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Effect of chronic ethanol on enkephalin in the hypothalamus and extra-hypothalamic areas

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Abstract

Background—Ethanol may be consumed for reasons such as reward, anxiety reduction, or caloric content, and the opioid enkephalin (ENK) appears to be involved in many of these functions. Previous studies in Sprague-Dawley rats have demonstrated that ENK in the hypothalamic paraventricular nucleus (PVN) is stimulated by voluntary consumption of ethanol. This suggests that this opioid peptide may be involved in promoting the drinking of ethanol, consistent with our recent findings that PVN injections of ENK analogues stimulate ethanol intake. To broaden our understanding of how this peptide functions throughout the brain to promote ethanol intake, we measured, in rats trained to drink 9% ethanol, the expression of the ENK gene in additional brain areas outside the hypothalamus, namely, the ventral tegmental area (VTA), nucleus accumbens shell (NAcSh) and core (NAcC), medial prefrontal cortex (mPFC), and central nucleus of the amygdala (CeA).

Methods—In the first experiment, the brains of rats chronically drinking 1 g/kg/day ethanol, 3 g/kg/day ethanol, or water were examined using real-time quantitative polymerase chain reaction (qRT-PCR). In the second experiment, a more detailed, anatomical analysis of changes in gene expression, in rats chronically drinking 3 g/kg/day ethanol compared to water, was performed using radiolabeled *in situ* hybridization (ISH). The third experiment employed digoxigenin-labeled ISH (DIG) to examine changes in the density of cells expressing ENK and, for comparison, dynorphin (DYN) in rats chronically drinking 3 g/kg/day ethanol vs. water.

Results—With qRT-PCR, the rats chronically drinking ethanol plus water compared to water alone showed significantly higher levels of ENK mRNA, not only in the PVN but also in the VTA, NAcSh, NAcC and mPFC, although not in the CeA. Using radiolabeled ISH, levels of ENK mRNA in rats drinking ethanol were found to be elevated in all areas examined, including the CeA. The experiment using DIG confirmed this effect of ethanol, showing an increase in density of ENK-expressing cells in all areas studied. It additionally revealed a similar change in DYN mRNA in the PVN, mPFC, and CeA, although not in the NAcSh or NAcC.

Conclusions—While distinguishing the NAc as a site where ENK and DYN respond differentially, these findings lead us to propose that these opioids, in response to voluntary ethanol

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consumption, are generally elevated in extra-hypothalamic as well as hypothalamic areas, possibly to carry out specific area-related functions that, in turn, drive animals to further consume ethanol. These functions include calorie ingestion in the PVN, reward and motivation in the VTA and NAcSh, response-reinforcement learning in the NAcC, stress reduction in the CeA, and behavioral control in the mPFC.

Keywords

Enkephalin; Dynorphin; Hypothalamus; Ethanol; Corticolimbic

The consumption of ethanol may be due to any of its multiple effects, from calorie ingestion to reward enhancement. Previous studies have demonstrated that the opioid enkephalin (ENK), in the hypothalamic paraventricular nucleus (PVN), is stimulated by chronic consumption of ethanol (Chang et al., 2007a; Oliva and Manzanares, 2007), suggesting that this peptide may be involved in promoting ethanol drinking. This is supported by our recent studies in rats, showing a stimulatory effect of PVN injections of ENK analogues on the consumption of ethanol (Barson et al., 2009b). The opioid dynorphin (DYN) is similarly stimulated in the PVN by chronic ethanol intake (Chang et al., 2007a), and PVN injection of a kappa receptor agonist, the receptor preferentially targeted by DYN, also stimulates the intake of ethanol (Barson et al., 2009b). In addition to their expression in the PVN, that may reflect their role in controlling ingestive behavior, both ENK and DYN are expressed in multiple extra-hypothalamic, mesocorticolimbic nuclei that are believed to play a major role in reward. The purpose of this study was to further characterize changes in the opioid systems of these different nuclei that may occur in response to voluntary, chronic consumption of ethanol.

Through the association of opioids in various brain regions with reward and ingestive behavior, studies suggest that ENK and, in some cases, DYN may also participate in voluntary ethanol intake. The PVN is important in energy homeostasis and, in particular, controlling the consumption of palatable food (Sawchenko, 1998; Williams et al., 2001). Injections of opioid agonists in this nucleus enhance food intake, while antagonists suppress it (Glass et al., 2000; Naleid et al., 2007). The ventral tegmental area (VTA) is a reward center that is believed to mediate the reinforcing effects of ethanol (Koob and Bloom, 1988; Soderpalm et al., 2009) as well as other drugs of abuse (Koob, 1992; Wise and Hoffman, 1992). Injection of ENK analogues in this nucleus, like the PVN, potentiates food intake (Cador et al., 1986; Jenck et al., 1987b; Noel and Wise, 1995), and it additionally produces place preferences (Bals-Kubik et al., 1993; Phillips and LePiane, 1982). Kappa agonists injected in the VTA also potentiate food intake (Jenck et al., 1987a; Noel and Wise, 1995), although in contrast to ENK produce place aversions (Bals-Kubik et al., 1993). The nucleus accumbens (NAc) similarly mediates the reinforcing effects of drugs of abuse (Di Chiara, 2002; Di Chiara and Bassareo, 2007). Eating, especially of highly palatable foods rich in fat or sucrose, appears to be driven by ENK in the NAc (Kim et al., 2004; Zhang et al., 1998). In addition, accumbal ENK has anxiolytic properties, as suggested by the finding that NAc injection of the opioid antagonist naloxone decreases time spent in the open arms of an elevated plus maze (Zarrindast et al., 2008). These functions of ENK in the NAc contrast with those described for DYN. This opioid in the NAc appears to mediate aversive states,

based on the findings that its expression is enhanced by immobilization stress (Shirayama et al., 2004) and by food restriction (Berman et al., 1994). Thus, opioids in the hypothalamic PVN and mesolimbic VTA and NAc may participate in controlling voluntary consumption of ethanol.

