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Disturbances in behavior and cortical enkephalin gene expression during the anticipation of ethanol in rats characterized as high drinkers

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Abstract

The process of ethanol anticipation is a particularly important phenomenon that can determine subsequent drug-taking behavior. Recent studies suggest that systems within the medial prefrontal cortex (mPFC), during anticipation, may contribute to the goal-directed seeking of ethanol. The current investigation examined the possibility that the opioid peptide enkephalin (ENK), known to mediate some of the reinforcing properties of ethanol, may function in the mPFC during the anticipation of ethanol access. Using a limited access (3 h/d) paradigm for 10 days with 20% ethanol, Sprague-Dawley rats were first identified either as low drinkers (LD, <1.0 g/kg/3 h) or as high drinkers (HD, >2.0 g/kg/3 h) that exhibited a long-term phenotype of high ethanol consumption and a significant ethanol deprivation effect. During the anticipation period immediately preceding daily ethanol access, the HD rats compared to LD or Control animals with *ad libitum* ethanol access exhibited increased anticipatory behaviors, including greater exploratory behavior in a novel open field as revealed by significantly more time spent in the rearing position (+53–65%, $p < 0.05$) and increased number of rears made (+33–44%, $p < 0.05$) and greater novelty-seeking behavior in a hole-board apparatus revealed by an increase in total (+50–52%, $p < 0.05$) and novel nose pokes (+45–48%, $p < 0.05$). In the HD rats, analysis of the mPFC using real-time quantitative PCR showed significantly greater mRNA levels of ENK ($p < 0.05$) and the mu-opioid receptor (MOR) ($p < 0.05$), but not delta-opioid receptor (DOR), and this increase in ENK expression was found, using *in situ* hybridization, to occur specifically in the prelimbic (PrL) subregion of the mPFC. When injected into the PrL during the anticipation period, a MOR agonist but not DOR agonist significantly increased consumption of 20% ethanol ($p < 0.05$). These findings support the role of ENK, acting through MOR within the PrL to promote the anticipation and excessive consumption of ethanol.

Keywords

Medial prefrontal cortex; Prelimbic cortex; Enkephalin; Ethanol anticipation; Mu opioid receptors; Delta opioid receptors

Introduction

Recent preclinical and clinical studies with alcohol addiction point to an important role of alcohol-related cues, such as certain environments or daily rituals, in triggering excessive consumption of ethanol as well as relapse and craving (Beck et al., 2009; Koob & Le Moal, 1997; Pickering & Liljequist, 2003). With anticipatory processes being essential in

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determining subsequent ethanol-seeking and -taking behaviors, it is important to examine the brain systems that are active during the specific period of heightened reward expectation.

Ethanol anticipation, described as a conditioned behavioral response that occurs during the expectation of a rewarding substance such as ethanol, is characterized by heightened exploratory and seeking behaviors (Melendez et al., 2002). Investigations to date of the neurochemical mechanisms contributing to ethanol anticipation have focused attention on the mesolimbic dopamine (DA) system, composed of the ventral tegmental area (VTA) and nucleus accumbens (NAc). Studies in inbred ethanol-preferring animals or in outbred rats trained chronically to self-administer high amounts of ethanol have revealed a significant increase in the release of dopamine as well as excitatory amino acids in the NAc during the anticipation or seeking phase of ethanol acquisition, (Doyon et al., 2003; Katner, Kerr, & Weiss, 1996; Li et al., 2008; Melendez et al., 2002). While these neurotransmitters in the accumbens are known to play an important role in reward-related processes of ethanol anticipation, there are other brain areas involved in goal-directed behaviors that are also likely to contribute to the seeking aspect of ethanol anticipation. One such area is the medial prefrontal cortex (mPFC), which is anatomically positioned to integrate sensory and limbic information and has recently received attention in terms of its involvement in food as well as drug anticipation and excessive consumption (Goldstein & Volkow, 2011; Mitchell et al., 2012; Ng, Stice, Yokum, & Bohon, 2011). Human imaging studies demonstrate that cues related to palatable foods or drugs of abuse increase neural activity within this brain area and that this activation is, in turn, related to enhanced craving or consumption of these substances (Dagher, Tannenbaum, Hayashi, Pruessner, & McBride, 2009; Grusser et al., 2004; Killgore et al., 2003; Ng et al., 2011). Similarly, animal studies have provided evidence suggesting that palatable food cues stimulate the activation of neurons within the mPFC (Schiltz, Bremer, Landry, & Kelley, 2007; Schroeder, Binzak, & Kelley, 2001), while also increasing the release of DA in this area (Merali, McIntosh, & Anisman, 2004). Of particular note is the very recent finding, with drugs of abuse such as heroin, that cue-induced relapse behavior is closely related to the neuronal activation of specific subregions of the mPFC (Bossert et al., 2011). While this evidence strongly suggests a functional role for the mPFC in the anticipatory process related to food and drugs, there is little information on possible neurochemical systems in this area that may mediate the anticipation of ethanol.

With recent clinical evidence showing an important role of cortical opioid release in heavy alcohol drinking (Mitchell et al., 2012), one possible candidate may be the opioid peptide, enkephalin (ENK). While not yet studied in the mPFC of rodents, ENK in other brain areas has been related to behaviors characteristic of anticipation, as well as to the consumption of ethanol. Central injections of ENK analogs, in mesolimbic and more recently hypothalamic regions, provide strong evidence for its role in mediating the rewarding mechanisms of ethanol consumption (Barson, Carr et al., 2009; Barson et al., 2010), and rodents that prefer and consume large amounts of ethanol, compared to non-preferring animals, have greater endogenous expression of this peptide in the NAc and other limbic regions (Jamensky & Gianoulakis, 1999; Marinelli, Kiianmaa, & Gianoulakis, 2000). Specific ENK receptors, such as mu opioid receptors (MOR) and delta opioid receptors (DOR) are particularly important in mediating this phenomenon, with ethanol sensitivity and consumption found to be reduced in mice lacking either of these two receptor subtypes (Blednov, Walker, Martinez, & Harris, 2006; Hall, Sora, & Uhl, 2001). The possibility that ENK in the mPFC may be contributing to the high drinking phenotype is supported by the finding that selectively-bred, ethanol-preferring animals under naïve conditions exhibit high mRNA levels of both ENK and MOR in this region (Marinelli et al., 2000) and that ethanol exposure in preferring and non-preferring animals can stimulate the expression of this peptide in the mPFC (Chang, Karatayev, Barson, Chang, & Leibowitz, 2010; Mendez, Leriche, & Calva, 2001; Mendez & Morales-Mulia, 2006). Whereas the function of ENK

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within the mPFC has yet to be investigated in relation to ethanol consumption or anticipation, a recent study showed the injection of a MOR-specific agonist in select subregions of the mPFC to enhance exploratory behavior as well as the consumption of a palatable high-energy diet (Mena, Sadeghian, & Baldo, 2011).

With this evidence suggesting the possibility that opioid mechanisms within the mPFC may be related to anticipatory behavior and ethanol consumption, the current study was designed, first, to establish a model for characterizing the anticipatory behaviors of Sprague-Dawley rats identified by their high versus low drinking behavior using a limited access paradigm. These subgroups were then examined during the period of ethanol anticipation for their mRNA expression of ENK as well as MOR and DOR within the mPFC using quantitative PCR and also of ENK in specific ventral and dorsal subregions of the mPFC using *in situ* hybridization. Finally, central microinjection procedure was used to investigate the effect of specific ENK analogs within the mPFC and the possible function of this opioid in driving the consumption of ethanol during the same anticipatory period.

Materials and methods

Subjects

Adult, male Sprague-Dawley rats (Charles River Breeding Labs, Kingston, NY) were housed individually, on a 12-h reversed light/dark cycle in a fully accredited American Association for the Accreditation of Laboratory Animal Care facility, according to institutionally approved protocols as specified in *the NIH Guide to the Care and Use of Laboratory Animals* and also with the approval of the Rockefeller University Animal Care Committee. The rats in each set of ethanol-drinking groups were approximately matched for body weight, with an overall range of 300–350 g at the start of the experiment. All animals were allowed 1 week to acclimate to their individual housing conditions, during which time they were given *ad libitum* access to standard rodent chow (LabDiet Rodent Chow 5001, St. Louis, MO; 12% fat, 60% carbohydrate, and 28% protein) and water offered in two sipper tubes.

