



Drinking Pattern in Intermittent Access Two-Bottle-Choice Paradigm in Male Wistar Rats Is Associated with Exon-Specific BDNF Expression in the Hippocampus During Early Abstinence

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Abstract

Outbred rats differentially consume alcohol when having free access to it. Among others, BDNF (brain-derived neurotrophic factor) is believed to control voluntary ethanol intake in rodents. Meanwhile, expression of BDNF exons in brain regions and epigenetic mechanisms underlying alcohol intake pattern remain obscure. The main goal was to study whether voluntary alcohol drinking pattern affects expression of BDNF exons in selected rat brain regions during early abstinence. Intermittent access to 20% ethanol in a two-bottle-choice procedure (IA2BC) was used as a model of voluntary ethanol intake. Male Wistar rats ($n = 24$) had twenty 24-h sessions of free access to two-bottle choice (water or 20% ethanol) with 24-h withdrawal periods (water only). Control animals had access to water only ($n = 11$). After finishing IA2BC, the animals were divided according to the compliance of ethanol intake pattern with gradual escalation, a key feature of the paradigm. To assess potential behavioral disturbances during the early abstinence, rats were consequently tested in the open field test, the elevated plus-maze, and the sucrose preference test. On the third day after the last drinking session, expression of BDNF exons and polypeptide was measured in the frontal cortex, hippocampus, striatum, and midbrain using quantitative PCR and Western blotting, respectively. Additionally, chromatin immunoprecipitation was performed to analyze enrichment of positive Ph-CREB (Ser133) and negative EZH2 transcriptional regulators as well as markers of active H3K9ac and repressed H3K27me3 chromatin at exon-specific BDNF promoters in brain regions with affected BDNF expression. During the course of the IA2BC, one part of animals demonstrated gradual escalation from low to high alcohol intake and preference of alcohol over water (a typical pattern for IA2BC) while the other one consumed alcohol at a consistently high level (an unusual pattern for IA2BC). Drinking pattern in the IA2BC does not define differences of behavior in any of the tests during early abstinence. Finally, the IA2BC rats with growing alcohol intake showed elevation of BDNF mRNA containing exon VI in the hippocampus associated with an enhanced H3K9ac occupancy at the respective promoter. Thus, rats differentially consuming alcohol in the IA2BC paradigm differ in epigenetically determined expression of BDNF exon VI in the hippocampus during early abstinence.

Keywords Male Wistar rats · Alcohol intake · Intermittent access two-bottle-choice paradigm · Early abstinence · Brain-derived neurotrophic factor · Hippocampus

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Introduction

Neuroplastic changes in mesocorticolimbic system underlie dependence on psychoactive drugs (Kalivas and O'Brien 2008). Neurotrophins are crucial players in the molecular machinery underlying alcohol use disorder (Ron and Barak 2016; Liran et al. 2020). Brain-derived neurotrophic factor (BDNF) is widely believed to be a key neurotrophin regulating molecular, structural, and functional disturbances in the CNS accompanying addiction to drugs of abuse including alcohol (Logrip et al. 2015; Ron and Berger 2018). BDNF may regulate both voluntary alcohol consumption and functional alterations in the abstinence; this was demonstrated using different models of alcohol use disorders. BDNF heterozygous (BDNF \pm) mice displayed increased ethanol intake in a two-bottle-choice procedure (Hensler et al. 2003); downregulation of BDNF level by 50% in these mice also enhanced voluntary ethanol consumption (McGough et al. 2004). On the other hand, some authors reported that signaling mediated by BDNF receptor TrkB does not affect voluntary ethanol intake but may regulate abstinent behavior after withdrawal. Thus, augmenting BDNF signaling by TrkB agonist 7,8-dihydroflavone (7,8-DHF) during ethanol consumption did not modify drinking pattern in the binge alcohol exposure paradigm; however, it abolished depression-like behavior after withdrawal (Briones and Woods 2013). Similarly, TrkB receptor antagonist ANA-12 administered daily during the whole stabilization period of the free-choice paradigm did not affect ethanol consumption (Stragier et al. 2015a). Particularly, the BDNF levels changed in the dorsal striatum (McGough et al. 2004), cortex (Logrip et al. 2009), and hippocampus (Briones and Woods 2013; Stragier et al. 2015a; Roni and Rahman 2017) of rodents consuming alcohol on a free-choice basis.

BDNF expression is a complex process due to the presence of several regulatory regions within the gene. Dynamic epigenetic changes within BDNF gene such as rearrangements of proteins regulating transcriptional activity and histone modifications underlie BDNF expression alterations and subsequent neuroplasticity both in physiological and pathological contexts (Mitchellmore and Gede 2014). CREB (cAMP-responsive element-binding protein) is the most studied transcriptional regulator of the BDNF gene. After phosphorylation at Ser-133, CREB recruits the histone acetyl transferase CREB-binding protein (CBP), which may act as a transcriptional co-activator through acetylation of lysine residues within histones to remodel chromatin structure into a form accessible to active transcription (West et al. 2001). CREB-haplodeficient mice were reported to consume significantly higher amounts of alcohol solution and show a higher preference for ethanol over water in a two-bottle free-choice paradigm compared with the respective wild-type mice (Pandey et al. 2004). At least partially, CREB-mediated voluntary alcohol intake may be regulated by BDNF since CREB-haplodeficient mice have decreased

BDNF content in the brain. In addition to myriads of transcriptional regulators of the BDNF gene activity, recently, EZH2 (enhancer of zeste 2), a polycomb repressive complex 2 (PRC2) subunit, has been described as a negative regulator of BDNF expression both in vitro (Qi et al. 2014; Palomer et al. 2016) and vivo (Koo et al. 2015). However, EZH2 reduces BDNF mRNA level in the ventral tegmental area and robustly increases morphine-conditioned place preference in mice (Koo et al. 2015); its role in the context of voluntary alcohol abuse has not been studied yet.

Among the animal models of alcohol abuse based on voluntary intake, intermittent access to 20% ethanol in a two-bottle-choice procedure (IA2BC) attracts special attention (carefully reviewed by Carnicella et al. 2014). According to the model, rats receive 24-h sessions of free access to two-bottle choice (water vs. 20% ethanol) with 24-h withdrawal periods for several weeks. Exposure of outbred rats to repeated cycles of free-choice ethanol intake and withdrawal using the IA2BC has been shown to induce a gradual escalation of voluntary ethanol intake and preference. However, genetic variability of outbred animals may underlie a significant diversity of ethanol intake within a population, intake pattern deviating from a gradual escalation. The gradual increase from moderate to excessive ethanol drinking can potentially model the transition from moderate “social”-like drinking to excessive alcohol drinking in humans. Molecular and biochemical adaptations as well as behavioral alterations were reported after training in the IA2BC procedure following a short period of abstinence. According to both experimental and clinical data, motor hyperactivity, anxiety, and depression may manifest during early abstinence period after prolonged alcohol exposure (Holleran and Winder 2017; Jesse et al. 2017).

We supposed that a population of outbred Wistar rats might be heterogeneous in alcohol drinking pattern in the IA2BC, these differences being detectable in the behavior during early abstinence. In the present study, we used a retrospective analysis to differentiate rats according to their drinking patterns and to estimate anxiety and depression-like behavior during the early abstinence period. We also tried to address the issue of the involvement of exon-specific BDNF expression and epigenetic changes at the respective promoters in several brain regions of male Wistar rats with different patterns of voluntary alcohol intake during early abstinence.

Methods

Animals and Procedures

Animals

Male outbred Wistar rats from the “Stolbovaya” Breeding Center (Moscow region, Russia) were used in the study. At

the beginning of the experiment, the average weight of 12-week-old animals was 295 ± 7 g. The rats were housed in individual cages under 12:12-h light/dark cycle (5 am “light on”/5 pm “light off”) and free access to water and food. Behavioral testing was conducted during the light phase of the daily cycle. The rats were not handled before the start of or between the experiments with the exception of the needs of regular cleaning. Table 1 represents a scheme of the experimental protocol.