In addition to these areas, the medial prefrontal cortex (mPFC) and central nucleus of the amygdala (CeA) may also contribute to ethanol intake. The mPFC, like the VTA and NAc, is implicated in reward, particularly in reward-oriented behavior (Steketee, 2003; Volkow et al., 2005). Although injection of an ENK analogue in this region does not appear to be rewarding, as indicated by little change in place preference (Bals-Kubik et al., 1993) or self-stimulation threshold (Shaw et al., 1984), injection of a kappa agonist in the mPFC is found to produce place aversion (Bals-Kubik et al., 1993). With regard to the amygdala, studies have implicated this structure in the emotional aspects of consuming palatable food (Will et al., 2004). Injection of opioid agonists in the central nucleus of the amygdala (CeA) increases anxiety behavior (Wilson and Junor, 2008) in addition to food intake (Gosnell, 1988; Kim et al., 2004; Levine et al., 2004), and opioid antagonists decrease anxiety (Land et al., 2008) and intake of palatable food (Glass et al., 2000) or ethanol (Foster et al., 2004; Heyser et al., 1999). The expression of ENK is also increased in response to stress, but this response is diminished in mice with high anxiety, suggesting that ENK mRNA in the CeA reflects a coping mechanism under stressful conditions (Hebb et al., 2004). Together, these findings indicate that opioids may have multiple functions throughout the brain, mediating food and ethanol intake through their effects in the PVN on caloric intake, in the VTA and NAc on reward properties, in the mPFC on reward-related behavior, and in the CeA on anxiety.

The present study was carried out to further examine the roles of ENK and, for comparison, DYN in relation to the voluntary, moderate drinking of ethanol. These two opioid peptides may take on different roles as an individual progresses from social use to abuse of ethanol. Whereas ENK is believed to mediate some of the rewarding and reinforcing aspects of acute ethanol intake (Herz, 1997; Weiss and Porrino, 2002), DYN may mediate the aversive aspects of withdrawal with repeated ethanol exposure (Shippenberg et al., 2007; Walker and Koob, 2008), driving ethanol intake as animals become dependent (Walker and Koob, 2008). In the current study, Sprague-Dawley rats were trained over several weeks to chronically consume 9% ethanol, and using different techniques, the gene expression of these two opioids was examined in mesocorticolimbic nuclei as well as the hypothalamic PVN. Although ethanol intake might enhance the expression of both ENK and DYN in certain brain areas, as we have shown in the PVN (Chang et al., 2007a), the different roles played by ENK and DYN in ethanol intake might lead them to respond differently in other areas.

MATERIALS AND METHODS

Subjects

Adult, male Sprague–Dawley rats (275-375g, Charles River Laboratories International, Inc., Wilmington, MA) were housed individually, on a 12-hour reversed light/dark cycle. The rats in each set of water-drinking and ethanol-drinking groups were matched for body weight.

All animals were allowed 1 week to acclimate to their individual housing conditions, during which time they received *ad libitum* access to standard rodent chow (LabDiet Rodent Chow 5001, St. Louis, MO) and water, which was delivered via two plastic 8 oz water bottles at the top of the cage (PETCO Animal Supplies, Inc, San Diego, CA). The housing facility was fully accredited by AAALAC. Behavioral protocols were approved by the Rockefeller University Animal Care Committee.

Test procedures

In all experiments, rats were given *ad libitum* access to lab chow, water, and in some cases ethanol. The water-drinking control rats (n = 5 – 10/experiment) were maintained on chow with water provided in two plastic, 8 oz bottles with a steel ball as a tip valve to prevent spillage (PETCO Animal Supplies, Inc, San Diego, CA). The ethanol-drinking experimental groups (n = 10 – 15/experiment) were additionally given access to ethanol diluted with tap water, presented in place of one of the two water bottles. These bottles were placed on the top of the home cage, and the relative position of the water- and ethanol-containing bottles was alternated each day to prevent place preference. Access to the ethanol-containing bottle was provided for 12 hours each day for 22 days, with ethanol presented at dark onset. The concentration of ethanol was increased stepwise, every 4 days, from 1% to 2%, 4%, 7%, and then 9% v/v. With this limited-access paradigm, animals tend to consume a large percentage (30-35%) of their daily intake during the first two hours of access. On the final day of each experiment, after 1 hour of access to ethanol, rats drinking ethanol or water only were sacrificed by rapid decapitation. In Experiment 1, the ethanol groups included those drinking approximately 1 g/kg/day (ranging from 0.7 to 1.6 g/kg/day) and 3 g/kg/day (ranging from 2.3 to 3.5 g/kg/day). In Experiments 2 and 3, the ethanol groups included only those drinking an average of 3 g/kg/day.

In Experiment 1, the PVN, VTA, NAc shell (NAcSh), NAc core (NAcC), mPFC, and CeA were microdissected for measurements of ENK mRNA using quantitative real-time polymerase chain reaction (qRT-PCR). In addition, trunk blood was collected for measurements of blood ethanol concentration (BEC). In Experiment 2, the whole brain was removed and placed in a 4% paraformaldehyde solution for ENK measurements using radiolabeled *in situ* hybridization histochemistry (ISH). In Experiment 3, the whole brain was removed and placed in a 4% paraformaldehyde solution to examine gene expression of ENK and, for comparison, DYN using digoxigenin-labeled *in situ* hybridization histochemistry (DIG).

