. It has been shown that DPP-IV is involved in formation of ethanol tolerance and dependence [11].

Prolyl endopeptidase (PEP) participates in degradation of numerous peptide hormones and neuropeptides. Role of PEP in effects of ethanol on organisms of humans and animals is still poorly investigated.

Activities of DPP-IV and PEP could serve as markers of aggressive behavior [12], levels of anxiety [13] and depression [14]. It was shown that the individuals diagnosed with alcoholism have extremely low levels of DPP-IV and PEP [15].

Alcohol dehydrogenase (ADG) is the enzyme cleaving endogenous and exogenous ethanol. Role of this enzyme in formation of ethanol sensitivity has been investigated in great detail [16].

Based on the information presented above, goal of this study was investigation of the level of motor activity, anxiety-like and depression-like behavior, sensitivity to analgesic effect of ethanol, as well as activity of the DPP-IV, PEP, and ADG enzymes in the blood of rats conceived by the fathers that consumed ethanol immediately prior to mating.

MATERIALS AND METHODS

Animals. Wistar rats obtained from Stolbovaya Laboratory Animal Facility (Moscow, Russia) were used in the study. Animals were kept in ventilated Tecniplast green line 1500U cages (Tecniplast, Italy) with natural corn bedding (Zolotoy Kot, Russia), 4-5 animals per cage with free access to water and combined food (3 kcal/g; Profgryzun, Moscow, Russia) at average temperature 21°C, average humidity 20%, with illumination (90 lux) from 20:00 to 08:00.

Mating procedure. Rats with body mass 210-240 g (males) and 170-200 g (females) at the age of 2-3 months were used in the experiments. Three groups of males and females (4 animals of each sex per group) were formed with equal average level of motor activity in the groups. A female in the estrus state was placed with a male for 24 h. At the time of mating each cage contained 1 male and 1 female.

Males of the 1st (control) group were administered water intragastrically. Males of the 2nd and 3rd groups received immediately prior to mating a 20% ethanol solution at a dose 0.5 or 1.5 g/kg intragastrically through a gastric tube. Males received solution immediately after they started exhibiting mating behavior, and were placed back to the cage after ethanol administration. Female rats did not receive anything. In each group, three females out of four produced offsprings.

Examination of offsprings. As a result of mating of control animals 15 males and 16 females were born. Mating of the fathers that received 0.5 g/kg of ethanol produced 16 males and 16 females. Only males were used in further experiments. Behavior of the 46 males conceived under conditions described above was examined from the age of 2 months to the age of 3 months. Levels of motor activity was tested sequentially every 3-5 days, as well as their behaviour in the open field test, elevated plus maze test, and tail suspension test was examined. And finally, sensitivity of the rat offsprings to analgesic effects of ethanol was tested in the hot plate test. Seventy-two hours after administration of ethanol and determination of its analgesic effect, animals were decapitated and blood samples were taken for biochemical tests.

Determination of the level of motor activity. Rats were individually placed into experimental Phenomaster chambers (TSE Systems, Germany) for 60 min, where their total horizontal motor activity was determined automatically, as well as motor activity in the center and number of rearing during 60 min with 10 min intervals. The experimental chambers were identical to the housing cages in which rats were kept to minimize effects of stress. Experiments were carried out from 11:00 to 15:00 in the absence of illumination. Simultaneously 8 Phenomaster chambers were used.

Open field test behavioral assessment. "Open field" comprised a round 98-cm diameter arena surrounded with 31-cm high walls (OpenScience, Russia, model TS0501-R). Arena was separated with lines into 7 central and 12 peripheral sectors. Arena was illuminated uniformly with a medical lamp (arena surface illuminance 450 lux). At the beginning a rat was placed into the center of arena and horizontal motor activities were monitored (total number of crossed sections), number of rearings, and number of center location within five minutes.