Intermittent Access to 20% Ethanol in a Two-Bottle-Choice Procedure

The IA2BC procedure was performed according to Simms and co-authors (Simms et al. 2008). Briefly, rats ($n = 24$) received twenty 24-h sessions of free access to two-bottle choice (water and 20% (v/v) ethanol) with 24-h withdrawal periods (water only). The placement of the ethanol bottle was alternated in each ethanol drinking session to control for side preferences. Control rats ($n = 11$) had a continuous access to two bottles with water. Liquids were presented in the dark period. Bottles were weighed before and after each drinking session. The rats were weighed before each drinking session. Ethanol intake is presented as grams of alcohol per kilogram of body weight per drinking session. Spillage of water and ethanol solution (loss of fluid in a cage without rat) was recorded on daily basis, and subsequently subtracted from individual fluid intakes. The preference for ethanol over water was calculated in percent of ethanol intake to total liquid intake. Gradual increment of alcohol intake over time course is a key feature of the IA2BC (Carnicella et al. 2014). After finishing the procedure, correlation analysis was used to check the relationship between drinking session number and alcohol intake level. The drinking pattern was considered as typical for

IA2BC if the analysis demonstrated statistically significant positive correlation between drinking session number and alcohol intake level.

Open Field Test

The open field test (OFT) was performed on day 1 after the last drinking session in the IA2BC using a round arena of 120 cm in diameter with a wall of 40 cm in height. The floor of the arena was divided into center (60 cm in diameter) and border zones. The rat was placed into the central zone of the arena and allowed to explore it for 5 min. The distance traveled (horizontal activity) and total number of rearing and climbing (total vertical activity) were assessed as measures of ambulatory activity. The level of anxiety-like behavior was determined by counting the number of entries into the center and time spent in the center. Animal behavior was recorded with a digital video camera and estimated visually by a blinded investigator.

Elevated Plus-Maze Test

The elevated plus-maze (EPM) test was performed on day 2 after the last drinking session. The EPM apparatus (Open Science, Russia) consisted of four arms (50×14 cm) forming a cross centered on a central area (14×14 cm): two open arms with a perimeter wall 1.5 cm high and the two closed arms with a perimeter wall 20 cm high. The maze was elevated to a height of 50 cm from the floor. The EPM was located in a room illuminated with eight, 20-W luminescent overhead lights, which produced consistent illumination within the room (1000 lm each). The EPM was placed close to the center of the room, and had similar levels of illumination on both open and closed arms. Animals were placed in the central area with the nose to the open arm and allowed to freely move for 5 min. Animal behavior was recorded using a digital video camera and the number of entries into open and closed (an entry was counted when all four paws were placed into a section) and time spent in the arms were estimated visually by a blinded investigator.

Sucrose Preference Test

On the first day after the last drinking session in the IA2BC, the rats were allowed to choose between a bottle filled with water and a bottle with 1% sucrose solution during a 24-h period (acclimation for sweet solution). Subsequently, on the second day after the last drinking session in the IA2BC, 5% sucrose solution and water were presented for 24-h period. The measurement of 5% sucrose consumption was performed on day 3 after the last drinking session. Bottle positions were changed

Table 1 Experimental design

Day	Procedure
1–40	Intermittent access to 20% ethanol in the two-bottle-choice procedure (IA2BC). Male Wistar rats received twenty 24-h sessions of free access to two-bottle choice (water and 20% ethanol) with 24-h withdrawal periods (water only).
+ 1	OFT Access to 1% sucrose solution and water
+ 2	EPM Access to 5% sucrose solution and water
+ 3	SPT (measurement of 5% sucrose solution consumed) Brain sampling for biochemical analysis: <ul style="list-style-type: none"> • BDNF expression (RT-qPCR, WB) in the frontal cortex, hippocampus, striatum, and midbrain; • ChIP analysis for positive Ph-CREB (Ser133) and negative EZH2 transcriptional regulators as well as marks of active H3K9ac and repressed H3K27me3 chromatin at BDNF promoters in brain regions with altered BDNF expression

every 12 h in order to prevent the development of place preference. All bottles were weighted prior to the start and after 5% sucrose solution presentation. Sucrose consumption is presented as grams of sucrose per kilogram of body weight per day. The preference of sucrose solution was calculated as percent of 5% sucrose solution intake to total liquid intake.

Biochemical Studies

After the end of the sucrose preference test (SPT), brains were removed and the samples of the frontal cortex, hippocampus, striatum, and midbrain were taken for biochemical measurements. These main mesocorticolimbic structures were chosen since they are regarded as responsible for motivational and (or) aversive aspects of alcohol misuse (Gilpin and Koob 2008). One hemisphere from each brain was randomly assigned for reverse transcription real-time polymerase chain reaction (RT-qPCR) and western blotting (WB) while the second hemisphere was used for chromatin immunoprecipitation (ChIP) analysis. The samples were frozen in liquid nitrogen and stored at -80°C until use.

Reverse Transcription Real-Time Polymerase Chain Reaction

The content of BDNF mRNAs specific for exons I, IV, and VI as well as common exon IX was estimated using RT-qPCR. In the rat brain, BDNF mRNAs containing exons I, IV, and VI are sensitive to different stimuli, such as contextual fear learning (Lubin et al. 2008), social defeat stress (Duclot and Kabbaj 2013), morphine withdrawal (Peregud et al. 2015), and chronic unpredictable stress (Stepanichev et al. 2018). Total RNA was extracted from the samples using ExtractRNA reagent (#BC032; Evrogen, Russia). The purity and concentration of the total RNA were measured by recording the optical density at 260 and 280 nm. Only samples with a ratio of optical density of 260/280 within the range of 1.8–2.1 were used for the study. Three samples out of 35 (one sample from each of the groups) did not pass this criterion and were excluded from further biochemical analysis. The integrity of the total RNA was estimated using electrophoresis in 1.5% agarose gel. Isolated total RNA was purified from genomic DNA using 2.5 U DNase I (#EN0521; Thermo Fisher Scientific, USA). Total RNA (0.5 μg) was reverse transcribed in the presence of 50 U of reverse transcriptase MMLV (#SK022; Evrogen, Russia) and a mixture of 1 μM each of oligo (dT)15 and random decamer oligonucleotides (#SB001 and #SB002, respectively; Evrogen, Russia) as primers. qPCR was performed in two parallel samples using a master mix containing Taq DNA-polymerase with antibodies inhibiting the activity of the enzyme and SYBR GREEN intercalating fluorescent dye (#PK147; Evrogen, Russia) in the presence of 5 pmol of synthetic oligonucleotide primers. Sequences of primers used for amplification of BDNF exons I, IV, VI (Schmidt et al. 2012),

IX (Tsankova et al. 2004), ACTB (Peregud et al. 2016), and rpS18 (Peregud et al. 2015) were previously described. The oligonucleotides were synthesized by Evrogen (Russia). qPCR was performed using the ANK-32 thermocycler (Institute for Analytical Instrumentation, Russian Academy of Sciences and Bauman Moscow State Technical University, Russia) according to the following protocol: 1, 10 s at 95°C ; 2, 10 s at 65°C ; and 3, 40 s at 72°C (fifty cycles). The contents of the specific mRNAs were calculated according to the threshold cycle method (C_t) using ANK32 software for Windows, version 1.1 (Institute for Analytical Instrumentation, Russian Academy of Sciences, Russia) and it was followed by comparison of ACTB and rpS18 mRNAs content of as reference genes by the $2^{-\Delta\Delta C_t}$ method (Livak and Schmittgen 2001).

Western Blotting

To analyze content of pro-BDNF protein in tissue extracts, the standard western blotting procedure was performed. Briefly, brain tissue was homogenized in 10 volumes of phosphate-buffered saline (PBS), containing 1% NP-40, 10% glycerol, 5 mM EDTA, 50 U/mL of RNase inhibitor (#RNI0100; Sileks, Russia), and a cocktail of protease and phosphatase inhibitors (#78440; Thermo Fisher Scientific, USA). Homogenates were centrifuged at 13,000g for 20 min at 4°C , and the supernatants stored at -80°C until use. Total protein concentrations in the homogenates were determined using BCA Protein Assay Reagent (#23227; Pierce, USA). Equal amount of proteins (25 μg per well) were separated by SDS-PAGE on 13% polyacrylamide gels. Then, proteins were transferred electrophoretically to PVDF membranes and blocked with 2% non-fat dry milk. After blocking, the membranes were incubated overnight at 4°C with rabbit polyclonal anti-BDNF antibody diluted to 1:500 (#sc-546; SantaCruz Biotechnology, USA). After incubation with secondary anti-IgG antibody conjugated to horseradish peroxidase (Bio-Rad, USA), immunoreactive proteins were detected by SuperSignal West Pico Chemiluminescent Substrate (#34077; Pierce, USA), and then quantified by scanning membranes on C-DiGit Blot Scanner (LI-COR Biosciences, USA) and densitometry analysis using TotalLab v. 2.00 software (Nonlinear Dynamics Ltd., USA). BDNF was detected from the band density at 32 kDa and considered to be corresponding to the pro-form. After stripping, BDNF-stained membranes were re-probed with anti-beta-tubulin loading control antibody (#MA5-16308; Thermo Fisher Scientific, USA) (1:40,000) for following normalization procedure.