Blood ethanol assessment

To determine blood ethanol concentration (BEC), trunk blood from animals in Experiment 1 was collected at the time of sacrifice. Serum was analyzed using an Analox GM7 Fast Enzymatic Metabolic Analyser (Luneburg, MA).

Brain dissection

Immediately after sacrifice, the brain was placed in a matrix slicing guide with the ventral surface facing up. A total of five coronal cuts, yielding four slices, were made rostrally. The first cut was made in the anterior middle optic chiasm (Bregma −0.8 mm), according to the

atlas of Paxinos and Watson (1998). The second cut was made 1.5 mm rostral to this, yielding the first slice (Bregma −0.8 to 0.7 mm), and this was followed by the third and fourth 1.0 mm cuts, yielding the second (Bregma 0.7 to 1.7 mm) and third (Bregma 1.7 to 2.7 mm) slices, and a final cut 0.5 mm further rostral, which yielded the fourth slice (Bregma 2.7 to 3.2 mm). The second and fourth slices were used for microdissection, respectively, of the NAcSh and NAcC (Bregma 0.7 to 1.7 mm) and of the mPFC (Bregma 2.7 to 3.2 mm). Caudal to the original slice, two additional 1.0 mm slices (Bregma −0.8 to −2.8 mm) were made, with the first used for microdissection of the PVN (Bregma −0.8 to −1.8 mm) and the second for the CeA (Bregma −1.8 to −2.8 mm). Further caudally, one 0.5 mm slice was made by cutting between the caudal boundary of the mamillary bodies and the rostral boundary of the pons, which was used for microdissection of the VTA (Bregma −5.6 to −6.1 mm).

These sections were placed on a glass slide and rapidly dissected under a microscope. The NAcSh was dissected bilaterally in a moon shape, with the dorsal tip beginning at the lateral ventricle, the medial aspect at the semilunar nucleus, and the ventral edge along the ventral pallidum. The NAcC was dissected bilaterally as an oval, lateral to the lateral ventricle and medial to the lateral stripe of the striatum. The mPFC was dissected in a roughly diamond shape, bilateral from the dorsomedial tip of the slice to the corpus callosum and then rostrally to 0.2 mm dorsal to the olfactory ventricle. The PVN was dissected as a reversed isosceles triangle, 1.0 mm bilateral to the third ventricle and between the fornix structures (Chang et al., 2004). The CeA was dissected bilaterally as a circle, immediately dorsomedial to the basolateral amygdala and 0.2 mm dorsolateral to the optic tract. The VTA was also dissected bilaterally as a circle, lateral to the interfascicular nucleus, medial to the medial lemniscus, ventral to the red nucleus, and dorsal to the paranigral nucleus.

Real-time quantitative PCR

As previously described (Chang et al., 2004), total RNA from pooled microdissected samples was extracted with TRIzol reagent. RNA was treated with RNase-free DNase I before RT. For qRT-PCR, cDNA and minus RT were synthesized using an oligo-dT primer with or without SuperScript II Reverse Transcriptase. The SYBR Green PCR core reagents kit (Applied Biosystems, Foster City, CA) was used, with cyclophilin as an endogenous control. qRT-PCR was performed in MicroAmp Optic 96-well Reaction Plates (Applied Biosystems). This was done on an ABI PRISM 7900 Sequence Detection system (Applied Biosystems), under the condition of 2 minutes at 50°C, 10 minutes at 95°C, and 40 cycles of 15 seconds at 95°C and 1 minute at 60°C. Each study consisted of 4 independent runs of qRT-PCR in triplicate, and each run included a standard curve, a nontemplate control, and a negative RT control. The levels of target gene expression were quantified relative to the level of cyclophilin, using the standard curve method. The primers, designed with ABI Primer Express V.1.5a software from published sequences, were: (1) cyclophilin: 5’-GTGTTCTTCGACATCACGGCT -3’ (forward) and 5’-CTGTCTTTGGAACTTTGTCTGCA -3’ (reverse); and (2) ENK: 5’-GGACTGCGCTAAATGCAGCTA-3’ (forward) and 5’-GTGTGCATGCCAGGAAGTTG-3’ (reverse). The concentrations of primers were 100 nM. All reagents, unless indicated, were from Invitrogen (Carlsbad, CA).

Radiolabeled in situ hybridization histochemistry

Besides qRT-PCR, mRNA levels of ENK were measured with radiolabeled ISH in rats drinking 3 g/kg/day of ethanol or water only. This technique allows for more anatomically precise measurements of gene expression than qRT-PCR. Antisense and sense RNA probes were labeled with ^{35}S-UTP (Amersham Biosciences, Piscataway, NJ), as previously described (Chang et al., 2008; Lucas et al., 1998). Alternate free-floating coronal sections were consecutively processed as follows: 10 minutes in 0.001% proteinase K, 5 minutes in 4% paraformaldehyde, and 10 minutes each in 0.2 N HCl and acetylation solution, with a 10-minute wash in PB between each step. After the wash, the sections were hybridized with a ^{35}S-labeled probe (10^3 cpm/mL) at 55°C for 18 hours. Following hybridization, the sections were washed in 5X sodium chloride and sodium citrate (SSC), and the nonspecifically bound probe was removed by RNase (Sigma) treatment for 30 minutes at 37°C. Sections were then run through further stringency washes with 0.1 M dithiothreitol (Sigma) in 2X SSC and 1X SSC and 0.1X SSC at 55°C. Sections were finally mounted, air-dried, and exposed to a Kodak BioMax MR film for 18 to 24 hours at −80°C, when films were developed and microscopically analyzed. The sense probe control was performed in the same tissue, and no signal was found.