Test procedures

The first three experiments examined the anticipatory behaviors and neurochemical profile of the mPFC of animals during the period of expecting a large versus small bout of ethanol access, compared to animals under non-anticipating conditions, while the last experiment tested whether central injections of specific opioid agonists into the mPFC during the period of ethanol anticipation can, in turn, affect ethanol drinking behavior.

In Experiment 1, rats ($N = 32$) were trained over 10 days to consume a 20% ethanol solution using a 2-bottle choice paradigm similar to the drinking in the dark paradigm often employed in mice (Rhodes, Best, Belknap, Finn, & Crabbe, 2005). In the “anticipation” group, rats ($n = 24$)were placed on a limited access ethanol schedule, in which they were given ethanol for 3 h/d starting at dark onset, while having *ad libitum* access to chow and water. The “non-anticipation” group (Control) ($n = 8$), in contrast, had *adlibitum* access to the 20% ethanol solution, together with chow and water. The ethanol (95% ethanol, David Sherman Corp., St. Louis, MO) diluted in tap water was presented in the home cage in a plastic bottle at the top of the cage (PETCO Animal Supplies, Inc.) which was fitted with a sipper tube containing a steel ball as a tip valve to prevent spillage. Ethanol consumption was recorded daily and body weights twice a week. Beginning on the 7th day of drinking, ethanol consumption showed an increasingly stronger, positive correlation from day to day (days 7–8: $r = +0.58$; days 8–9: $r = +0.67$; days 9–10: $r = +0.83$), which allowed the animals to be sub-grouped based on their intake values during the last 4 days of drinking. The non-

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anticipation rats consumed an average of 1.3 ± 0.2 g/kg/day, while the anticipation rats drank a range of 0.4–3.2 g/kg/3 h, allowing them to be further subdivided into low drinkers (n = 8, LD, lowest 33%) consuming an average of 0.6 ± 0.1 g/kg/3 h and high drinkers (n = 8, HD, highest 33%) consuming 2.3 ± 0.3 g/kg/3h, with the middle group omitted from the study. On the 10th day of ethanol drinking, tail vein blood was also collected at the end of the 3rd hour and measured for blood ethanol content (BEC). On day 11, the Control, LD and HD rats were further examined during the period of ethanol anticipation at dark onset, for 5 min in a novel open field activity chamber first without and then with a hole board apparatus installed for exploratory and novelty-seeking behaviors, with food removed 1 h prior to testing. An additional set of HD and LD anticipation rats (Experiment 1b, n = 8/group) were further examined for their long-term drinking patterns when allowed to consume ethanol 12 h/d for an additional 2 weeks and then, 1 week after ethanol withdrawal, for their ethanol deprivation effect (EDE), an indicator of relapse-like behavior (McBride, Le, & Noronha, 2002). To measure the EDE, a post-deprivation test was conducted on three consecutive days, during which animals were allowed to consume the 20% ethanol or water solution for 12 h/d, and these values were compared to ethanol intake at baseline.

For Experiment 2, a new set of rats (N = 32) was trained to consume a 20% ethanol solution as described for Experiment 1, and after 10 days, they were classified in an identical manner into Control, LD and HD groups (n = 8/group). The average drinking values for days 7–10 were similar to those obtained in Experiment 1, with the HD rats consuming 2.1 ± 0.22 g/kg/3 h ethanol, the LD rats consuming 0.8 ± 0.17 g/kg/3 h, and the Control rats consuming 1.5 ± 0.19 g/kg/24 h. In order to measure the neurochemical mechanisms possibly contributing to the anticipation of ethanol, the rats were sacrificed on the 11th day of training immediately at dark onset, when they were expecting their daily ethanol access and when their behavioral measures of anticipation (exploration and novelty-seeking) were recorded in Experiment 1. Chow was removed 1 h before dark onset, in order to prevent any effects that food intake might have on peptide expression. Although ethanol itself is known to stimulate the expression of ENK in the mPFC (Chang, Barson, et al., 2010; Mendez et al., 2001; Mendez & Morales-Mulia, 2006), it is important to note that the anticipating animals in this paradigm had no ethanol available for 20 h prior to sacrifice. Also, the Control animals were sacrificed at the onset of the dark cycle, making it unlikely that they consumed high amounts of ethanol as rats typically consume most of their daily ethanol several hours into the dark cycle. After sacrifice, the brains were rapidly removed, and the mPFC was micro-dissected and processed for measurements of ENK, MOR and DOR mRNA expression using quantitative real-time polymerase chain reaction (qRT-PCR), as described below.

In Experiment 3, a new set of animals (N = 24) was used to confirm and provide more anatomical precision to the qRT-PCR results obtained in Experiment 2. The rats were trained to consume the 20% ethanol solution and then classified into HD and LD animals as described in Experiments 1 and 2 (see Table 1). The HD and LD rats were sacrificed on the 11th day of training, at dark onset when they were expecting their daily ethanol access, and their whole brains were collected and processed for measurements of ENK in the specific subregions of the mPFC using digoxigenin-labeled *in situ* hybridization (DIG). For this experiment, the Control group was not tested, as it showed no difference from the LD rats in terms of its level of ENK expression, as described in the results below.

The purpose of Experiment 4 was to examine the effect that injection of specific ENK agonists, during the anticipatory period, have on the drinking of ethanol. Rats (N = 8) were first trained for 10 days to consume 20% ethanol solution for 3 h/d, as described above, and were then surgically implanted with cannulas aimed at the prelimbic subregion (PrL) of the mPFC. They were allowed to recover for one week, while still being maintained on their normal, limited access schedule of ethanol and *ad libitum* schedule of food and water. With

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water and ethanol solution available, animals were then injected (0.5 μl) into the mPFC with either saline vehicle or two doses of the MOR-specific agonist, [D-Ala2, N-MePhe4, Gly-ol]-enkephalin (DAMGO 1.8 nmol, 3.6 nmol), during the first week of testing and with either saline or two doses of the DOR-specific agonist, [D-Ala2, L-Leu5]-enkephalin (DALA 7.1 nmol, 14.2 nmol), during the second week. Measurements of water and ethanol intake were recorded for 3 h after injection, and the effects produced by each drug injection were compared to vehicle given on counterbalanced consecutive days using a within-subject design.

Blood ethanol concentration

In Experiment 1, tail vein blood was collected from Control, LD and HD rats on the 10th day of ethanol training and used for BEC measurements. BEC measurements were made using the Analox GM7 Fast Enzymatic Metabolic Analyzer (Lunenburg, MA) and reported as mg/dl.

Brain dissections

Immediately after sacrifice, the brains for Experiment 2 were removed for peptide measurements using qRT-PCR. Brains were placed with the ventral surface facing up in a matrix and three 1.0 mm coronal sections were made, with the middle optic chiasm as the posterior boundary (Paxinos & Watson, 1986). For micro-dissection, the sections were placed on a glass slide and the mPFC (Bregma 2.8–3.6 mm) was removed under a microscope, using the forceps minor of the corpus collosum as a landmark. These dissections were stored in RNA*later* (Sigma–Aldrich Co., St. Louis, MO) until processed.

Quantitative Real-time PCR analysis

In Experiment 2, qRT-PCR was used to measure ENK, MOR and DOR mRNA levels in them PFC. As previously described (Morganstern et al., 2010), total RNA from individual microdissected cortical samples was extracted with the RNAEasy Mini Kit (Qiagen, CA) using 1.0 mm zirconia/silica beads (Biospec Products, OK). For all groups, the cDNA and minus RT were synthesized using an oligo-dT primer with or without SuperScript II reverse transcriptase. The qRT-PCR experiments were conducted with Applied Biosystems (ABI) system. With Applied Biosystems Primer Express V1.5a software, primers were designed to have a melting temperature of 58–60 °C and to produce an amplicon of 50–160 base pairs. The last five bases on the 3′ end contained no more than 2 G and/or C bases, to reduce the possibility of nonspecific product formation.