Chromatin Immunoprecipitation

ChIP was performed to analyze enrichment of positive Ph-CREB (Ser133) and negative EZH2 transcriptional regulators

as well as marks of active H3K9ac and repressed H3K27me3 chromatin at the exon-specific BDNF promoters. The hippocampus was minced on ice, placed into a tube containing 1% freshly prepared formaldehyde in PBS, and incubated 10 min at room temperature. After adding glycine to final concentration of 125 mM, cross-linked tissue was washed in PBS and homogenized in SDS lysis buffer (50 mM Tris pH 8.1, 1 %SDS, 10 mM EDTA, and a cocktail of protease and phosphatase inhibitors (#78440; Thermo Fisher Scientific, USA)). The lysates were sonicated to shear DNA into 100–700 bp fragments (DNA length was verified on a 1% agarose gel). The resulting homogenates were centrifuged for 20 min at 13,000g at 4 °C. Aliquots of total chromatin (20% of lysate volume used for immunoprecipitation) were saved and designated as “Input.” Before immunoprecipitation, lysates were diluted ($\times 10$) with buffer containing 16.7 mM Tris pH 8.1, 1.1% Triton X100, 167 mM NaCl, 0.01% SDS, 1.2 mM EDTA, and a cocktail of protease and phosphatase inhibitors. Diluted samples were incubated overnight at 4 °C in the presence of ChIP-validated antibodies (EMD Millipore Corporation, USA): 2 μ g anti-Ph-CREB (# 17-10131), 1 μ g anti-EZH2 (#17-662), 2 μ g anti-histone H3 trimethylated at Lys27 – H3K27me3 (#17-622), or 2.5 μ g anti-histone H3 acetylated at Lys9 - H3K9ac (#17-658). Immunocomplexes were harvested by adding pre-blocked with 0.5 mg/ml bovine serum albumin (#A1391; AppliChem, Germany) and 0.2 mg/ml salmon sperm DNA (#15632011; Thermo Fischer Scientific, USA) Magna ChIP™ protein A+G magnetic beads (#16-663; Millipore, USA) followed by incubation at room temperature for an hour. Beads were washed with low-salt buffer containing 20 mM Tris pH 8.1, 1% Triton X100, 0.1% SDS, 2 mM EDTA, and 150 mM NaCl, followed by washing with high-salt buffer containing 500 mM NaCl. DNA-protein complexes were eluted from the beads by buffer containing 50 mM Tris pH 8.1, 1% SDS, and 10 mM EDTA. Complexes were digested by 0.25 mg/ml proteinase K (#P8102; NEB, USA) for 4 h at 65 °C. DNA was isolated using DNAzol reagent (#DN217, MRC, USA) in the presence of linear acrylamide Satellite Red (#BC001; Evrogen, Russia) as a co-precipitant. DNA quantities in the immunoprecipitated samples were detected by qPCR using primers encompassing promoters of BDNF exons I, IV (Schmidt et al. 2012), and VI (Lubin et al. 2008). C_t values were used to calculate immunoprecipitated DNA quantities as percentage of corresponding inputs.

Statistics

The data are presented as $M \pm SEM$. All biochemical analyses were performed in at least two technical replicates. Distribution of behavioral and biochemical data corresponded to normal according to Kolmogorov-Smirnov test. Grubbs test did not reveal statistically significant outliers within analyzed data. Pearson correlation was used to analyze the relationship

between drinking session and alcohol intake (test for conformity with typical IA2BC drinking pattern—gradual escalation). The results obtained from the IA2BC procedure were analyzed for statistical significance of between group differences by ANOVA for repeated measures (RM-ANOVA) followed by Tukey’s honestly significant difference (HSD) post hoc test. The rest of the data (results of the OFT, the EPM, the SPT, and biochemical studies) was analyzed by one-way ANOVA followed by Tukey’s HSD post hoc test. Statistical analysis of the data was performed using STATISTICA 7.0 software (StatSoft Inc., USA) or Prism 6 for Windows (GraphPad Software Inc., USA). The results are presented by means of Microsoft Excel 2016 (Microsoft Corporation, USA) and GIMP 2.8.20 (Spencer Kimball, Peter Mattis and the GIMP Development Team). Differences were considered significant at p values less than 0.05.

Results

Male Outbred Rats Exhibit Different Patterns of Alcohol Intake in the IA2BC Procedure

Calculation of Pearson correlation coefficient r for relationship between drinking session number and level of alcohol intake showed that a part of animals demonstrated positive correlation of these variables conforming to gradual escalation of alcohol intake over time, a typical IA2BC drinking pattern, while another part of the animals did not meet this criterion. These differences in alcohol intake patterns in IA2BC allowed dividing animals into two groups. Group I consisted of the rats demonstrating a gradual escalation of alcohol intake over the course of the procedure (pattern typical for IA2BC); Group II included the rats consuming ethanol at stable high level (pattern unusual for IA2BC) (Fig. 1). RM-ANOVA revealed a significant main effect of drinking session number on ethanol intake $F_{(19,380)} = 5.737$, $p = 1.273 \times 10^{-12}$ and no effect of drinking pattern $F_{(1,20)} = 0.117$, $p = 0.736$, though significant interaction between these two factors occurred, $F_{(19,380)} = 3.906$, $p = 1.208 \times 10^{-7}$. According to subsequent post hoc analysis, only the animals of Group I consumed significantly lower ethanol during the first four drinking sessions as compared with the drinking sessions 10–20 (Fig. 1a). Particularly, ethanol intake after sessions 1 and 2 was lower as compared with sessions 10–20 ($p < 0.0005$), while after sessions 3 and 4 rats consumed less alcohol as compared with sessions 10–16 ($p < 0.05$) and 17–20 ($p < 0.005$). As expected, RM-ANOVA revealed similar trends for alcohol preference data. Analysis of alcohol preference demonstrated a significant main effect of drinking session $F_{(19,380)} = 9.315$, $p < 0.0001$, no effect of drinking pattern $F_{(1,20)} = 0.001$, $p = 0.971$, but significant interaction of these factors $F_{(19,380)} = 4.901$, $p = 2.4 \times 10^{-10}$. Similar to ethanol intake, alcohol preference as indicated by post hoc analysis in