Computer-assisted microdensitometry of autoradiographic images was determined as described (Reagan et al., 2004) on the MCID image analysis system (Image Research Inc., St. Catherines, ON, Canada). Microscale ^{14}C standards (Amersham Biosciences) were exposed on the same Kodak film with the sections and digitized. Gray-level/optical density calibrations were performed with a calibrated film-strip ladder (Imaging Research Inc.) for optical density. This was plotted as a function of microscale calibration values. All subsequent optical density values of digitized autoradiographic images fell within the linear range of the function. The values obtained represent the average of measurements taken from 10 sections per animal. Within each section, the optical density for the nucleus was recorded, from which the background optical density from a same-size area in the corpus callosum was subtracted. The mean value of the ethanol-drinking group in each experiment was reported as a percentage of the water-drinking control group.

Digoxigenin-labeled in situ hybridization histochemistry

In situ hybridization histochemistry with digoxigenin-labeled probes was used to quantify both ENK and DYN gene expression in rats drinking 3 g/kg/day of ethanol or water only. 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 and DYN were synthesized by *in vitro* transcription as previously described (Chang et al., 2008; Lucas et al., 1998). Free-floating coronal sections were processed for DIG-ISH as for radiolabeled ISH until the high stringency wash, with the exception of replacement with the DIG-labeled probe. 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 as described (Chang et al., 2008). Gene expression level was measured by semiquantification with Image-Pro Plus software

(Version 4.5, Media Cybernetics Inc., Silver Spring, MD) as described (Leibowitz et al., 2007) and expressed as the density of mRNA-containing cells, "cells/mm^2".

Data analysis

The data in the figures and table, for circulating ethanol and peptides, are expressed as mean ± SEM. Statistical analyses of these data were performed using a 1-way ANOVA, followed by Neuman-Keuls post-hoc tests for multiple comparisons between groups, or using an unpaired *t*-test where appropriate.

RESULTS

Experiment 1: Effect of chronic voluntary ethanol consumption on ENK mRNA measured by qRT-PCR

Building on previous work showing 9% ethanol intake to stimulate ENK expression in the PVN (Chang et al., 2007a), this experiment used a similar paradigm, to confirm this prior finding in the PVN and also to determine whether this opioid peptide in mesocorticolimbic areas is similarly affected by the consumption of ethanol. Three groups of rats were tested (n=5/group), a water-drinking control group and two experimental groups voluntarily drinking ethanol at approximately 1 g/kg/day (1.2 ± 0.1 g/kg/day) or 3 g/kg/day (3.2 ± 0.4 g/kg/day). With the limited-access paradigm, the animals tended to consume a large percentage (30-35%) of their daily intake during the first two hours of access. They decreased their water intake, from 15.6 ± 3.3 ml in the water group to 11.7 ± 1.1 ml in the 1 g/kg/day group and 3.8 ± 0.7 ml in the 3 g/kg/day group, to compensate for the added daily liquid consumed. Blood ethanol content was significantly different across these three groups [$F(2, 14) = 120.64$, $p < 0.001$)]. Post-hoc tests showed that, compared to water-drinking rats (0.5 ± 0.5 mg/dl), BEC values were significantly higher in rats drinking 1 g/kg/day ethanol (24.8 ± 1.8 mg/dl, $p < 0.001$) or 3 g/kg/day ethanol (48.5 ± 2.4 mg/dl, $p < 0.001$), with the latter having significantly higher BEC compared to the 1 g/kg/day group ($p < 0.001$).

In the qRT-PCR analysis, ENK was found to be elevated by chronic voluntary consumption of ethanol in nearly every area examined (Fig. 1). The stimulation of ENK in the PVN was confirmed with a 1-way ANOVA [$F(2, 14) = 22.99$, $p < 0.001$]. Post-hoc tests revealed that levels of ENK mRNA in the PVN were significantly higher in the 1 g/kg/day ($p < 0.05$) and 3 g/kg/day ($p < 0.001$) groups compared to the water group and also in the 3 g/kg/day compared to the 1 g/kg/day group ($p < 0.001$). This effect was similarly seen in the VTA [$F(2, 14) = 35.27$, $p < 0.001$], although the increase was greater in the 1 g/kg/day compared to 3 g/kg/day group ($p < 0.001$). In the NAc, the ENK mRNA was significantly elevated in both the shell [$F(2, 14) = 14.97$, $p < 0.001$] and core [$F(2, 14) = 12.43$, $p < 0.01$] compared to the water group, although this occurred only in the 3 g/kg/day group ($p < 0.05$ for NAcSh, $p < 0.01$ for NAcC), with the 1 g/kg/day showing a tendency to reduce ENK mRNA levels ($p < 0.05$ for NAcSh, *ns* for NAcC). In the mPFC, ENK mRNA was stimulated by chronic ethanol intake [$F(2, 14) = 40.75$, $p < 0.001$], with levels markedly enhanced in both ethanol groups compared to the water group ($p < 0.001$). In contrast to these different areas, the CeA was the only nucleus that failed to show a change in ENK mRNA in response to chronic ethanol intake in either group [$F(2, 14) = 0.14$, *ns*].