Determination of anxiety-like and depression-like behavior in the elevated plus maze test (EPM). "The Elevated Plus Maze" apparatus (Columbus Instruments, USA) was used in the study. It comprises a cross-shaped platform with four arms (length – 50 cm, width – 15 cm). Two opposite arms have non-transparent high walls, while other two are open. Height of the walls in closed arms is 43 cm. Labyrinth is raised to the height of 75 cm. Surface area of the central part of the apparatus is 15 cm^2. Illumination of the labyrinth surface is 90 lux. A rat was placed

for 5 min into the platform and number of crossing from section to section was measured, as well as time spent in the center, in the closed and open arms of the apparatus.

Determination of the level depression-like and in the tail suspension test. The test involves hanging a rat with adhesive tape and special attachment upside down by the tail for 6 min (TSE Systems, Tail Suspension Monitor, 303590 series). Duration(s) and number of immobilization periods and passive movements were detected. Energetic whole-body movements and rotation around the axis were considered as active movement. Slow movements with one or two paws and head movement, such as swaying and whisker twitching were considered as passive movements. All other motion-fewer periods were considered as immobilization periods.

Determination of sensitivity to analgesic effect of ethanol. Animals from all three groups for this test were randomly classified according the level of motor activity and separated into two subgroups: 20% ethanol solution at the dose 1.5 g/kg was intragastrically administered to the rats from the first subgroup 30 min prior to the test. Rats from the second subgroup received isotonic solution of sodium chloride. Each animal was placed onto the surface of a "Hot plate" instrument (TSE Systems) heated to 56°C. Latency period until animal started licking paws was measured.

Determination of enzyme activity. *Sample preparation*. Activities of ADG, PEP, and DPP-IV were determined in blood plasma samples collected into tubes with EDTA. Samples were centrifuged for 10 min at 3000 rpm. Plasma samples were transferred into clean tubes and stored at –70°C before use in the experiments.

Fluorometric method for determination activity of PEP and DPP-IV. Method is based on fluorometric determination of released in the course of enzymatic reaction 7-amino-4-methylcoumarin (AMC) from the peptide Z-Ala-Pro-AMC (for PEP) or from the peptide Gly-Pro-AMC (for DPP-IV), which has different fluorescence spectrum from the fluorescence spectra of the peptides. Hydrolysis of the substrates was recorded after 30-min incubation at 37°C using a LS-5B spectrofluorimeter (Perkin-Elmer, USA). Amount of the released 7-amino-4-methylcoumarin was determined from the level of fluorescence. Specific activity of enzymes was calculated using equation (1):

$$A = [(E - C)/(S - B)]*t - 1*v - 1, \quad (1)$$

where E – fluorescence of the sample (380/460 nm); C – fluorescence of the mixture containing 0.05 ml of each substrate and enzyme, 1.9 ml of Tris-HCl-buffer (prepared from Tris base Serva, Germany) (pH 8.0) supplemented with 1 mM of each EDTA-Na_2 (Reanal, Hungary), dithiothreitol (Serva) and NP-40 (Sigma, USA) and 1 ml of acetate buffer (pH 4.0); B – fluorescence of the mixture containing 0.05 ml of substrate solution, 1.95 ml Tris-HCl-buffer (pH 8.0) supplemented with 1 mM of each EDTA-Na_2, dithiothreitol, and NP-40 and 1 ml of acetate buffer (pH 4.0); S – fluorescence of the mixture containing 0.05 ml of substrate solution, 1.93 ml of Tris-HCl-buffer (pH 8.0) supplemented with 1 mM of each EDTA-Na_2, dithiothreitol, and NP-40, 1 ml of acetate buffer (pH 4.0), and 0.02 ml of 7-amino-4-methylcoumarin solution (2 nmol, Serva). Reaction was stopped by addition of 1 ml of acetate buffer (pH 4.0) to the incubation mixture.

