Alcohol 46 (2012) 559–568

Contents lists available at SciVerse ScienceDirect

Alcohol

journal homepage: http://www.alcoholjournal.org/

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

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ARTICLE INFO

Article history:
Received 30 December 2011
Received in revised form
3 May 2012
Accepted 10 May 2012

Keywords:
Medial prefrontal cortex
Prelimbic cortex
Enkephalin
Ethanol anticipation
Mu opioid receptors
Delta opioid receptors

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.

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 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

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doi:10.1016/j.alcohol.2012.05.003

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 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 *ad libitum* 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-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/3 h, 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