Experiment 2: Effect of chronic ethanol intake on ENK mRNA measured by radiolabeled ISH

This experiment was conducted to confirm the results of Experiment 1 using radiolabeled ISH. The same test paradigm was used as in Experiment 1, except that only one ethanol group (n = 5) drinking 3 g/kg/day was tested and compared to a water-drinking, control group (n = 5). In this experiment, the VTA was not examined, since the signal was too low to be quantified with this method. Using radiolabeled ISH, ENK expression was found to be significantly increased by chronic voluntary ethanol consumption in every area examined (Fig. 2). Thus, compared to the water-drinking group, the rats drinking 3 g/kg/day of ethanol demonstrated a 17-48% increase in ENK mRNA in the PVN ($p < 0.001$), NAcSh ($p < 0.01$), NAcC ($p < 0.001$), and mPFC ($p < 0.001$), similar to results obtained with qRT-PCR, and a small but statistically significant increase (+13%) in the CeA ($p < 0.01$). The finding here of enhanced ENK in the CeA using radiolabeled ISH, but not qRT-PCR as in Experiment 1, suggests that the microdissected samples examined using the latter technique may have included tissue outside of the nucleus that diluted the effect. These changes in ENK mRNA in response to ethanol consumption are illustrated in photomicrographs of the PVN and NAc (Fig. 3). They clearly demonstrate how ENK is affected in a broad range of brain areas that have diverse functions related to the consumption and rewarding properties of ethanol intake.

Experiment 3: Effect of chronic ethanol intake on ENK and DYN mRNA measured by DIG

To confirm the results of Experiment 2 with ENK and extend this analysis to include DYN, this experiment used the same paradigm to examine, using DIG, the expression of both opioid peptides in rats chronically drinking ethanol (3 g/kg/day) compared to water control (n = 5/group). This technique, which allows a better visualization and counting of ENK- and DYN-expressing neurons, revealed a dense concentration in the NAc, moderate concentration in the PVN, mPFC, and CeA, and relatively low concentration in the VTA. It also showed different patterns of ENK and DYN expression in the areas examined. Specifically, in the PVN, ENK was expressed primarily in the medial parvicellular part throughout the PVN, although some neurons were seen in the lateral magnocellular area of the posterior PVN. In contrast, DYN was most strongly expressed in the lateral magnocellular part of the PVN, with the medial parvicellular area showing only moderate expression. In the NAc, ENK was densely expressed in the core and only lightly in the shell, whereas DYN exhibited the reverse pattern, with expression occurring densely in the shell and sparsely in the core. In the mPFC, the expression of ENK was moderate in the external pyramidal lamina of the prelimbic and anterior cingulate cortex, dense in the deep lamina of the infralimbic, prelimbic, and anterior cingulate cortex, and sparse in the external lamina of the infralimbic cortex. In contrast, DYN was lightly- to moderately-expressed in the deep lamina and sparsely expressed in the external layers of the mPFC. In the CeA, ENK was strongly, densely expressed in the lateral and capsular part, and moderately or sparsely in medial part. In contrast to ENK, DYN was moderately expressed throughout the CeA. In the VTA, the ENK- and DYN-expressing neurons were only sparsely expressed and thus could not be analyzed.

This analysis of the opioid peptides using DIG confirmed the stimulatory effects of ethanol on ENK mRNA in every area examined and, in addition, showed a similar change in DYN mRNA in these areas, with one notable exception (Table 1). Thus, the rats drinking 3 g/kg/day of ethanol exhibited an increased density of ENK-expressing cells in the PVN ($p < 0.001$), NAcSh ($p < 0.001$), NAcC ($p < 0.001$), mPFC ($p < 0.001$), and CeA ($p < 0.01$). These same ethanol-drinking rats demonstrated a significant increase in density of DYN-expressing cells in the PVN ($p < 001$), mPFC ($p < 0.01$), and CeA ($p < 0.001$). However, in contrast to ENK, no change in DYN was detected in the NAcSh (*ns*) or NAcC (*ns*). These effects revealed by DIG are illustrated in the photomicrographs of ENK and DYN mRNA in the PVN and CeA (Fig. 4). Thus, of the density of cells expressing both opioid peptides was similarly increased by consumption of ethanol in all areas, with the exception of the NAcSh and NAcC where DYN mRNA was unresponsive to ethanol.

DISCUSSION

The results of this study in Sprague-Dawley rats demonstrate that chronic, moderate voluntary ethanol intake, in addition to stimulating ENK expression in the hypothalamic PVN, has a similar effect in multiple extra-hypothalamic, mesocorticolimbic nuclei, namely, the VTA, NAc, mPFC, and CeA. The expression of DYN is similarly affected in these areas, except for the NAc. These findings support the involvement of these opioid peptides in the consumption of ethanol in addition to reward-related functions. While the mechanism of these effects is most likely indirect since a specific ethanol receptor has yet to be identified, it is notable that even moderate levels of blood ethanol were able to induce these changes in gene expression.

One goal of this paper was to corroborate our prior finding that chronic consumption of ethanol in Sprague-Dawley rats stimulates the expression of ENK and DYN in the PVN (Chang et al., 2007a). Using qRT-PCR, radiolabeled ISH, and DIG, the results confirm that ENK mRNA in the PVN is stimulated by chronic ethanol intake. As demonstrated by qRT-PCR, the increase in ENK mRNA occurs in a dose-dependent manner, with rats drinking 3 g/kg/day of ethanol exhibiting a significantly greater increase (+46%) compared to those drinking 1 g/kg/day (+12%). This change in mRNA expression was confirmed using radiolabeled ISH, with rats consuming 3 g/kg of ethanol exhibiting a similar magnitude of change (+40%). This effect has also been observed in Wistar rats drinking approximately 3.5 g/kg/day ethanol (Oliva and Manzanares, 2007), although the increase did not attain statistical significance in ethanol-preferring, Fawn-Hooded rats (Cowen and Lawrence, 2001). It was further validated here with DIG, which revealed an increase (+88%) in the density of ENK-expressing cells concentrated in the medial parvicellular part of the PVN. The analysis of DYN in neurons concentrated in the lateral magnocellular PVN revealed a smaller but statistically significant increase in cell density (+28%), consistent with our early findings (Chang et al., 2007a). Together with our recent finding that injections of ENK and DYN agonists in the PVN increase ethanol intake (Barson et al., 2009b), these changes in gene expression further corroborate the existence of a positive feedback circuit between PVN opioids and ethanol drinking (Leibowitz, 2007). A similar relationship appears to exist between dietary fat and these PVN opioids, which are endogenously stimulated by ingestion of a high-fat diet (Chang et al., 2007b) and, when injected in the PVN, produce an increased