The SYBR Green PCR core reagents kit (ABI, CA) was used with cyclophilin (cyc) as an endogenous control. PCR was performed in MicroAmp Optic 96-well Reaction Plates (ABI) on an ABI PRISM 7900 Sequence Detection system, with the condition of 2 min at 50 °C, 10 min at 95 °C, then 40 cycles of 15 s at 95 °C and 1 min at 60 °C. Each study consisted of 4 independent runs of PCR in triplicate, and each run included a standard curve, non-template control, and negative RT control. The levels of target gene expression were quantified relative to the level of cyc by the relative quantification method, based on threshold with Ct value of 18–25 for the different genes. For our initial experiments, we used cyc, β-actin and GAPDH as controls. Since cyc gave the most reliable data with no region or treatment specific changes in quantity, we continued to use cyc to normalize our data for ENK, MOR and DOR expression. The primers, designed with ABI Primer Express V.1.5a software based on published sequences, were: 1) cyc: 5′-GTGTTCTTCGACATCACGGCT -3′ (forward) and 5′-CTGTCTTTGGAACTTTGTCTGCA -3′ (reverse); 2) ENK: 5′-GGACTGCGCTAAATGCAGCTA -3′ (forward) and 5′- GTGTGCATGCCAGGAAGTTG -3′ (reverse); 3) MOR: 5′- TGCATCCCAACCTCGTCCACGA - 3′ (forward) and 5′-

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TCTGCCTCCAGATTTTCTAGCTGGT - 3′ (reverse); and 4) DOR: 5′-CCCCGGGATGGAGCAGTGGTATG -3′ (forward) and 5′-GGGCACCACGAAGGCGAAGAG -3′ (reverse). The concentrations of primers were 100–200 nM, and all reagents, unless indicated, were from Invitrogen (Carlsbad, CA). The specificities of RT-PCR products were confirmed by both a single dissociation curve of the product and a single band with a corresponding molecular weight revealed by an agarose gel electrophoresis. In addition to the non-template control and a negative RT control, the specificity of the quantitative PCR was verified with an anatomical negative control by using the corpus callossum in the same brain. No signals above threshold of all targeted genes were detected by qRT-PCR in all of the controls.

Digoxigenin-labeled in situ hybridization histochemistry

In situ hybridization histochemistry with DIG-labeled probes was used to quantify ENK mRNA in specific subregions of the mPFC. This technique specifically measures the density of neurons expressing the peptide gene above threshold levels. Brains were cut into 30 μm thick sections with a cryostat. DIG-labeled cRNA probes of ENK were synthesized by *in vitro* transcription as previously described (Chang, Barson, et al., 2010; Chang, Gaysinskaya, Karatayev, & Leibowitz, 2008). Alternate free-floating coronal sections were consecutively processed as follows: 10 min in 0.001% proteinase K, 5 min in 4% paraformaldehyde, and 10 min each in 0.2 N HCl and acetylation solution, with a 10-min wash in PB between each step. After the wash, the sections were hybridized with a DIG-labeled probe at 55 °C for 18 h. Following hybridization, the sections were washed in 5X sodium chloride and sodium citrate (SSC), and the nonspecifically bound probe was removed by RNase (Sigma–Aldrich, St. Louis, MO) treatment for 30 min at 37 °C. Sections were then run through further stringency washes with 0.1 M dithiothreitol (Sigma–Aldrich, St. Louis, MO) in 2X SSC and 1X SSC and 0.1X SSC at 55 °C. After the high stringency wash, the sections were blocked and incubated in AP-conjugated sheep anti-digoxigenin antibody (Sheep Anti-DIG-AP, Fab fragments, 1:1000; Boehringer Mannheim) overnight. After washing in Tris buffer (0.1 M, pH 9.5), the signal was revealed with NBT/BCIP and the sections mounted, dehydrated and coverslipped. Gene expression level was measured by semi-quantification with Image-Pro Plus software (Version 4.5, Media Cybernetics Inc., Silver Spring, MD) as described (Leibowitz, 2007) and expressed as the density of mRNA-containing cells, “cells/mm^2”.

Open field measurements

The open field activity chamber consisted of a computerized Plexiglas box (43.2 × 43.2 cm) with a white floor, black walls, and infrared photocells (MED Associates, Georgia, VT). Behavioral testing occurred in a separate room under dim red light. Measurements of rearing behavior, which is often associated with enhanced exploration of the environment (Prut & Belzung, 2003), were analyzed by recording vertical movements (time and count) in the novel open field for 5 min. Immediately after this test, a hole-board apparatus was installed into the open field and animals were tested for novelty-seeking behavior as represented by the total number of nose-pokes and number of novel nose-pokes made within 5 min. After each testing period, the test chambers were thoroughly cleaned and dried to avoid non-specific exploration and seeking of certain odors.

Surgery

Subjects were anesthetized with a combination of ketamine (80 mg/kg, i.p.) and xylazine (10 mg/kg, i.p.), supplemented with ketamine when necessary. Guide shafts (21-gauge stainless steel, 10 mm in length) were implanted perpendicularly according to the atlas of Paxinos and Watson (Paxinos & Watson, 1986), in the mPFC (AP: +3.0; M-L: 0.6; D-V: −2.5), with reference to bregma, the midsaggital sinus, and the level of skull surface. The bilateral

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injectors protruded 1.5 mm beyond the guide shafts for injection. Other than the time of injection, stainless steel stylets were left in the guide shafts to prevent occlusion.

Microinjection procedures

All solutions were delivered through concentric microinjectors made of 26-gauge stainless steel outside with fused-silica tubing inside (74 μm ID, 154 μm OD, Polymicro Technologies, Phoenix AZ). The silica injector tip protruded beyond the implanted guide shaft to reach into the region of interest (D-V −4 for PrL). The drug dose was chosen based on our preliminary tests and a recent publication with feeding behavior (Mena et al., 2011). The DAMGO (1.8 nmol, 3.6 nmol; Sigma–Aldrich Co., St. Louis, MO) and DALA (7.1 nmol, 14.2 nmol; Sigma–Aldrich Co., St. Louis, MO) was dissolved in preservative-free 0.9% NaCl solution (Hospira Inc, Lake Forest, IL) and prepared fresh immediately prior to microinjection.

To minimize stress from the injections, the animals were handled extensively throughout their ethanol training prior to the initiation of the tests. Injections were counterbalanced so that each animal received vehicle or two doses of drug (DAMGO, DALA) in opposite order on three consecutive days, and they were given immediately at the onset of the dark cycle, a period of ethanol anticipation. All injections were made using a syringe pump, which infused 0.5 μl during 47 s at a flow rate of 0.6 μl/min, and the microinjector was kept in place for another 47 s, to allow diffusion before removal. Ethanol and water intake were measured at one and 3 h after injections and ethanol access.

Histology

Injection sites were verified by injecting 0.25 μl methylene blue dye (Sigma, St. Louis, MO). Brains were kept in formalin for a minimum of 1 week prior to slicing, then cut in 40 μm sections on a cryostat and slide-mounted for microscopic verification. Behavioral data from animals with injector tips in the region of interest were included in the analysis, while those with probes 0.5 mm or farther from the target regions were discarded from the analysis and used as negative control sites.

Data analysis

Statistical analyses of behavior and peptide expression were performed using a one-way analysis of variance (ANOVA), followed by post-hoc tests (Holm-Sidak for behavior and blood ethanol) for multiple comparisons between groups. For the injections, data were compared between drug and vehicle. Changes in ethanol consumption after the one-week deprivation period were compared by a two-way ANOVA with group (HD versus LD) as the between-subject factor and time (pre and post deprivation) as the within-subject factor. Ethanol and water intake data after microinjections were each analyzed separately using a two-way repeated measures ANOVA, with three levels of treatment (2 doses of drug and saline vehicle) and three levels of time (1–3 h) as within-subject factors. Follow-up pairwise comparisons were made using paired two-tailed *t*-tests, where $p < 0.05$ was considered significant. Since we knew *a priori* that time could be a confounding factor in the injection study, follow-up statistical tests were used in one case despite a negative main result, to determine significance at specific time points. Data in all figures are expressed as mean ± SEM.