Spectrophotometric method for determination of ADG activity. Activity of ADG in blood plasma was determined with the modified method suggested by Mezey et al. [17]. For this a 50-µl plasma samples were mixed with 400 µl of glycine buffer (pH 8.8), 3% ethanol solution, and NAD solution (3 mg/ml, Fluka, Switzerland); next each sample was incubated in a water bath for 60 min at 37°C followed by measuring optical density at 340 nm with a DU-50 spectrophotometer (Beckman, USA). Activity of ADG (µmol NADH/ml*min) was calculated using molar extinction coefficient of the reduced form of pyridine nucleotides (6.22 mmol/ml/cm).

Statistical data processing. Statistical data processing was carried out according to algorithms of Statistica 13.0 program with testing normality of data distribution using Shapiro–Wilk test. In the case of normal distribution one-way ANOVA test was used to compare mean values of several independent data sets with following comparison of the mean values using Duncan's test; in the case of non-normal data distribution non-parametric one-way Kruskal–Wallis test was used followed by *post hoc* analysis using non-parametric Mann–Whitney U-test. Data are presented as a M ± SEM, where M – is a mean value, SEM – standard error of the mean.

RESULTS

Behavioral changes. It was shown that the males conceived by the fathers in the state of intoxication exhibited significant differences in behavior in comparison with the normal animals. In particular, motor activity of the rats conceived by the fathers in the state of intoxication was 2-2.5-fold less intense: U = 36.00, p = 0.000997 in comparison of the 'Control' group with the 'Ethanol 0.5 g/kg' group and U = 62.00, p = 0.001642 – in comparison with the 'Ethanol 1.5 g/kg' group (Fig. 1a).

It was also found out in the 'Open field' test that the rats conceived by the ethanol-affected fathers