preference for a high-fat diet (Arjune et al., 1991; Barton et al., 1995; Ookuma et al., 1997). This evidence suggests that the PVN opioids may have a particular role in enhancing intake of hedonically-valuated nutrients, ethanol as well as fat, and by responding positively to signals produced by this ingestive behavior, they may provide a mechanism for promoting consumption of these nutrients.

Since ethanol is better known as a drug than a nutrient, our next goal was to investigate its effects on ENK and DYN in different mesocorticolimbic areas. The VTA is a reward center that is believed to mediate the reinforcing effects of ethanol (Koob and Bloom, 1988; Soderpalm et al., 2009). The present results using qRT-PCR demonstrate that chronic voluntary consumption of ethanol enhances the expression of ENK in this area. Whereas this effect could not be confirmed with radiolabeled ISH or DIG because of the relatively low mRNA levels in the VTA (Garzon and Pickel, 2002; Nylander et al., 1995), the effect observed here with qRT-PCR was replicated in two groups of ethanol-consuming rats and was unusually large (+100%) in the animals consuming 1 g/kg/day of ethanol. This appears to be the first demonstration of an ethanol-induced increase in ENK mRNA in this brain area. There is one other report (Lindholm et al., 2000) that measured met5-enkephalin-Arg6-Phe7 in the VTA after daily i.p. injections of ethanol 2 g/kg in Sprague-Dawley rats, and this study showed little change in peptide levels, perhaps due to the stress produced by the injections that may have masked the effect of ethanol. The increase in ENK expression in rats voluntarily drinking ethanol may reflect the important role of VTA opioids in positive reinforcement. There are several studies showing VTA injection of μ- and δ-agonists to enhance food intake (Jenck et al., 1987a; Mucha and Iversen, 1986; Noel and Wise, 1995) and produce place preferences (Bals-Kubik et al., 1993; Phillips and LePiane, 1982). This reinforcement from opioids in the VTA is likely due to their known stimulatory effects on dopamine (DA) release (Devine et al., 1993; Latimer et al., 1987). Together with enhanced ENK during voluntary ethanol consumption, this increase in DA very likely stimulates reward that could drive further consumption (Hoebel et al., 2007). It is noteworthy that the stimulatory effect of ethanol on DA release (Di Chiara and Imperato, 1985; Yoshimoto et al., 1992) diminishes with prolonged exposure to ethanol (McBride et al., 1990). This gradual decrease in DA reward may be related to the present finding that the stimulatory effect of chronic ethanol on ENK mRNA also becomes smaller in rats chronically consuming the greater amount of ethanol (3 g/kg/day). This evidence supports the idea that ENK in the VTA drives ethanol intake due to positive reinforcement.

The NAc also plays an important role in drug reward and reinforcement (Di Chiara, 2002; Di Chiara and Bassareo, 2007). In the NAc, the present qRT-PCR results demonstrate that chronic voluntary consumption of ethanol at 3 g/kg/day enhances the mRNA expression of ENK in the NAcSh and NAcC, while 1 g/kg ethanol reduces it in the NAcSh. Results using radiolabeled ISH confirm this finding for 3 g/kg ethanol, demonstrating an increase in both the NAcSh and NAcC. These findings are further validated using DIG in rats consuming 3 g/kg/day of ethanol, which show enhanced density of ENK neurons in the NAcSh, where it is only lightly expressed, and also the NAcC, where it is densely expressed. The results agree with the stimulatory effect on ENK in the NAc observed with acute injections of ethanol at 1.6 or 2.5 g/kg (de Gortari et al., 2000; Li et al., 1998; Marinelli et al., 2005; Mendez and Morales-Mulia, 2006), although there are other studies of this nucleus showing

no change in ENK mRNA with repeated injections of ethanol at 0.5 or 4 g/kg/day (Lindholm et al., 2000; Mathieu-Kia and Besson, 1998) or a decrease in ENK mRNA with voluntary consumption of a greater amount (6 g/kg/day) of ethanol (Cowen and Lawrence, 2001; Oliva and Manzanares, 2007). Although the explanation for these different findings remains unclear, the amount of ethanol consumed appears to be an important factor, with the NAc showing greater sensitivity than other areas to inhibitory signals from ethanol and ENK in the NAc responding according to an inverse, U-shaped curve. This nucleus also revealed a difference between the two opioid peptides that was not evident in the other areas, with NAc expression of DYN unaffected by chronic ethanol consumption. This agrees with previous studies showing no change in DYN mRNA in ethanol-preferring animals (Cowen and Lawrence, 2001; Ploj et al., 2000). Although our results suggest that the NAcSh and NAcC respond similarly to ethanol, other studies of these subregions suggest that they play different roles in behavior. In particular, the NAcSh, where DYN is more heavily expressed, is believed to play a greater role in motivational valence, whereas the NAcC, where ENK is more heavily expressed, is thought to be more important in response-reinforcement learning (Di Chiara and Bassareo, 2007; Kelley, 1999; Shirayama and Chaki, 2006). The present findings, together with evidence that injection of ENK agonists in the NAc enhances the intake of ethanol (Zhang et al., 1998), suggest that ENK in the NAc, like that of the VTA, may drive ethanol intake due to positive reinforcement mechanisms. This is in contrast to DYN, which showed no change in gene expression in response to ethanol intake in the present study and failed to stimulate ethanol intake when injected in the NAc (Barson et al., 2009a).