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Results

Experiment 1: patterns of ethanol consumption and anticipatory behaviors in high versus low drinkers

The first experiment examined the patterns of ethanol consumption and anticipatory behaviors in the HD ($n = 8$), LD ($n = 8$) and Control ($n = 8$) rats during the expectation of daily ethanol access. These three subgroups of animals each showed a very distinct pattern of ethanol consumption over the 10-day limited access period (Fig.1). While the Control rats initially (days 1–5) consumed relatively greater amounts of ethanol compared to the HD and LD rats, the HD animals subsequently (days 6–10) showed a marked increase in their ethanol consumption, drinking an average of 2.3 ± 0.3 g/kg/3 h compared to only 0.6 ± 0.1 g/kg for the LD group and 1.3 ± 0.2 g/kg for the Control group. With their greater ethanol intake, the HD rats had significantly higher BEC levels (65 ± 9 mg/dl, $p < 0.05$) than the LD (21 ± 6 mg/dl) and Control (38 ± 7 mg/dl) rats. With measurements of their exploration and novelty-seeking in an open field activity chamber during ethanol expectation, the HD rats also exhibited clear differences in these behaviors related to anticipation. Analyses of their exploration in a novel open field using a one-way ANOVA revealed a significant main effect for vertical time [$F(2, 21 = 3.92$, $p < 0.05$] and vertical counts [$F(2, 21) = 4.50$, $p < 0.05$], which in the HD rats reflected a significant increase in vertical time (+65%, $p < 0.05$) and vertical counts (+44%, $p < 0.05$) compared to the LD rats and a 53% increase in vertical time ($p < 0.05$) and 33% increase in vertical counts ($p < 0.05$) compared to the Control rats, with the LD and Control animals exhibiting similar behaviors (Fig. 2A). A significant main effect was also found for the two measures of novelty seeking, total nose pokes [$F(2, 23) = 4.14$, $p < 0.05$] and novel nose-pokes [$F(2, 23) = 3.86$, $p < 0.05$], with the HD animals showing a greater number of total hole entries (+50%, $p < 0.05$) and novel hole entries (+48%, $p < 0.05$) as compared to the LD animals and the LD rats making more total (+52%, $p < 0.05$) and novel entries (+45%, $p < 0.05$) compared to the Control group (Fig. 2B). An additional group of rats examined in parallel for their long-term behaviors (Experiment 1b), during 4 weeks of continued ethanol access, showed the HD rats to consume significantly greater (+92%) amounts of ethanol compared to LD rats [$F(1, 14 = 3.96$, $p < 0.05$] and to exhibit a significant ethanol deprivation effect [$F(2, 14) = 3.96$, $p < 0.05$] after a one-week withdrawal period, consuming 40%more ethanol than during the pre-deprivation period ($p < 0.05$), an effect not seen in the one-week deprived LD rats (Table 1). These behavioral measures demonstrate that the HD rats, characterized by their greater 3 h ethanol intake during the limited access period, show greater anticipatory behaviors during the daily expectation of ethanol solution and over the long term consume greater amounts of ethanol and exhibit increased relapse-like behavior after a one-week withdrawal period.

Experiment 2: ENK, MOR and DOR mRNA in mPFC during ethanol anticipation measured by qRT-PCR

This experiment was designed to determine whether the HD rats, prone to consuming excess ethanol and showing relapse-like behavior, exhibit changes in endogenous gene expression of ENK, MOR and DOR in the mPFC during the period of anticipation of ethanol access. A new set of rats was subgrouped as HD ($n = 8$), LD ($n = 8$) and Control ($n = 8$), as described in Experiment 1 (Table 1), and was examined for their peptide expression in the mPFC immediately before the scheduled access. Measurements of mRNA levels performed using qRT-PCR revealed a significant main effect on ENK [$F(2, 21 = 6.25$, $p < 0.05$] and MOR [$F(2, 21) = 5.97$, $p < 0.05$], in contrast to DOR [$F(2, 21) = 2.97$, not significant, *n.s*]. In the HD rats, post-hoc comparisons revealed a 57% increase ($p < 0.05$) in ENK expression levels compared to the LD rats and 43% increase ($p < 0.05$) compared to the Control group, and they also showed a 30% increase ($p < 0.05$) in MOR expression compared to the LD group as well as a 59% increase ($p < 0.05$) compared to the Control group, with no differences

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detected between the LD and Control rats (Fig. 3). These results show that, during the period of ethanol anticipation, the expression of both ENK and MOR receptors in the mPFC is significantly greater in rats expecting to consume a large bout of ethanol.

Experiment 3: ENK mRNA in specific subregions of mPFC during ethanol anticipation measured by ISH

To better visualize and anatomically differentiate the specific areas of the mPFC where ENK-producing cells are affected during ethanol anticipation, DIG-labeled ISH was performed in an additional set of HD ($n = 8$) and LD ($n = 8$) rats identified as described in Experiments 1 and 2 (Table 1). In both subgroups, cells expressing ENK were found to be broadly distributed throughout the dorsal (Cg and PrL) and ventral (IL) subregions of the mPFC. When compared to the LD rats, the HD animals showed a site-specific increase in the density of ENK-expressing neurons (Table 2), as illustrated in the photomicrographs (Fig. 4). This greater density of ENK cells in the HD rats was evident in the PrL (+27% $p < 0.05$), with no effect seen in the IL (−15%, *n.s.*) with a slight trend seen in the Cg (−10%, $p = 0.058$] subregion of the mPFC. These results underscore the site-specificity of this change in ENK mRNA and focus attention on the PrL subregion as playing an important role during the period of high ethanol anticipation.

Experiment 4: effects of ENK agonists in the PrL subregion of the mPFC on ethanol drinking behavior

Based on the expression results from Experiment 3 indicating that endogenous ENK is elevated in the PrL region of HD rats where it may drive their higher ethanol intake, this experiment tested the function of ENK in this area through local injection of the MOR-specific agonist, DAMGO (1.8 nmol, 3.6 nmol), or the DOR-specific agonist, DALA (7.1 nmol, 14.2 nmol), compared to saline vehicle and subsequent measurements of ethanol consumption on a similar 3 h/d limited access schedule. When injected into the PrL region, DAMGO compared to vehicle showed a significant and specific increase in 20% ethanol consumption [$F(2,14 = 4.15, p < 0.05$], while having no effect on intake of water [$F(2,14) = 1.82$, *n.s.*]. Pairwise comparisons showed this increase in ethanol consumption to occur with the high dose at 2 h ($p < 0.05$) and 3 h ($p < 0.05$) (Fig. 5), with injections made at three anterior–posterior levels of the PrL subregions of the mPFC (Fig. 6). In contrast to DAMGO, injection of DALA into the PrL did not significantly affect the intake of ethanol [$F(2,14) = 2.15$, *n.s.*] or water [$F(2,14) = 2.23$, *n.s.*]. However, comparison of the drinking data at each individual hour showed a non-significant trend for the 14.2 nmol dose of DALA to reduce ethanol consumption during 2 h ($p = 0.08$) and 3 h ($p = 0.09$) (Fig. 5). These results with specific ENK agonists focus attention on MORs in the PrL as being important in promoting the consumption of ethanol and DORs in this same region possibly having the opposite effect of slightly decreasing ethanol drinking behavior.

Discussion

The current findings demonstrate that animals consuming larger amounts of ethanol, in a brief, 3 h period of daily access, exhibit distinct patterns of anticipatory behaviors immediately prior to ethanol availability and significant neurochemical changes in the mPFC in association with these behaviors. In addition to this anticipatory behavior, the HD animals defined by their daily consumption of over 2.0 g/kg/3 h, more than 2-fold greater than the LD rats, are found over the long term to drink more ethanol when offered the solution for 12 h/day and to demonstrate a strong deprivation effect after one-week withdrawal period. On the limited access paradigm, the HD rats while anticipating ethanol access exhibit a neurochemical profile in the mPFC characterized by a significant increase in mRNA levels of both ENK and MOR. This increased expression of endogenous ENK is

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anatomically localized to the PrL subregion of the mPFC, where this peptide may act specifically through MOR to stimulate the drinking of ethanol.