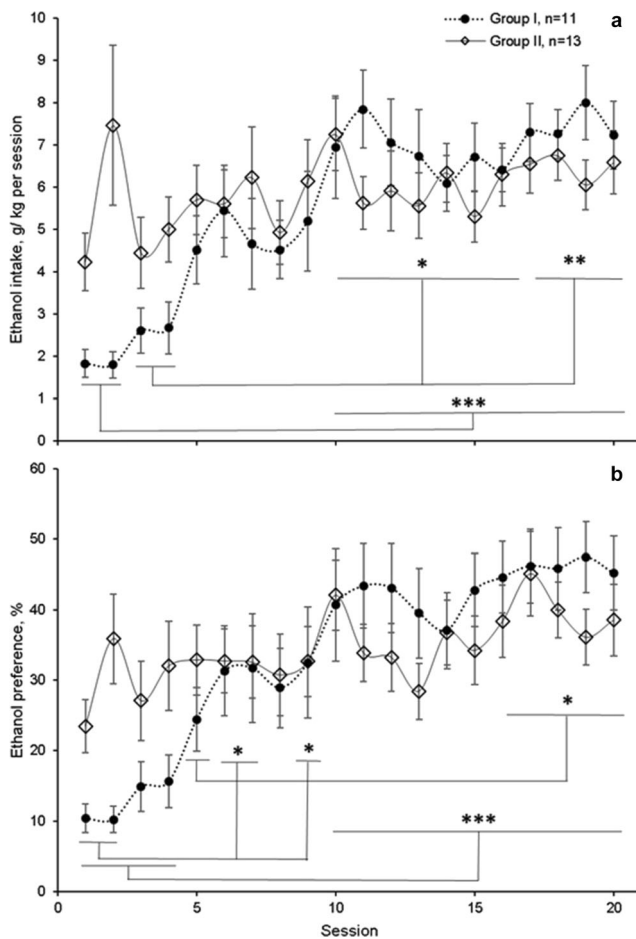


Fig. 1 Male Wistar rats differently consume alcohol in IA2BC paradigm. A half of animals demonstrated gradual increase from low to high level of alcohol intake (a) and preference alcohol over water (b), Group I, while other consumed alcohol at the stable high level, Group II. Rats received twenty 24-h sessions of free access to two-bottle choice (water and 20% ethanol) with 24-h withdrawal periods (water only). Brackets indicate statistically significant differences of alcohol intake and preference between indicated sessions within the group demonstrating increasing level of alcohol intake (Group I). RM-ANOVA followed by Tukey’s HSD post hoc test. * $p < 0.05$; ** $p < 0.005$; *** $p < 0.0005$

earlier drinking sessions was lower as compared with the final ones only in Group I (Fig. 1b). Specifically, ethanol preference after sessions 1–4 was lower as compared with sessions 10–20 ($p < 0.0005$); additionally, ethanol preference after sessions 1 and 2 was lower as compared with sessions 6, 7, and 9 ($p < 0.05$). Moreover, ethanol preference after session 5 was lower as compared with sessions 16–20 ($p < 0.05$). However, despite quite different patterns of alcohol intake clearly observed in the initial period of drinking, the final result was similar in both experimental groups. According to the absence of main effect of drinking pattern in RM-ANOVA, neither ethanol intake nor preference differed between groups as revealed by post hoc analysis. Individual drinking data are present in the [supplementary table](#). RM-ANOVA revealed a significant main effect of drinking session number on total liquid (water

and ethanol) intake $F_{(19,418)} = 2.344, p = 0.001$ and no effect of group $F_{(1,22)} = 0.007, p = 0.935$, these two factors not interacting, $F_{(19,418)} = 1.043, p = 0.410$. As expected, subsequent post hoc analysis did not reveal differences in total liquid intake between experimental groups (Fig. 2a). Analysis of water intake showed a significant main effect of drinking session number $F_{(19,418)} = 9.534, p = 1.012 \times 10^{-12}$ and no effect of ethanol drinking pattern $F_{(1,22)} = 0.026, p = 0.873$, though significant interaction between these two factors occurred, $F_{(19,418)} = 3.505, p = 1.271 \times 10^{-6}$. Contrary to ethanol intake, animals of Group I consumed significantly more water during the first four drinking sessions as compared with drinking sessions 10–20 (Fig. 2b). Water consumption did not differ between Groups I and II (Fig. 2b). According to post hoc analysis, water intake after sessions 1–4 was higher as compared with sessions 15–20 ($p < 0.0005$), while after sessions 1–3 the rats consumed more

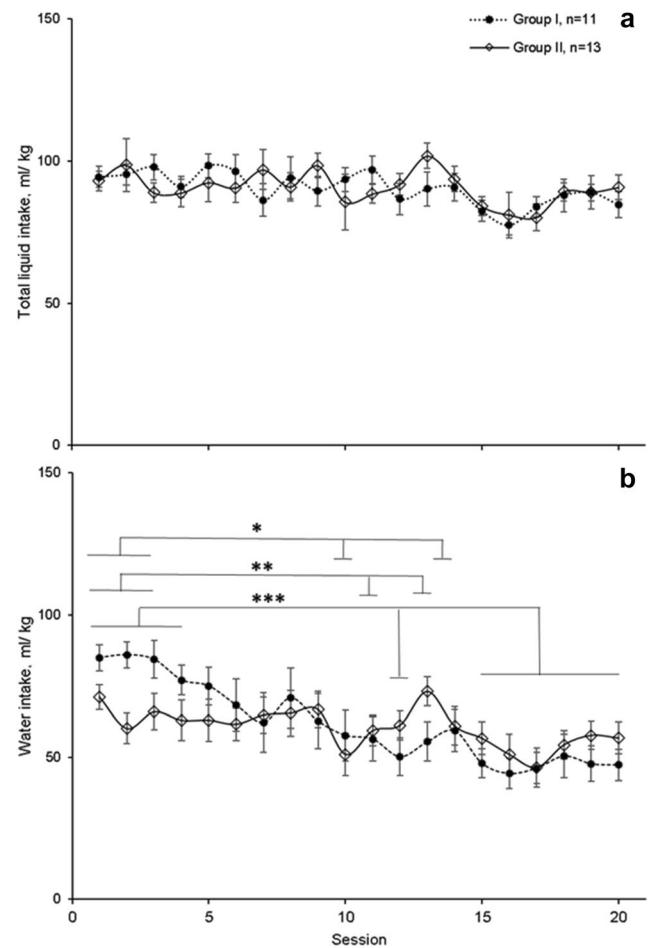


Fig. 2 The animals consume water in the IA2BC paradigm in a manner opposite to ethanol intake. While total liquid volume consumed does not differ between groups (a), Group I gradually increasing ethanol intake in the IA2BC paradigm, on the contrary, decreases water intake with time (b). Rats received twenty 24-h sessions of free access to two-bottle choice (water and 20% ethanol) with 24-h withdrawal periods (water only). Brackets indicate statistically significant differences of water intake between indicated sessions within Group I. RM-ANOVA followed by Tukey’s HSD post hoc test. * $p < 0.05$; ** $p < 0.005$; *** $p < 0.0005$

water as compared with sessions 10 and 14 ($p < 0.05$) and 11 and 13 ($p < 0.005$).

Different Patterns of Alcohol Intake Are Not Associated with Anxiety and Depression-Like Behavior During the Early Abstinence Period

Next, we studied affective behavior in rats with different patterns of alcohol intake during the early abstinence period. We did not find any significant effect of voluntary alcohol consumption or differential patterns of intake on locomotor behavior of rats in the OFT on the first day after the IA2BC procedure completion (Fig. 3; one-way ANOVA main group effect on distance traveled $F_{(2,32)} = 0.156$, $p = 0.856$; on vertical activity $F_{(2,32)} = 0.900$, $p = 0.417$). The indices of anxiety-like behavior in the OFT were also similar in different groups of animals (main group effect on entries into the center zone $F_{(2,32)} = 1.299$, $p = 0.287$; on the time spent in the center zone $F_{(2,32)} = 1.962$, $p = 0.157$; data not shown).

On the second day after the last drinking session, the rats were tested in the EPM (Fig. 4). Although the rats of Group II entered the open arms more rarely and spent less time therein, one-way ANOVA did not reveal statistical significance of group effect for the number of entries into the open $F_{(2,32)} = 2.826$, $p = 0.079$ or closed arms $F_{(2,32)} = 1.413$, $p = 0.263$.