Analysis of an additional mesocorticolimbic area, the mPFC, revealed that chronic voluntary consumption of ethanol robustly enhances ENK mRNA as shown by qRT-PCR. This effect occurred in both the 1 and 3 g/kg/day ethanol-drinking rats. It was confirmed in additional groups using radiolabeled ISH and DIG, which also revealed a somewhat smaller increase in DYN mRNA in the mPFC. To date, there is only one other study that has examined the effect of chronic ethanol on opioid peptides in this nucleus, and in rats consuming relatively low levels of 8% ethanol, it demonstrated an increase in met5-enkephalin-Arg6-Phe7 levels but a decrease in dynorphin-B peptide (Gustafsson et al., 2005). Whereas little is known about the role of ENK or DYN in the mPFC, enhanced glutamatergic output from this area is associated with drug-seeking behavior (McFarland et al., 2003). Injection of opioid agonists in the mPFC has a local inhibitory effect on cellular activity (Giacchino and Henriksen, 1998), including both GABAergic interneurons and glutamatergic projection neurons (Tanaka and North, 1994). Since this inhibition of glutamate output suggests that the opioids may enhance behavioral control while the inhibition of GABAergic interneurons suggests that they have the opposite effect, we propose that both ENK and DYN in the mPFC may be fine-tuning this inhibitory control such that the ethanol-induced increase in ENK and DYN mRNA drives further ethanol-seeking behavior.

The CeA is another mesocorticolimbic nucleus implicated in addiction (See, 2005). The present results demonstrate that chronic voluntary consumption of ethanol enhances expression of ENK and DYN in the CeA. While not detected with qRT-PCR, this increase in ENK mRNA was revealed using the more anatomically precise *in situ* hybridization, both

radiolabeled ISH and DIG. There is only one other study of ENK in this brain region after chronic ethanol intake (Cowen and Lawrence, 2001), and this report, conducted in ethanol-preferring Fawn-Hooded rats, described a similar enhancement of ENK mRNA. The DIG analysis in rats consuming 3 g/kg/day ethanol also revealed a significant increase in the density of DYN-expressing cells in the CeA. While not seen in selectively-bred ethanol-preferring rats (Cowen and Lawrence, 2001) that may have higher basal levels of DYN than outbred animals (Jamensky and Gianoulakis, 1997), this change agrees with another study in outbred Sprague-Dawley rats showing injection of ethanol to stimulate extracellular levels of DYN (Lam et al., 2008). The significance of these effects with respect to ethanol-drinking is suggested by reports showing opioids in the CeA to mediate stress and conditioned learning (Koob, 2009; See, 2005), both of which may be important in ethanol self-administration. It is known that general opioid antagonists injected in the CeA decrease operant responding for ethanol (Foster et al., 2004; Heyser et al., 1999). Also, the expression of ENK and DYN mRNA in the CeA is enhanced by stressful experiences (Berman et al., 1997; Wiedenmayer et al., 2002), possibly as a coping mechanism. This suggests that the ethanol-induced enhancement of ENK and DYN expression in the present study reflects the increased anxiety, stress and corticosterone levels associated with chronic ethanol consumption (Patterson-Buckendahl et al., 2005; Silvestre et al., 2002) and a possible role for the CeA opioid peptides in mediating these effects.

The results of this study show that chronic consumption of ethanol enhances ENK and DYN expression in both hypothalamic and extra-hypothalamic nuclei. This suggests that ENK and DYN in these different brain areas are involved in multiple functions affected by ethanol, including feeding signals, reward and reward-related learning, inhibitory control of behavior, and anxiety. Each of these functions is likely to play a role in stimulating the consumption of ethanol and is similarly evident in humans drinking alcohol. Moderate amounts of alcohol consumption in humans not only enhance food intake (Yeomans, 2004) and decrease inhibitory control of behavior (Weafer and Fillmore, 2008), they also enhance reward that can be blocked or decreased with opiate antagonist medication (Yeomans and Gray, 2002). Studies with ENK knockout mice suggest that this peptide is important in decreasing anxiety reactions (Bilkei-Gorzo et al., 2007; Bilkei-Gorzo et al., 2008; Ragnauth et al., 2001) while increasing incentive motivation (Hayward et al., 2002). Studies with DYN knockout mice, in contrast, indicate that this peptide enhances anxiety reactions (Land et al., 2008; McLaughlin et al., 2003; Wittmann et al., 2009) while increasing preference for ethanol (Blednov et al., 2006; Kovacs et al., 2005). These functions, particularly those of ENK, may be important in driving the consumption of ethanol. Therefore, the results of the present study, showing enhanced ENK and DYN expression in extra-hypothalamic, mesolimbic nuclei in addition to the PVN, suggest that these opioids may function in concert to drive an animal to further consume ethanol. While we found ENK mRNA to be enhanced by ethanol in the NAc similar to the other areas, DYN mRNA in the NAc shell or core was notably unaffected in the ethanol-drinking rats. These results, like those with knockout mice models, suggest that ENK and DYN play a somewhat different role in their relation to ethanol consumption.