Greater anticipatory behaviors in animals identified as high drinkers

The protocol used in this study, which allows one to characterize rats based on their initial pattern of ethanol consumption during a period of limited access, provides a useful method for examining the behavioral and neurochemical correlates of high ethanol anticipation. Our results clearly demonstrate that the HD rats compared to LD rats, while anticipating their daily ethanol access, exhibit increased exploratory as well as novelty-seeking behaviors. The increased exploration in an open field is similar to that described both in ethanol-preferring animals anticipating their daily administration period (Melendez et al., 2002) and in outbred rats anticipating a palatable snack (Merali et al., 2004). While the increase in novelty-seeking behavior in a hole-board apparatus is the first to be reported in animals anticipating substance access, it is consistent with other studies showing animals, identified as high responders to novel environments or objects, to have a greater propensity to consume certain drugs, such as ethanol and amphetamines (Blanchard, Mendelsohn, & Stamp, 2009; Karatayev et al., 2010; Nadal, Armario, & Janak, 2002). At the same time, HD rats may have a general disturbance in motivational processes similar to that observed in alcohol preferring rats that, in addition to drinking high amounts of ethanol, also consume excessive amounts of other naturally rewarding substances such as sucrose and saccharine (Sinclair, Kampov-Polevoy, Stewart, & Li, 1992; Stewart, Russell, Lumeng, Li, & Murphy, 1994). Overall, our findings from the first experiment show that novelty-seeking and exploration may both be characteristics of the ethanol anticipatory process in a limited access paradigm. The additional finding, that HD rats also exhibit prolonged increases in ethanol consumption during chronic access and a significant deprivation effect after brief withdrawal, further validates the utility of this model in studying the mechanisms that mediate high ethanol anticipation and perhaps the anticipation of other reinforcing substances.

Increased expression of ENK and MOR in the mPFC of animals anticipating a large bout of ethanol

The results described here provide evidence for a neurochemical mechanism that may underlie these behavioral characteristics of high ethanol anticipation. In the HD compared to LD and Control rats, endogenous expression of both ENK and MOR was found to be significantly increased in the mPFC during the period of anticipating brief access to ethanol. While the mPFC is known to have an important function in mediating goal-oriented behaviors such as food- or drug-seeking (Steketee,2003), the present results provide the first evidence suggesting a role for ENK in this area in mediating behaviors associated specifically with ethanol anticipation. Prior investigations have shown ENK and MOR in the mPFC (Marinelli et al., 2000), as well as other limbic regions (Karatayev et al., 2010; McBride et al., 2002; Nadal et al., 2002), to be increased in relation to ethanol preference in selectively-bred rodents under naïve conditions, and there are reports in genetically mutant mice demonstrating the importance of MORs throughout the brain in determining both ethanol preference and intake (Hall, Goeb, Li, Sora, & Uhl, 2004; Kovacs et al., 2005; Roberts et al., 2000). Also, feeding studies reveal greater expression of ENK and MOR within the hypothalamus and NAc in association with increased intake of and preference for palatable, fat-rich diets (Barnes, Holmes, Primeaux, York, & Bray, 2006; Chang, Karatayev, et al., 2010), suggesting a broad function of this opioid system in driving the consumption of rewarding substances. With additional evidence demonstrating increased neuronal activation of the mPFC in response to feeding-related cues (Schiltz et al.,2007; Schroeder et al., 2001), it is likely that specific mechanisms within this brain region, perhaps involving the opioids, have a function during the expectation of rewarding substances. Together with these published findings, our results suggest that the greater endogenous expression of ENK and

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MOR in the mPFC during anticipation of daily ethanol access may be an important factor in driving the greater consumption of ethanol seen in HD rats.

Increased ENK expression within the PrL of animals anticipating ethanol

The increase in ENK expression during ethanol anticipation observed specifically in the PrL, but not IL or Cg, of HD animals may be related to the involvement of this region in drug-seeking behavior. Recent findings have pointed to a functional dichotomy within the mPFC with regard to fear conditioning as well as drug-seeking paradigms. Both cocaine reinstatement after a period of extinction and expression of conditioned fear induced by a tone are enhanced by activation of the PrL subregion, while reduced by activation of the IL region (Di Ciano, Benham-Hermetz, Fogg, & Osborne, 2007; Peters, LaLumiere, & Kalivas, 2008; Vidal-Gonzalez, Vidal-Gonzalez, Rauch, & Quirk, 2006). Also, studies of heroin show inactivation of the PrL to reduce drug-seeking behavior and relapse (LaLumiere & Kalivas, 2008; Rogers, Ghee, & See, 2008), while inactivation of the IL region leads to a robust return to drug-seeking behavior with cocaine (Peters, LaLumiere, et al., 2008, Peters, Vallone, Laurendi, & Kalivas, 2008). The PrL region may be particularly important in drug-seeking behavior, as neuronal activity and expression of the immediate early gene, *c-fos,* is increased specifically in this region during the anticipation of drug reward with conditioned odors or contextual cues (Bossertet al.,2011; Bouret & Sara, 2004). The third subregion of the mPFC, the Cg area, while often examined together with the PrL given their close proximity, has been targeted with lesions and pharmacological manipulations that are found to have minimal effects on drug acquisition and fear responding (Vidal-Gonzalez et al., 2006; Weissenborn, Robbins, & Everitt, 1997). Together, these published studies suggest that the PrL subregion is more important in mediating the active seeking of commonly abused drugs, the IL area plays an opposite role in inhibiting such behavior, and the Cg alone has less of a role in the acquisition of conditioned behaviors related to drugs and fear. Our new findings of increased ENK expression specifically in the PrL region of HD rats during ethanol anticipation, together with evidence positively linking ENK peptide levels in limbic regions to increased ethanol preference and consumption (Jamensky & Gianoulakis, 1999; Karatayev et al., 2010; Marinelli et al., 2000), support an important role for this opioid peptide in the PrL in driving ethanol-seeking particularly during the initial anticipatory period.

Regulation of ethanol drinking by mu- and delta- opioid receptors in the PrL subregion of the mPFC

The results of this study suggest that ENK, in a limited access paradigm, may function specifically through MOR within the PrL area to increase ethanol consumption. In the injection experiment, we found the stimulation of drinking behavior by the MOR agonist in the PrL to be substance specific, occurring with ethanol and not water, and receptor specific, occurring with the MOR but not DOR agonist which tended to reduce ethanol intake. Although no studies have yet to examine the effects of MOR- and DOR-specific analogs in the mPFC on ethanol drinking behavior, a recent report suggests that DAMGO at a similar dose stimulates the consumption of energy-rich food and also rearing behavior, a characteristic of exploration, and that these effects are particularly strong in the ventro-medial mPFC that contains the PrL subregion and also with paradigms involving limited rather than *ad libitum* access (Mena et al., 2011). Consistent with our results here, this study found injection of the DOR agonist, DALA, to be ineffective in promoting the consumption of food and water, thereby highlighting the importance of MORs within the mPFC in both consummatory and anticipatory behaviors. Our findings are also in agreement with previous studies showing DAMGO to be effective in stimulating palatable food and ethanol consumption when injected into other limbic regions, such as the NAc and hypothalamus (Barson, Carr, et al., 2009; Barson, Karatayev, 2009; Will, Franzblau, & Kelley, 2003; Will,

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Pratt, & Kelley, 2006). In light of these published reports, our new evidence suggests that ENK, in addition to its important role in the reward-mediated mechanism of consummatory behavior, functions specifically through MOR in the PrL to activate seeking-related mechanisms that drive the excessive consumption of ethanol.

Proposed role of ENK and MOR in the mPFC in ethanol anticipation and consumption

Within the mPFC, ENK may function to stimulate ethanol drinking behavior by indirectly activating excitatory projections to the core of the NAc, an area closely related to drug seeking (McFarland, Lapish, & Kalivas, 2003). The mPFC is known to be composed of two major cell-types, the pyramidal cells that contain the excitatory amino acid, glutamate (GLUT), and project to multiple brain areas including the NAc and the non-pyramidal interneurons that contain the inhibitory amino acid, γ-aminobutyric acid (GABA), and also ENK that provides local control of the pyramidal cells. Studies indicate that the GLUT projection neurons of the mPFC are critical for behavioral control, with most neurons innervating and stimulating the NAc that mediates behaviors related to food- and drug-seeking (Carelli, 2002; Gass, Sinclair, Cleva, Widholm, & Olive, 2011; Maldonado-Irizarry & Kelley, 1994; Xie et al., 2011). With evidence suggesting that MOR are located on GABAergic bipolar interneurons within the mPFC (Taki, Kaneko, & Mizuno, 2000), one neurochemical mechanism that may underlie our finding of MOR agonist-induced increase in ethanol consumption may involve the disinhibition of these glutamatergic projections, perhaps by direct stimulation of MOR in this region that specifically reduces the activity of local, non-pyramidal interneurons (Witkowski & Szulczyk, 2006). These GLUT projections arising from the PrL subregion are known to project more densely to the core region of the NAc (NAcc), which is involved specifically in seeking behavior during drug anticipation (Ding, Gabbott, & Totterdell, 2001; Gorelova & Yang, 1997; Vertes, 2004). This relationship between the PrL and NAcc regions during anticipatory behavior is further supported by findings suggesting that GLUT released from the PrL to the NAcc can trigger relapse to drugs of abuse in response to certain cues (LaLumiere & Kalivas, 2008; Rocha & Kalivas, 2010). In light of these reports, our expression and injection studies showing specificity within the PrL subregion of the mPFC suggest that ENK through activation of MORs in this area promotes ethanol anticipation and excessive consumption through excitatory projections to the NAcc.