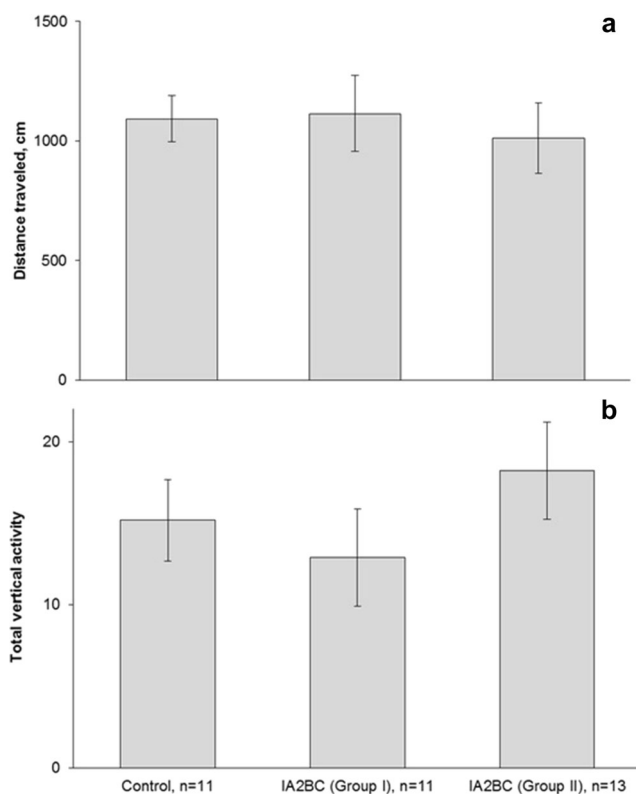


Fig. 3 The IA2BC procedure does not affect activity in the OFT during early abstinence. Horizontal (a) and vertical (b) ambulatory activity were measured on the first day after the last drinking session in the IA2BC

One-way ANOVA for the time spent in the closed or open arms did not show significant group effect either: $F_{(2,32)} = 0.533$, $p = 0.592$; $F_{(2,32)} = 0.866$, $p = 0.430$; $F_{(2,32)} = 0.533$, $p = 0.592$, respectively.

On the third day after the last drinking session, the rats were tested in the SPT. Though no difference in sucrose preference (Fig. 5a) or intake (Fig. 5b) was observed (main group effect $F_{(2,32)} = 0.533$, $p = 0.592$; $F_{(2,32)} = 0.408$, $p = 0.668$), a significant group effect on water intake was evident, $F_{(2,32)} = 3.4888$, $p = 0.043$ (Fig. 5c). Subsequent post hoc analysis demonstrated that water intake was higher in Group II rats as compared with control group (Fig. 5c).

Distinct Patterns of Alcohol Intake Are Associated with Minor Differences in BDNF mRNA Expression

BDNF mRNAs Content in the Brain Regions

Expression of BDNF transcripts containing exons I, IV, VI, and IX in the frontal cortex, hippocampus, striatum, and mid-brain was studied on the third day after the last drinking session in the IA2BC. One-way ANOVA revealed significant differences for expression of BDNF exon VI-specific transcript only in the hippocampus, while the level of other

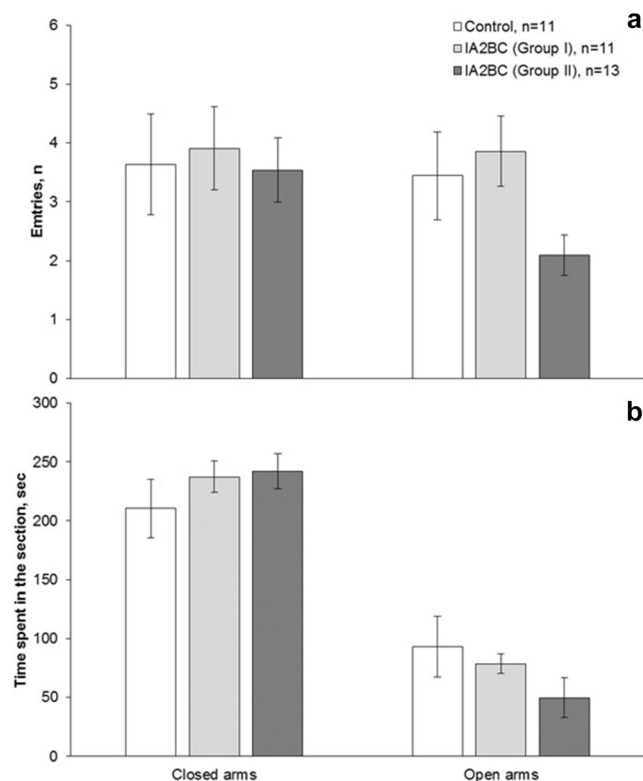


Fig. 4 The IA2BC procedure does not affect anxiety-like behavior in the EPM during early abstinence. The EPM test was performed on the second day after the last drinking session in the IA2BC. The number of entries into the closed or open arms (a) as well time spent in the section (b) were estimated

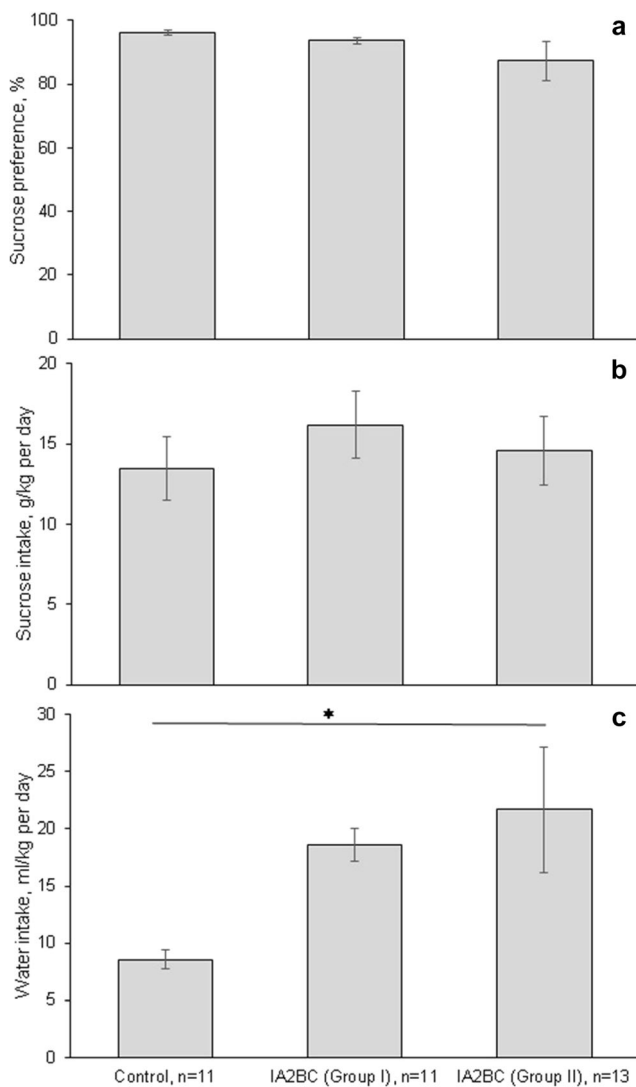


Fig. 5 Drinking pattern in the IA2BC paradigm is not associated with depression-like anhedonia state in the SPT during early abstinence. However, rats of Group II show increased water volume intake as compared with controls. The SPT was performed on the third day after the last drinking session in the IA2BC. Sucrose preference (a), sucrose intake (b), and water volume intake (c) were assessed. One-way ANOVA followed by Tukey’s HSD post hoc test. * $p < 0.05$

transcripts studied did not change (Table 2). According to post hoc analysis, expression of BDNF mRNA containing exon VI was significantly higher in the hippocampus of Group I as compared with control (Fig. 6b). No effect of group on the expression of BDNF mRNAs in the frontal cortex or midbrain could be revealed (Fig. 6a, c; Table 2). BDNF exons could not be reliably detected in the striatum.

Levels of Pro-BDNF Polypeptide in the Brain Regions

One-way ANOVA of pro-BDNF content in the brain regions on the third day after the last drinking session in the IA2BC did not reveal statistical significance for group effect in the

Table 2 One-way ANOVA statistics for biochemical data

Approach	Brain region	Parameter studied	$F_{(2,29)}$	p value
RT-qPCR	Frontal cortex	Exon I	0.864	0.433
		Exon IV	0.778	0.469
		Exon VI	1.264	0.298
		Exon IX	1.930	0.164
	Hippocampus	Exon I	1.395	0.264
		Exon IV	0.770	0.472
		Exon VI	4.300	0.024
		Exon IX	2.064	0.145
	Midbrain	Exon I	0.820	0.452
		Exon IV	0.127	0.881
		Exon VI	0.147	0.864
	WB	Frontal cortex	Pro-BDNF protein	2.436
Hippocampus			2.833	0.075
Striatum			0.622	0.544
Midbrain			0.312	0.734
ChIP	Hippocampus	Ph-CREB at pExI	0.607	0.552
		Ph-CREB at pExIV	0.883	0.424
		Ph-CREB at pExVI	0.205	0.815
		EZH2 at pExI	0.118	0.889
		EZH2 at pExIV	0.606	0.553
		EZH2 at pExVI	1.314	0.284
		H3K9ac at pExI	4.234	0.024
		H3K9ac at pExIV	1.012	0.376
		H3K9ac at pExVI	3.354	0.050
		H3K27me3 at pExI	2.700	0.084
		H3K27me3 at pExIV	0.077	0.926
		H3K27me3 at pExVI	5.079	0.013

brain regions studied (Table 2). Pro-BDNF level did not differ between groups in the frontal cortex, hippocampus, striatum, and midbrain (Fig. 7).