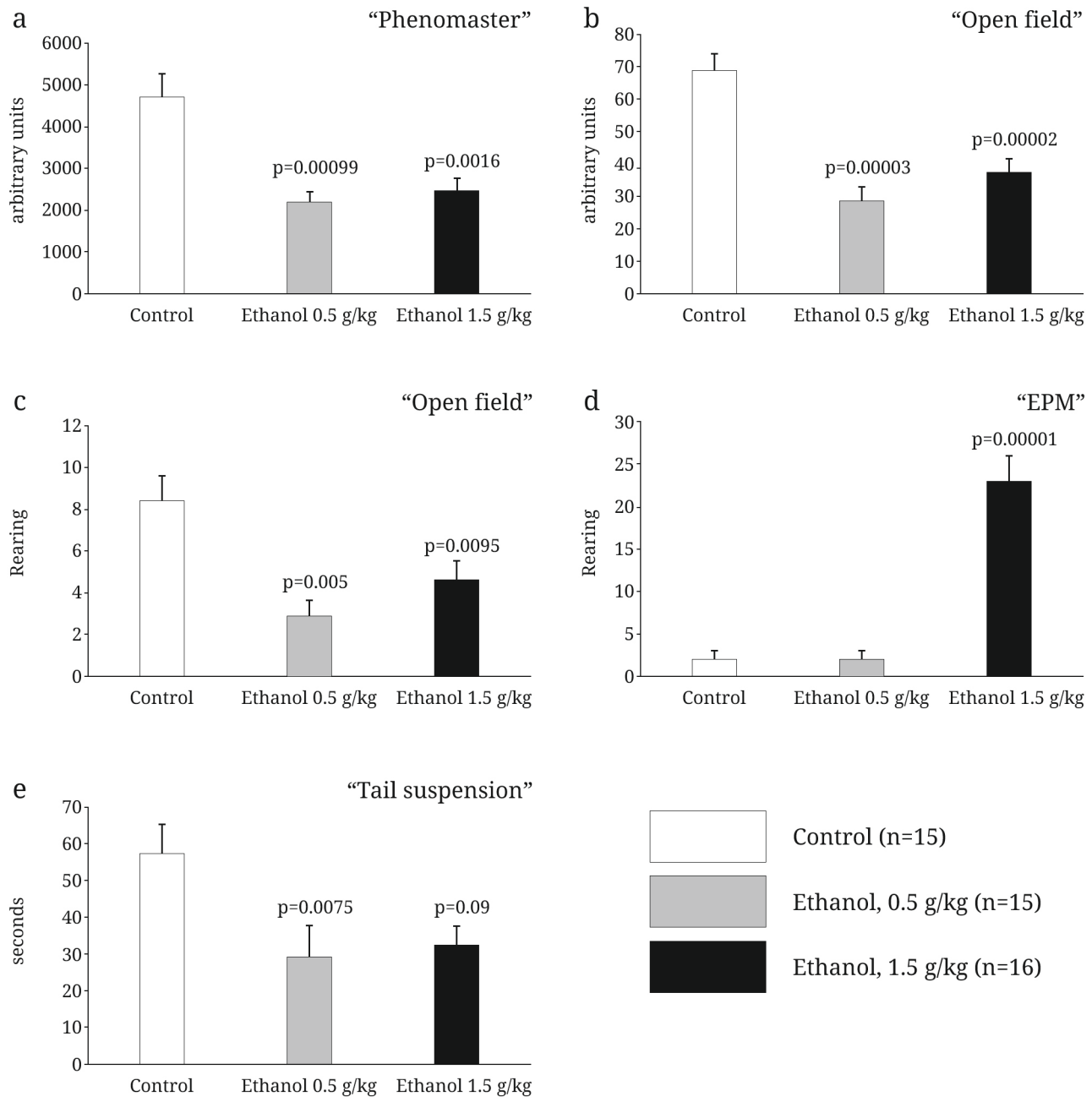

Fig. 1. Changes in behavior of rat males conceived by the fathers in intoxicated state. a) Motor activity (arb. units) in experimental chambers Phenomaster; b) horizontal motor activity (arb. unit) in the ‘open field’ test; c) vertical motor activity (rearing) in the ‘open field’ test; d) duration of staying in open arms of EPM (s); e) duration of rat immobilization in the tail suspension test (s). Significant differences from the control are presented (non-parametric Mann–Whitney U-test).

exhibited lower horizontal motor activity (significant difference from the control, U = 19.50, p = 0.000027 for the group ‘Ethanol 0.5 g/kg’; U = 33.50, p = 0.000018 for the group ‘Ethanol 1.5 g/kg’, Fig. 1b) and vertical motor activity (significant difference from the control, U = 32.50, p = 0.000478 for the group ‘Ethanol 0.5 g/kg’; U = 78.00, p = 0.00995 for the group ‘Ethanol 1.5 g/kg’, Fig. 1c).

Time spent in the open arms of the elevated plus maze (EPM) by the males conceived by the fathers exposed to 1.5 g/kg of ethanol increased more than 5-fold (U = 14.50, p = 0.00001) (Fig. 1d).

In the ‘Tail suspension test’, the rats conceived by the fathers intoxicated with alcohol demonstrated reduced immobilization time (significant difference from the control, U = 49.50, p = 0.007544 for the group ‘Ethanol 0.5 g/kg’; U = 77.50, p = 0.008991 for the group ‘Ethanol 1.5 g/kg’, Fig. 1e).

It was revealed that pain sensitivity of the rats conceived by the fathers under effect of alcohol