Summary and conclusion

In summary, exposure of animals to a limited ethanol access paradigm is an appropriate model for studying the behavioral and neurochemical correlates of anticipatory behavior. In high drinking animals, the anticipation of ethanol is characterized by distinct behaviors, increased exploration and novelty-seeking, and by a specific neurochemical phenotype, greater expression of ENK and- MOR within the mPFC. Our injection results for the first time demonstrate that ethanol drinking is enhanced by the stimulation of MOR in the PrL subregion of the mPFC. These expression and injection studies, highlighting the PrL, lead us to conclude that ENK acting through MOR within this specific subregion of the mPFC has an important role in mediating behaviors, such as active drug seeking, during periods of high ethanol anticipation and consumption. With the PrL area being especially important for relapse behavior, we further speculate that ENK neurons in this area, by activating excitatory projections to drug-seeking centers in the core of the NAc, may be contributing to the anticipation and thus reinstatement of drug-taking behavior. Further research should be aimed toward understanding this opioid-mediated mechanism as it controls the consumption of ethanol as well as other addictive drugs.

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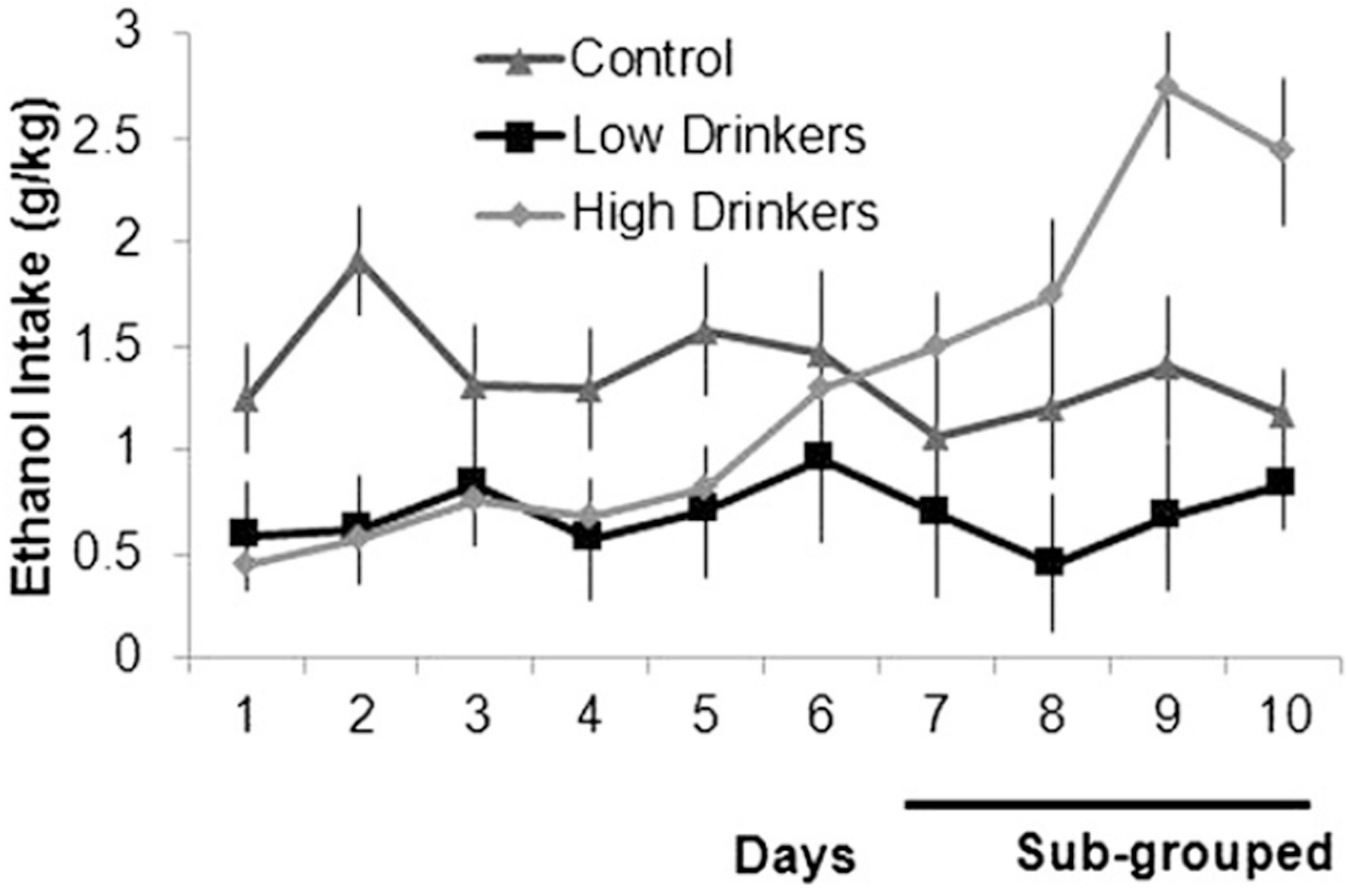

Fig. 1.
Patterns of daily ethanol consumption for rats characterized as Controls, Low Drinkers, and High Drinkers over the first 10 days of training.

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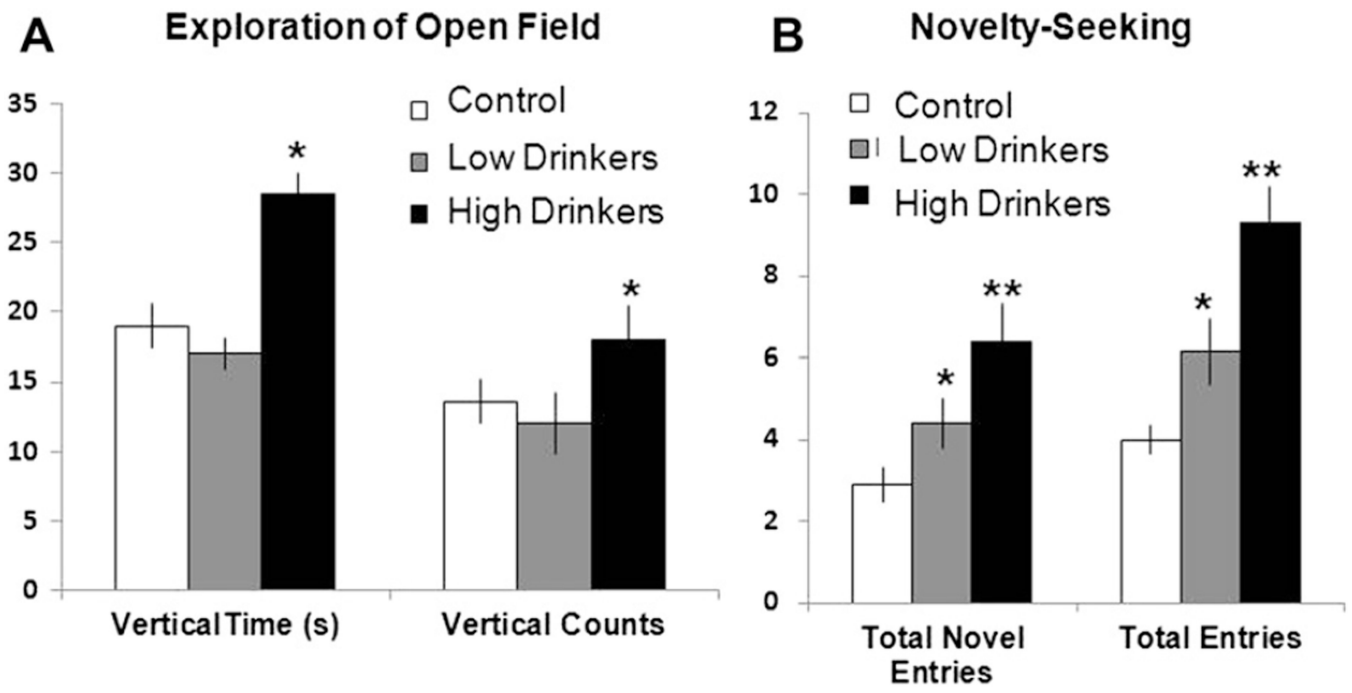

Fig. 2.