Epigenetic Aspects of BDNF Gene Promoters

Next, we tested whether upregulation of BDNF exon VI in the hippocampus of Group I rats was associated with epigenetic changes at the BDNF gene regulatory regions on the third day after the last drinking session in the IA2BC. We performed ChIP and estimated enrichments of positive Ph-CREB (Ser133) and negative EZH2 transcriptional regulators as well as marks of active H3K9ac and repressed H3K27me3 chromatin at the promoters of BDNF exons I, IV, and VI. One-way ANOVA of H3K9ac and H3K27me3 enrichment revealed that occupancy of both marks differed between groups at the promoters of exons I and VI (Table 2). According to post hoc analysis, H3K9ac occupancy was significantly higher at

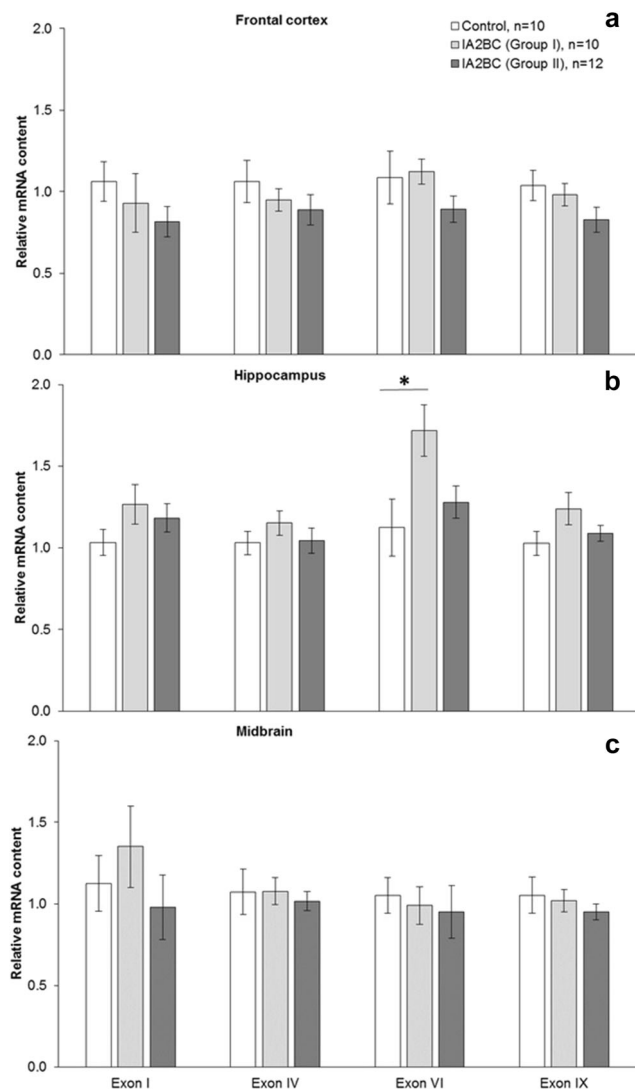


Fig. 6 Drinking pattern in the IA2BC is associated with elevation of hippocampal BDNF mRNA containing exon VI during early abstinence. Content of BDNF mRNAs in the frontal cortex (a), hippocampus (b), and midbrain (c) was determined using RT-qPCR on the third day after the last drinking session in the IA2BC. BDNF mRNA detection in the striatum was not reliable. One-way ANOVA followed by Tukey's HSD post hoc test. * $p < 0.05$

promoters of exons I and VI in the hippocampus of Group I rats as compared with controls (Fig. 8c). Similarly, H3K27me3 enrichment was significantly higher at the promoter of exon VI in Group I rats as compared with controls (Fig. 8d). The IA2BC experience did not affect enrichment of either Ph-CREB (Fig. 8a) or EZH2 (Fig. 8b) at BDNF promoters (Table 2).

Discussion

In the present study, male Wistar rats differently consumed alcohol in the IA2BC paradigm. Some rats exhibited a gradual

escalation of alcohol intake and preference of alcohol over water from low to high level (Group I) while others consumed alcohol at a consistently high level (Group II). During early abstinence period, the rats with different patterns of alcohol consumption demonstrated differences neither in ambulatory activity nor in anxiety-like nor in depression-like behavior. The pattern of alcohol consumption in Group I was associated with elevation of BDNF mRNA containing exon VI in the hippocampus accompanied by enhanced H3K9ac occupancy at the corresponding promoter. Thus, we report for the first time on the association between drinking pattern in the IA2BC procedure and brain region- and exon-specific BDNF expression during early abstinence.

Wistar Rats Differentially Consume Alcohol in the IA2BC Paradigm

Escalation of voluntary ethanol intake and preference over drinking sessions is the main feature of the IA2BC procedure (Carnicella et al. 2014), but some rats in a population may fail to escalate ethanol intake. For example, a subpopulation of high-drinking Fischer rats significantly rise ethanol consumption in the IA2BC whereas low-drinking rats do not (Mill et al. 2013). Similarly, high-drinking Wistar rats not only have a higher alcohol intake and preference compared with the low-drinking ones but also have an increased alcohol intake over time, which is not seen in low-drinking rats (Momeni and Roman 2014). As reviewed previously (Carnicella et al. 2014), at early stages of the IA2BC procedure, rats typically consume relatively little ethanol (< 2.5 g/kg/24 h); however, within 3–4 weeks of training, they gradually escalate consumption to considerably higher amounts of ethanol (5–6 g/kg/24 h), with ~50% ethanol preference. In the present study, only some male Wistar rats demonstrated a similar escalation pattern of ethanol intake and preference. However, another part of rats consumed ethanol at a consistently high level beginning from the first drinking session. We can assume that the stock of outbred Wistar rats used in the study may have a specific background affecting voluntary ethanol intake in such a manner. Indeed, Wise (1974, 1975) reported that Wistar rats purchased from two different breeders consumed alcohol differently on the every-other-day free-choice schedule without seasonal sensitivity.

Behavioral Alterations During Early Abstinence in Rats Differentially Consuming Alcohol in the IA2BC Paradigm

The IA2BC is characterized by specific behavioral alterations following a short period of abstinence. Specifically, acute (24–72 h) but not protracted (16–68 days) abstinence in rats trained in the IA2BC was reported to cause working memory

Fig. 7 IA2BC procedure does not affect pro-BDNF polypeptide content in the brain regions during early abstinence. The level of BDNF polypeptide in the brain regions was assessed on the third day after the last drinking session in the IA2BC. Upper panel show representative Western blots in hippocampus; bottom panel displays a diagram of respective densitometric analyses