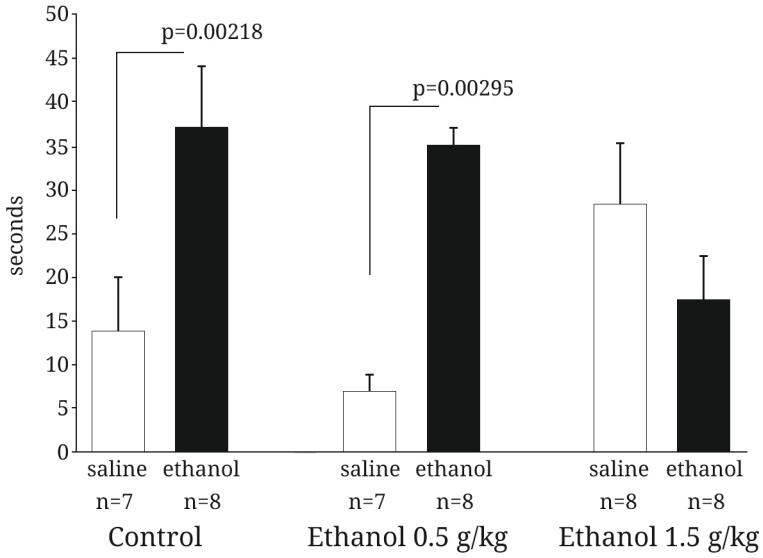

Fig. 2. Latency of paw licking in the 'Hot plate' test.

Table 1. Activity of the ADG, DPP-IV, and PEP enzymes in rat blood plasma

Saline/Ethanol	ADG (μmol/mg/min)		
	Control	Ethanol 0.5 g/kg	Ethanol 1.5 g/kg
Saline	19.073 ± 0.91	19.475 ± 0.70	21.798 ± 0.96* MS = 4.2465, df = 42.0 p = 0.0428
Ethanol 1.5 g/kg	17.896 ± 0.77# MS = 4.2465, df = 42.00 p = 0.0027	16.969 ± 0.61# MS = 4.2465, df = 42.00 p = 0.0104	19.819 ± 0.71
	DPP-IV (nmol/mg/min)		
	control	ethanol 0.5 g/kg	ethanol 1.5 g/kg
Saline	2.694 ± 0.15	2.943 ± 0.14	3.269 ± 0.09* U = 12.00, p = 0.0379
Ethanol 1.5 g/kg	3.077 ± 0.2# U = 13.00, p = 0.0488	3.134 ± 0.11	3.355 ± 0.12
	PEP (nmol/mg/min)		
	control	ethanol 0.5 g/kg	ethanol 1.5 g/kg
Saline	0.946 ± 0.04	0.955 ± 0.03	0.789 ± 0.01* U = 7.00, p = 0.0069
Ethanol 1.5 g/kg	0.774 ± 0.02# U = 6.00, p = 0.0046	0.804 ± 0.03# U = 5.00, p = 0.0029	0.799 ± 0.01

Note. * Significant differences between the groups of offsprings conceived by the fathers exposed to ethanol and conceived by the fathers that received water; # significant differences between the animals that received saline and ethanol in each group of offsprings.

does not differ significantly from the control rats. Administration of 1.5 g/kg of ethanol resulted in significant increase of the latency of paw licking (analgesia) both in the control animals and in the rats conceived by the fathers exposed to 0.5 g/kg of ethanol. However, no analgesic effect of ethanol was observed in the rats conceived by the fathers that received ethanol at the dose 1.5 g/kg (Fig. 2).

Changes in enzyme activities. Activity of ADG in the animals conceived by the fathers exposed to 1.5 g/kg of ethanol was significantly higher than in the animals of all other groups. Activity of ADG significantly decreased after ethanol administration in all groups (Table 1).

Activity of DPP-IV in the animals conceived by the fathers exposed to 1.5 g/kg of ethanol was significantly higher than in the animals of all other groups. Following ethanol administration activity of DPP-IV increased significantly only in the control animals (Table 1).

Activity of PEP in the animals conceived by the fathers exposed to 1.5 g/kg of ethanol was significantly lower than in the animals of all other groups. Following ethanol administration activity of PEP decreased significantly in the animals of the control group and in the animals conceived by the fathers exposed to ethanol at the dose 0.5 g/kg. No decrease of the activity of PEP after ethanol administration was observed in the rats conceived by the fathers exposed to 1.5 g/kg of ethanol (Table 1).

Comparison of the activities of ADG was carried out using one-way ANOVA, which followed by the comparison of mean values of dispersion complex using Duncan's test. Comparison of activities of other enzymes was carried out using non-parametric one-way dispersion analysis using Kruskal–Wallis test followed by posterior paired analysis based on Mann–Whitney *U*-test.