Measures of exploratory and novelty-seeking behaviors in an Open Field Activity Chamber. Compared to Controls and Low Drinkers, High Drinkers exhibited a significant increase ($p < 0.05$) with both measures of (A) exploration (vertical time and vertical counts) and (B) novelty-seeking (novel and total hole entries), with the Low Drinkers additionally showing increased novelty-seeking behavior compared to Control rats ($p < 0.05$).

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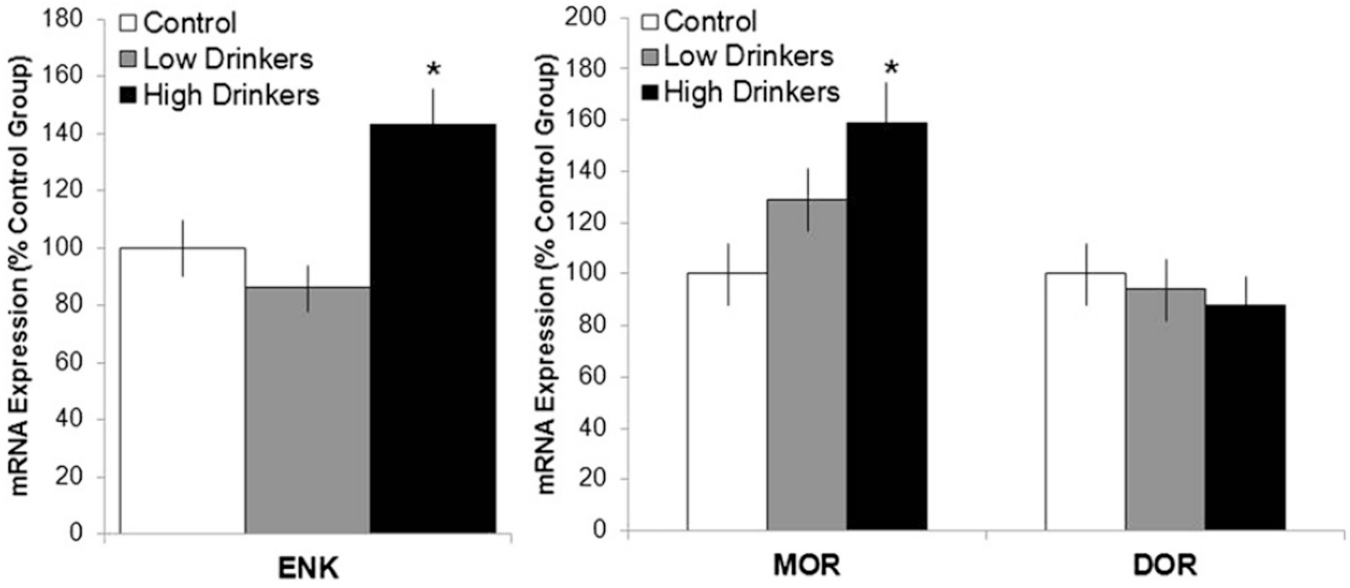

Fig. 3.

Gene expression of ENK, MOR and DOR as measured by qRT-PCR. The data showed a significant increase ($p < 0.05$) in mRNA levels of ENK and MOR in the mPFC of rats defined as High Drinkers compared to Low Drinkers and Controls.

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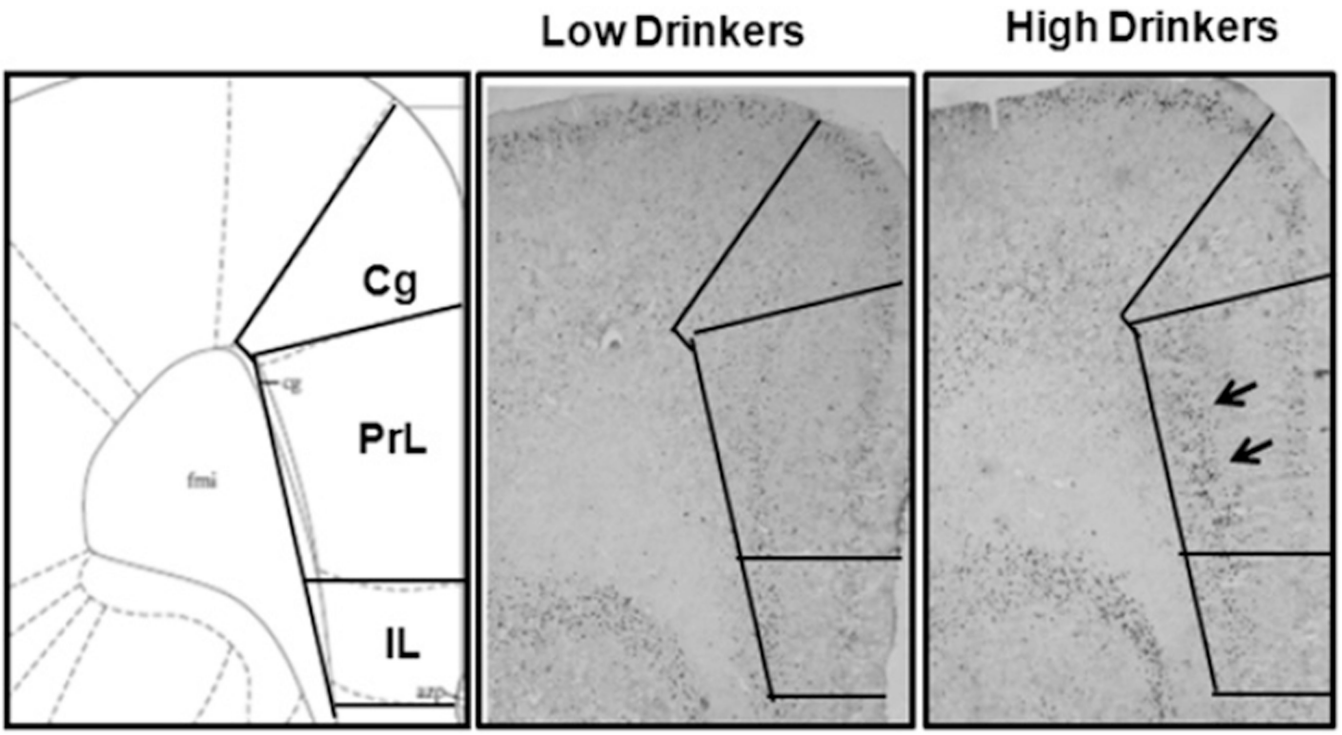

Fig. 4.

Photomicrographs illustrating the greater density of ENK-expressing cells in the PrL of High Drinkers compared to Low Drinkers as measured by digoxigenin-labeled ISH. Cg- cingulate cortex, PrL- prelimbic cortex, IL- infralimbic cortex.