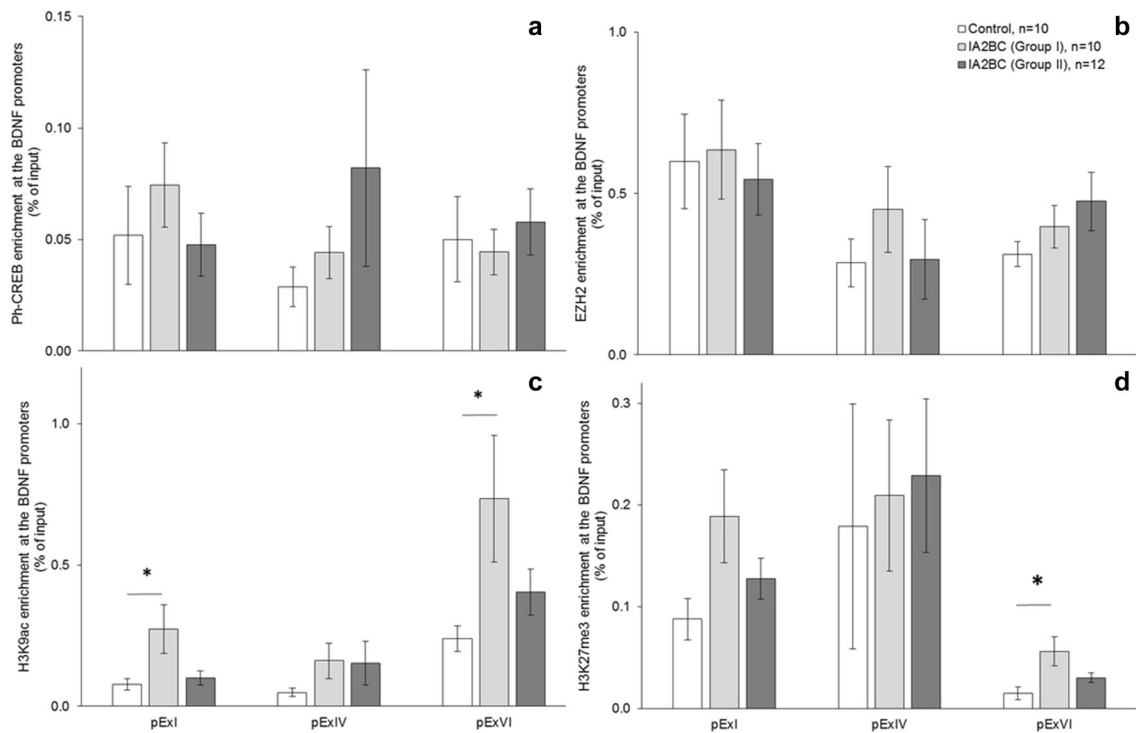
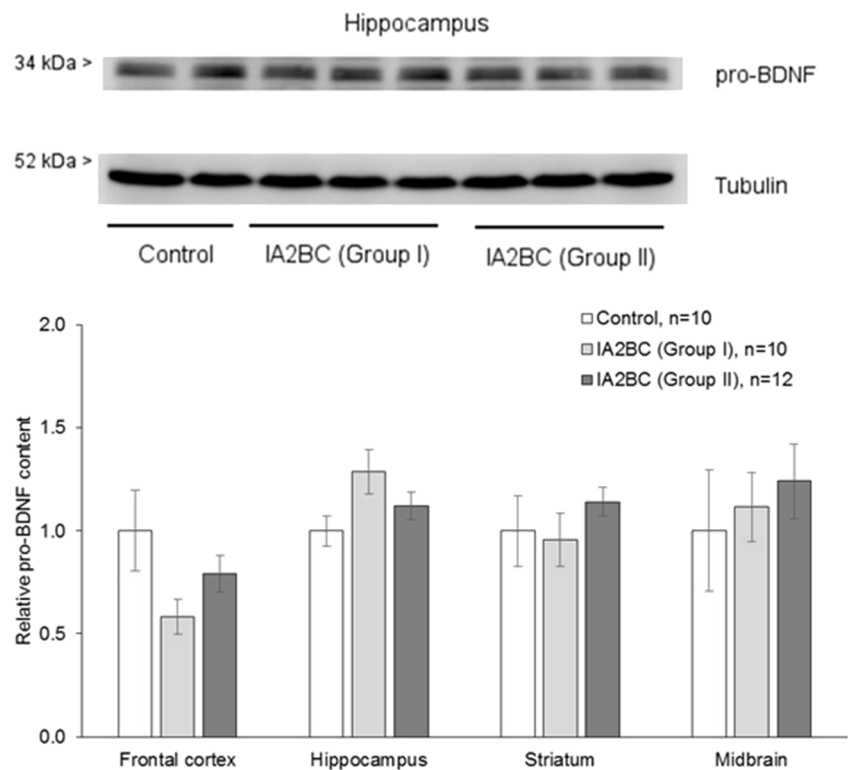


Fig. 8 Drinking pattern in the IA2BC is associated with specific post-translational modifications of histones H3 at the BDNF gene promoters in the hippocampus during early abstinence. ChIP followed by qPCR shows that on the third day after the last drinking session in the IA2BC, H3K9ac (c) occupancy demonstrates upregulation at promoters of exons I (pExI) and VI (pExVI) in the hippocampus of Group I rats as compared with

control animals, while H3K27me3 (d) occupancy increases at pExVI only. The IA2BC does not affect binding of positive Ph-CREB (Ser133) (a) and negative EZH2 (b) transcriptional regulators to BDNF promoters in the hippocampus. One-way ANOVA followed by Tukey’s HSD post hoc test. * $p < 0.05$

deficits in spontaneous alteration task and operant-based delayed non-matching to sample task (George et al. 2012). Interestingly, continuous chronic ethanol consumption in a free-choice paradigm (3–10% of ethanol for 3 weeks) also impaired learning and memory capacities analyzed in contextual fear conditioning test and novel object recognition task (Stragier et al. 2015b). In addition, withdrawal from chronic ethanol drinking under the IA2BC paradigm for 12 weeks could induce mechanical and thermal hypersensitivity in Sprague-Dawley rats starting at 12 h after ethanol removal and lasting for 7 days (Fu et al. 2015). In contrast, some studies demonstrated lack of anxiety- and depression-like behaviors, as respectively measured by the SPT and the EPM, as well as lack of deficit in object recognition memory during a week of abstinence after 6 weeks of drinking in the IA2BC (Nelson et al. 2018).

Rats demonstrating variability in voluntary ethanol intake in the IA2BC may demonstrate differences of some behaviors at the abstinence after the end of the IA2BC paradigm. Indeed, high-drinking in IA2BC outbred Wistar rats had lower risk-taking behavior prior to alcohol access and lower anxiety-like behavior after voluntary alcohol intake as compared with low-drinking rats (Momeni and Roman 2014). According to our results, Wistar rats do not demonstrate behavioral alterations in the OFT, the EPM, and the SPT during early abstinence after completion of the IA2BC in a manner independent on drinking pattern.

Briones and Woods (2013) demonstrated no significant group differences in the number of entries in the center area after 8 days of alcohol consumptions in binge-like manner, accompanied by significantly longer immobility time and decreased ambulation in the OFT as compared with controls. On the other hand, chronic ethanol consumption in a free-choice paradigm (3–10% of ethanol for 3 weeks) did not affect the ambulation and the time spent in the central area of the open field (Stragier et al. 2015b). We have revealed that in male Wistar rats, the IA2BC experience affects neither ambulatory activity nor anxiety-like behavior in the OFT.

Data from the experiments using different paradigms of alcohol drinking demonstrate various, often opposite associations of alcohol drinking patterns and anxiety-like behavior in the withdrawal period. The multivariate concentric square field test has revealed that high-drinking in the IA2BC outbred Wistar rats has lower anxiety-like behavior after voluntary alcohol intake compared with low-drinking rats (Momeni and Roman 2014). However, no changes of anxiety-like behavior as measured in the EPM were found at 24 h (George et al. 2012) or a week (Nelson et al. 2018) of abstinence after completing IA2BC procedure. Similarly, continuous ethanol access in a free-choice paradigm (3–10% of ethanol for 3 weeks) did not affect anxiety-like behaviors in the EPM (Stragier et al. 2015b). Thus, our data are, at least partially, in agreement with previous reports

demonstrating absence of anxiety-like alterations after completion of the IA2BC procedure.

Alcohol consumption in binge-like manner may result in depression-like anhedonia detected using a sucrose preference test (Briones and Woods 2013). However, Nelson and co-authors (Nelson et al. 2018) did not reveal changes of sucrose preference 1 week after IA2BC. In the present study, anhedonia was not observed during early abstinence after the IA2BC. Noteworthy, the level of sucrose preference revealed in the study was very high, thus preventing to reveal differences between experimental groups (ceiling effect). Yet, a significant increase in water intake was evident in Group II rats consuming totally more alcohol over the course of IA2BC. Water intake may be considered as an inverse measure of sucrose intake to some extent and, thus, it may indirectly point that these rats get more “anhedonic” during early abstinence.

Differential Region- and Exon-Specific Expression of BDNF mRNAs in Rats with Distinct Patterns of Alcohol in the IA2BC Paradigm

In the present study, the drinking pattern in the IA2BC paradigm was followed by specific modifications of BDNF expression in rat brain during early abstinence. First, the changes of BDNF level were specific for hippocampus. Second, the alterations of BDNF mRNA content were specific for exon VI.