DISCUSSION

We demonstrated pronounced differences in the behavior of male rats with fathers exposed to minor or moderate doses of ethanol prior to mating. In particular, decrease of the level of motor activity was observed both in the Phenomaster apparatus and in the 'Open field test'. Previously hypoactivity of mice was shown for the animals with fathers exposed to long-term action of ethanol prior to mating [4]. We also showed increase of the time spent in the open arms of the elevated plus maze by the animals with intoxicated fathers, which indicates reduced levels of anxiety. This phenomenon was also described previously for the offsprings with fathers chronically exposed to ethanol prior to mating [6]. In addition, the results of our experiments demonstrated that administration of ethanol at the dose 1.5 g/kg to the fathers prior to mating results in producing offspring with reduced depression-like response to stress and decrease of sensitivity to analgesic effect of ethanol. Decrease of sensitivity to ethanol was also shown previously for the offspring of chronically intoxicated mice [7]. Effects on depression have not been demonstrated previously. Hence, administration of a single minor or moderate dose of ethanol to the rat males immediately prior to mating cause the same behavioral changes as in the offspring of the fathers with chronic exposure to alcohol.

Activity of ADG in the blood of animals with fathers under the influence of alcohol was higher than in the control rats. It has been shown that ADG could participate in the mechanisms of analgesia induced by administration of ethanol [18], hence, decrease of sensitivity to analgesic effect of ethanol in the animals conceived by the intoxicated fathers could be explained by this fact.

The results of our experiments do not allow revealing causative association between the activity of enzymes and behavioral changes. However, we could suggest that the increase of activity of DPP-IV and decrease of activity of PEP in the blood of animals conceived by the fathers under the alcohol influence facilitate changes of the ratio of regulatory neuropeptides and peptide hormones, which, in turn, could cause behavioral changes. In order to find out what particular changes occur with the changes in activities of DPP-IV and PEP, further experiments are required. Nevertheless, certain suggestions could be made. In particular, decrease of the activity of PEP in blood could result in increase of the level of thyrotropin-releasing hormone in blood, which exerts an antidepressant effect [19, 20]. It was shown previously in the experiments with mice that the synthetic inhibitors of PEP have an antidepressant activity [21].

Increase of the DPP-IV activity in offspring males could indicate decrease of concentration of the substance P (SP) affecting perception of pain via modulation of SP concentration at the periphery and activation of the peripheral NK1 receptors by the peptide [22]. Endomorphin-2 is another important substrate of DPP-IV [23]. It is likely that DPP-IV modulates analgesia induced by endomorphin-2. The third important substrate of DPP-IV is neuropeptide Y (NPY) [24], which plays a very important role in regulation of affective state, reaction to stress, and pathogenesis of a number of diseases, including depression.

In addition, we have found out that the offspring males conceived by the fathers under alcohol influence exhibit suppression of the reaction of enzyme activities on ethanol administration. While in the control animals' administration of ethanol results in increase of the DPP-IV activity and decrease of the PEP activity, the offspring of the intoxicated fathers do not

exhibit such response. This could be manifestation of the general trend: offspring of the males exposed to ethanol prior to mating are less sensitive to most of the ethanol effects [7].

It was shown that chronic consumption of ethanol by the mouse males results in the decrease of methylation of imprinted genes in spermatozoa, including genes of neurotrophic factors [25]. This could result in the change of functioning of limbic system in the brain and, as a result, to the change in animal behavior. Considering that the single-dose administration of ethanol exerts the same effect as the chronic one, it cannot be ruled out that the observed in this study changes in the offspring of the intoxicated fathers could be associated with this epigenetic mechanism. As a result, activity of a number of enzymes could change, which, in turn, could result in the change of the balance of neuropeptides participating in mediation of the animal behavior.

Contributions. S.K.S. concept and supervision of the study; N.G.B. and G.A.N. conducting experiments; S.K.S. and N.N.Z. discussion of the results and writing text of the paper; N.G.B. editing text of the paper.

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