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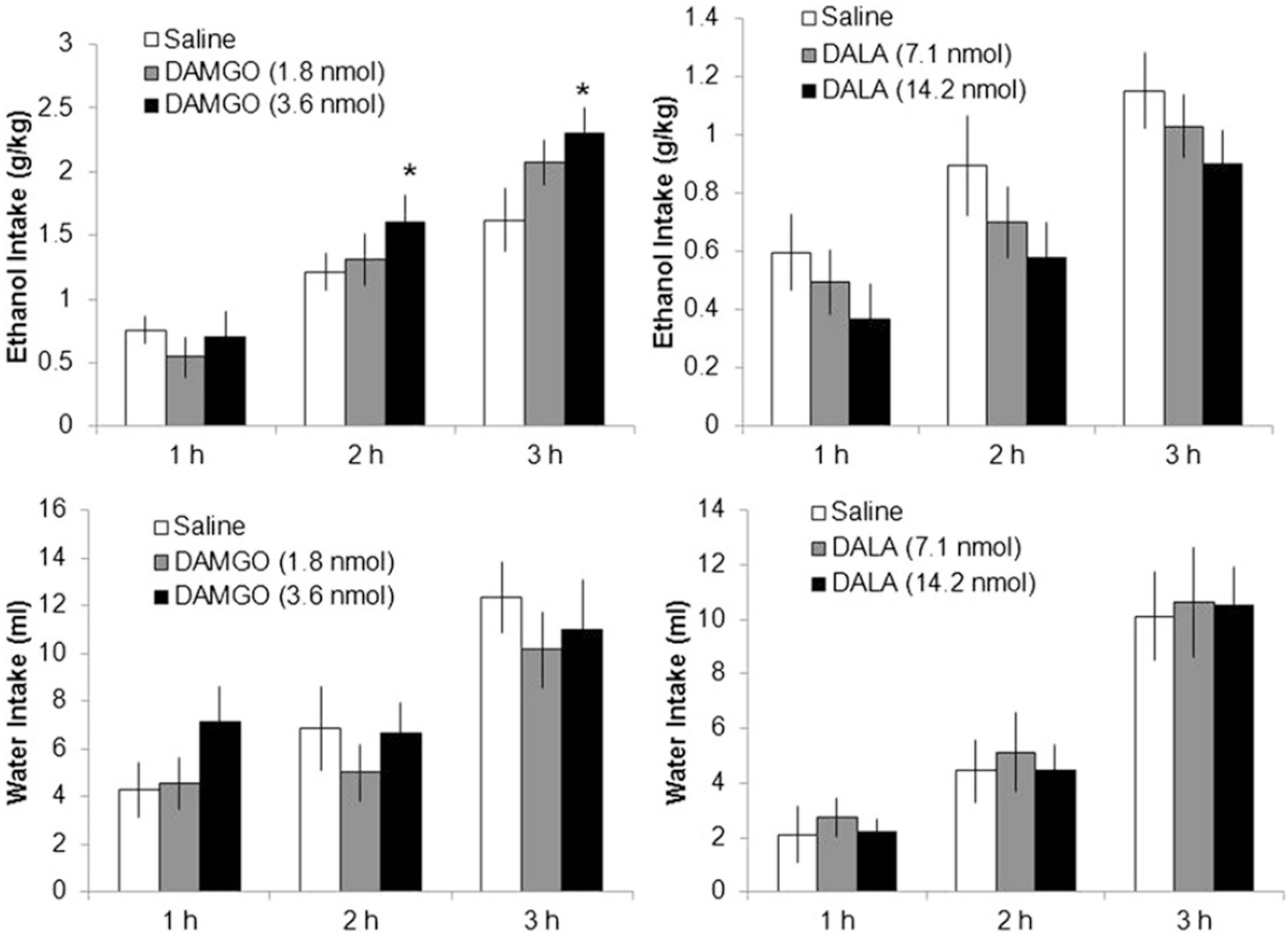

Fig. 5.

Measures of ethanol and water consumption after microinjection of DAMGO and DALA into the PrL region. The data demonstrated that compared to saline vehicle, DAMGO (3.6 nmol) significantly increased the drinking of 20% ethanol during the second and third hour of testing ($p < 0.05$), with no changes observed in water consumption with injections of DALA.

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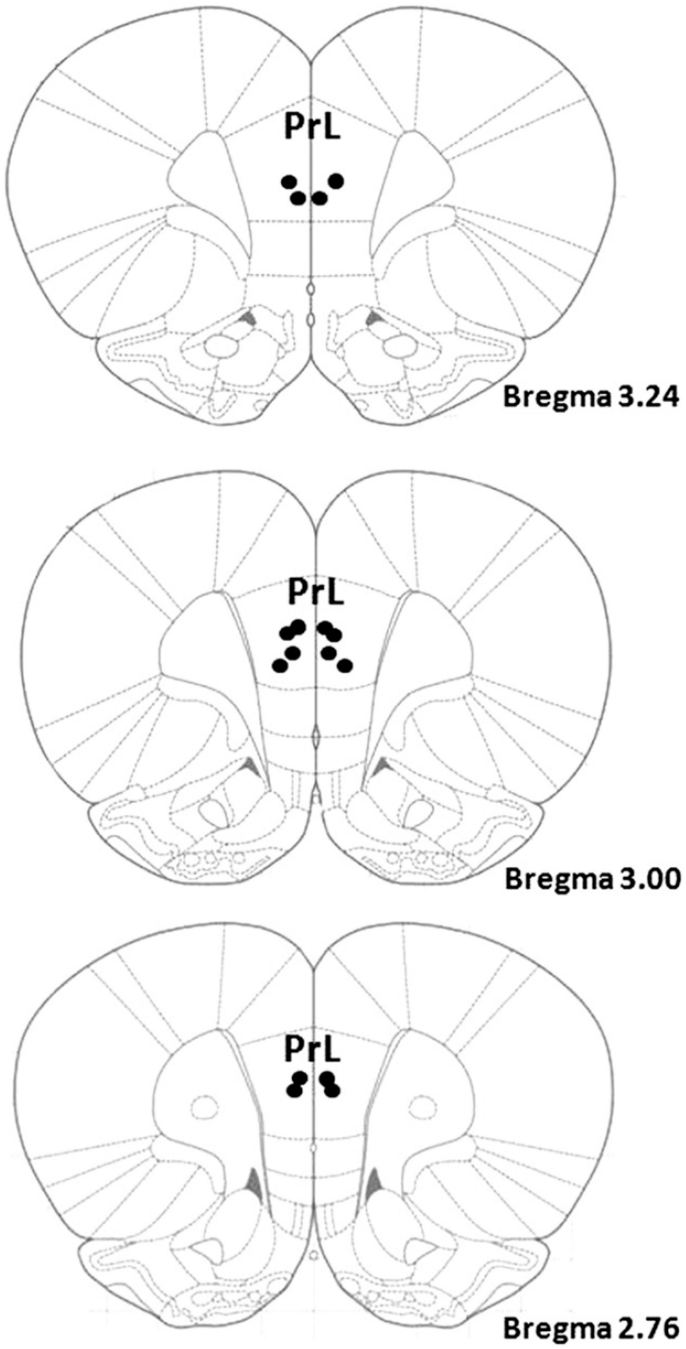

Fig. 6.

Black dots show the injection sites in the PrL region of the mPFC for all animals used in the experiments. Sections are labeled according to the distance from Bregma along the rostral–caudal axis. Adapted from *The Rat Brain*, compact 3rd edition, G. Paxinos and C. Watson, Copyright 1986, with permission from Elsevier. PrL- Prelimbic Cortex.

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Table 1

Measures of ethanol intake in Control, LD and HD rats. The rats were sacrificed during ethanol anticipation on day 11 in Experiments 2 and 3.

	Experiment 1b			Experiment 2	Experiment 3
	Ethanol intake for days 7–10 (g/kg)	**4-week ethanol intake (g/kg)**	**Post deprivation ethanol intake (g/kg)**	**Ethanol intake for days 7–10 (g/kg)**	**Ethanol intake for days 7–10 (g/kg)**
Control	–	–	–	1.5 ± 0.19	–
Low Drinkers (LD)	1.5 ± 0.19	1.3 ± 0.11	1.4 ± 0.13	0.8 ± 0.17	0.4 ± 0.08
High Drinkers (HD)	2.1 ± 0.25	2.5 ± 0.20[a]	3.5 ± 0.23[a,b]	2.1 ± 0.22	1.8 ± 0.14

[a] $p < 0.05$ compared to LD rats.

[b] $p < 0.05$ compared to ethanol intake pre-deprivaion.

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Table 2

The density of ENK-expressing cells in the subregions of the mPFC as measured by *in situ* hybridization (cells/um^2 × 10^{-4}).

	Low Drinkers	High Drinkers
Cg	2.35 ± 0.21	2.12 ± 0.23
PrL	1.90 ± 0.10	2.42 ± 0.17[a]
IL	1.81 ± 0.16	1.53 ± 0.13

PrL – prelimbic cortex, IL – infralimbic cortex, Cg – cingulate cortex.

[a] $p < 0.05$ compared to Low Drinkers.