There are plenty of data on BDNF expression at mRNA level (though mainly without exon specification) and/or protein level in rodent brain regions after voluntary alcohol consumption. However, region and exon specificity of BDNF expression in the IA2BC paradigm has not been studied yet. Furthermore, the available data differ significantly depending on the protocol of alcohol consumption. Continuous access to 10% ethanol in a two-bottle-choice paradigm for 4 weeks significantly increased BDNF mRNA expression in murine dorsal striatum, but not in the prefrontal cortex or hippocampus (McGough et al. 2004). No changes in hippocampal BDNF were also reported after 3 weeks of continuous access to alcohol (Gallego et al. 2015). Limited access (4 h in the dark period) to 10% ethanol in a two-bottle-choice paradigm for 6 weeks associated with ethanol intake escalation over time resulted in the downregulation of BDNF mRNA expression throughout murine neocortex cortex 24 h after cessation; however, it did not affect striatal BDNF (Logrip et al. 2009). Several groups reported a decrease in the BDNF protein level in the rat hippocampus during withdrawal period, independent on specific protocols (Briones and Woods 2013; Roni and Rahman 2017). In addition, ethanol intake without free choice also upregulated BDNF in brain regions, particularly in the hippocampus (Miller 2004). Early withdrawal (3 h) but not protracted abstinence (3 weeks) from intermittent ethanol exposure via vapor for 7 weeks increased BDNF

protein level in the hippocampus and medial prefrontal cortex of rat (Somkuwar et al. 2016). So far, only Stagier and co-authors (Stragier et al. 2015a) have studied epigenetic basis of BDNF expression after voluntary ethanol intake. BDNF mRNA containing exon VI, but not those containing exons I and IV, increases in the hippocampus of mouse after continuous access to ethanol in a two-bottle-choice procedure. In addition, they have demonstrated upregulation of BDNF protein in the murine hippocampus in these experimental settings (Stragier et al. 2015a). Despite significant differences in experimental conditions, our data regarding hippocampal BDNF exon VI upregulation after the IA2BC procedure generally correspond to the results received by Stagier and co-authors (2015a) after continuous access to 10% ethanol in a two-bottle-choice procedure.

Interestingly, expression of hippocampal BDNF exon VI is associated with vulnerability to social defeat in rats. Resilient animals exhibited a significant upregulation of both BDNF exon VI mRNA and protein levels following social defeat, while vulnerable rats failed to display this response (Duclot and Kabbaj 2013). Differential regulation of BDNF exon VI mRNA in resilient and vulnerable rats was associated with a coherent regulation of histone modifications at the corresponding promoter of the BDNF gene (Duclot and Kabbaj 2013). Resilient rats exhibited higher levels of both activation marks (H3K4me2 and H3K9K14Ac) following social defeat, whereas vulnerable did not. BDNF exon VI mRNA upregulation was associated with a reduced H3K9me2 level, a repressive histone mark, in resistant rats (Duclot and Kabbaj 2013). In contrast to social defeat, acute immobilization and footshock differently affected expression of exons I and IV but did not affect BDNF exon VI level in the hippocampus of rat (Fuchikami et al. 2010).

The expression of BDNF mRNA from of exon VI promoter is controlled by various neurotransmitters. BDNF mRNA containing exon VI was significantly reduced in the hippocampus and prefrontal cortex of serotonin transporter knockout rats and the downregulation of BDNF exon VI mRNA in prefrontal cortex correlated with an increase in DNA methylation at CpG sites of its promoter (Molteni et al. 2010). In cortical astrocytes, BDNF mRNA containing exon VI was induced by dopamine via CREB-dependent signaling (Koppel et al. 2018). In addition to CREB, expression of BDNF mRNA from the promoter of exon VI may require other transcription factors such as nuclear factor- κ B (Morioka et al. 2013), early growth response 3 (Meyers et al. 2018), or AP-1 (Tuvikene et al. 2016). Opposite to inducers, BDNF gene has the well-established repressor, i.e., EZH2. EZH2 knockdown or inhibition in N2A neuroblastoma cells was accompanied by upregulation of both total BDNF transcripts and those containing exon VI (Mallei et al. 2015). EZH2 overexpression in the rat ventral tegmental area increased H3K27me3 levels at all BDNF promoters and

decreased Ph-CREB binding to all of them (Koo et al. 2015). Importantly, differences in BDNF expression and possible epigenetic basis have been shown for human with early versus late onset alcohol use disorder. Particularly, amygdalar common coding BDNF exon IX and BDNF protein showed significant decrease in humans with early onset but not late onset alcohol use disorder compared with controls, occupancy of EZH2, and histone H3 trimethylation at BDNF gene regulatory loci underlying this process (Bohnsack et al. 2019).

Stagier and co-authors (Stragier et al. 2015a) demonstrated that increased level of acetylated histone H3 at the promoter of exon VI accompanied upregulation of BDNF exon VI mRNA in the mouse hippocampus after continuous ethanol access in a free-choice paradigm (3–10% of ethanol for 3 weeks). Knockdown of histone deacetylase 2, responsible for the deacetylation of histones including H3, in the amygdala attenuated anxiety-like behavior and voluntary alcohol intake in alcohol-preferring rats and increased levels of H3K9ac within promoter of BDNF exon IV with a resultant increase in protein levels (Moonat et al. 2013). Similarly, our data show that drinking pattern-specific upregulation of exon VI in the hippocampus is naturally accompanied by elevated active chromatin mark (H3K9ac) at the corresponding promoter. It is a predictable result since histone H3 acetylation at K9 position precedes gene expression. Interestingly, we also observed upregulation of H3K27me3 (mark of repressed chromatin) level at the promoter of exon VI. It is known that marks of active and repressed chromatin may coexist within BDNF promoters (Palomer et al. 2016). However, we have revealed that relative values of enrichment for active mark H3K9ac is higher than for repressed mark H3K27me3 which may allow expression of exon VI. Additionally, we found elevated occupancy of both active and repressed marks at exon I without alteration of expression of the respective exon. Since enrichment of repressed mark prevails over active mark, it may prevent expression of exon I. In contrast to histone modifications, we have not found enrichment changes for transcriptional regulators. Presumably, binding of transcriptional regulators to regulatory regions of BDNF gene and subsequent modification of H3 and chromatin state are temporally divided.

Drinking pattern-specific upregulation of hippocampal BDNF during early abstinence maybe not associated with behavior-related issues; however, BDNF may participate in other abstinence-related processes. Specifically, elevated BDNF level demonstrated in the present work may control morphological and (or) physiological processes by regulating translation of specific proteins in the hippocampus, specifically in animals escalating ethanol intake. Indeed, voluntary alcohol intake affects cell proliferation and differentiation in the hippocampus, while BDNF signaling controls this process. The specific blockade of TrkB by ANA-12 suppressed ethanol-induced (chronic ethanol consumption in a free-choice paradigm, 3–10% of ethanol for 3 weeks) increase of

the number of Ki-67 immunoreactive cells (Stragier et al. 2015a). Alcohol consumptions in a binge-like manner (4 h access in the dark period to 10% ethanol in a IA2BC for 12 days) resulted in a decrease in the number of bromodeoxyuridine-positive cells in the rat dentate gyrus, while daily administration 7,8-DHF prevented it (Briones and Woods 2013).

Conclusion

In summary, male Wistar rats differentially consume alcohol in the IA2BC paradigm: a half of them demonstrates gradual escalation from low to high level of alcohol intake and preference of alcohol over water while other animals consume alcohol at a consistently high level. Drinking pattern in the IA2BC is not associated with gross alterations in ambulatory activity and anxiety- or depression-like behavior during early abstinence. However, rats escalating alcohol intake in the IA2BC show elevation of BDNF mRNA containing exon VI in the hippocampus related to enhanced occupancy of active chromatin mark H3K9ac at the respective promoter. Thus, drinking pattern in the IA2BC is specifically associated epigenetically determined hippocampal BDNF expression from the exon VI promoter during early abstinence.

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Compliance with Ethical Standards

All experiments with animals were performed in accordance with the EU Directive 2010/63/EU. The experimental protocol was approved by the Ethical Commission of the Institute of Higher Nervous Activity and Neurophysiology, Russian Academy of Sciences.

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