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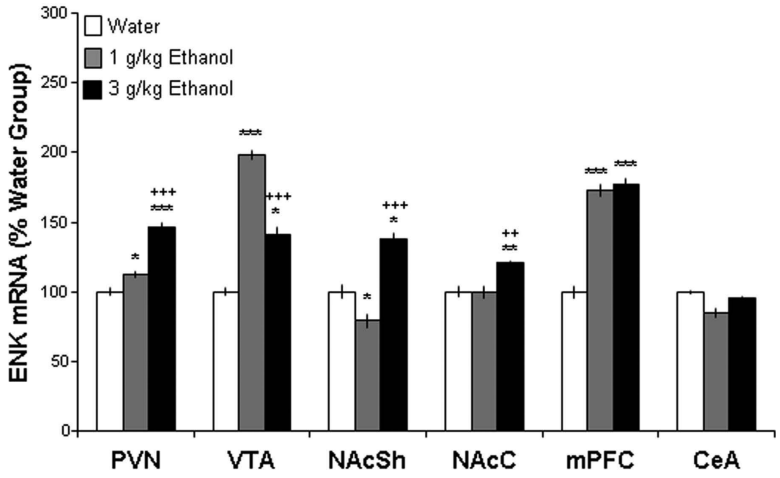

Fig. 1.

Increased levels of hypothalamic and extra-hypothalamic enkephalin (ENK) mRNA in rats drinking ethanol (n = 5/group) vs. water (n = 5), as assessed by qRT-PCR. Values are mean ± S.E.M.. For ethanol groups, ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$ vs. water. For the 3 g/kg/day ethanol group, $^{+++}p < 0.001$, $^{++}p < 0.01$ vs. 1 g/kg/day ethanol. CeA: central nucleus of the amygdala, mPFC: medial prefrontal cortex, NAcC: nucleus accumbens core, NAcSh: nucleus accumbens shell, PVN: hypothalamic paraventricular nucleus, VTA: ventral tegmental area.

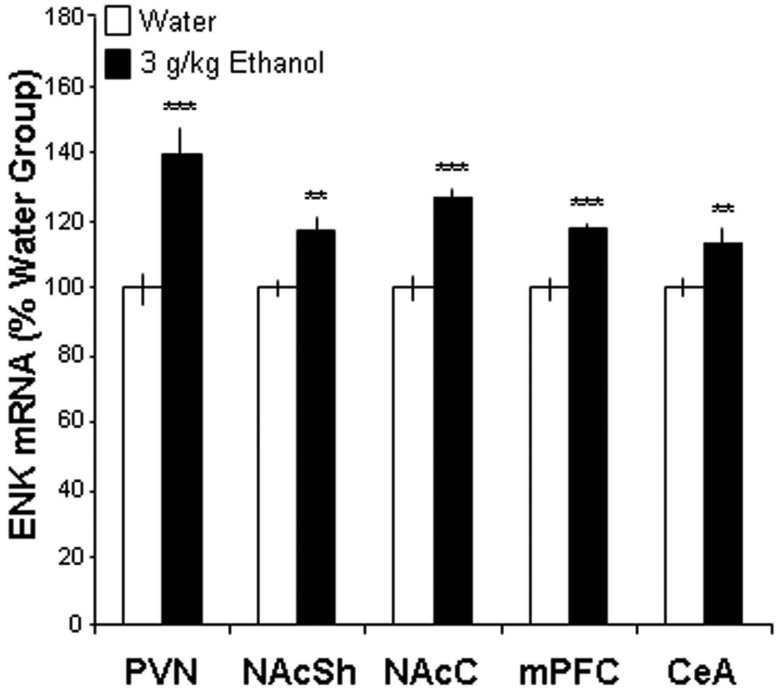

Fig. 2.

Increased levels of hypothalamic and extra-hypothalamic enkephalin (ENK) mRNA in rats drinking 3 g/kg/day ethanol (n = 5) vs. water (n = 5), as assessed by radiolabeled *in situ* hybridization. Values are mean ± S.E.M.; ***$p < 0.001$, **$p < 0.01$ vs. water. See legend to Fig. 1 for abbreviations.

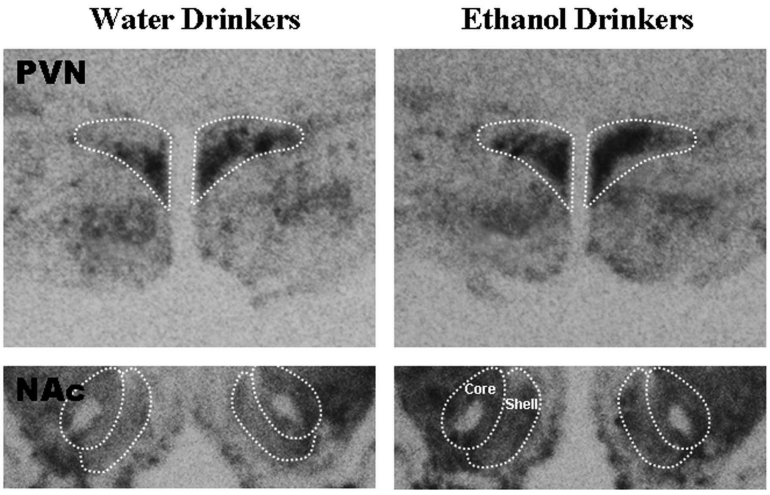

Fig. 3.

Photomicrographs showing radiolabeled *in situ* hybridization analysis of enkephalin (ENK) mRNA in the PVN and NAc of rats drinking water or 3 g/kg/day ethanol (4X magnification).

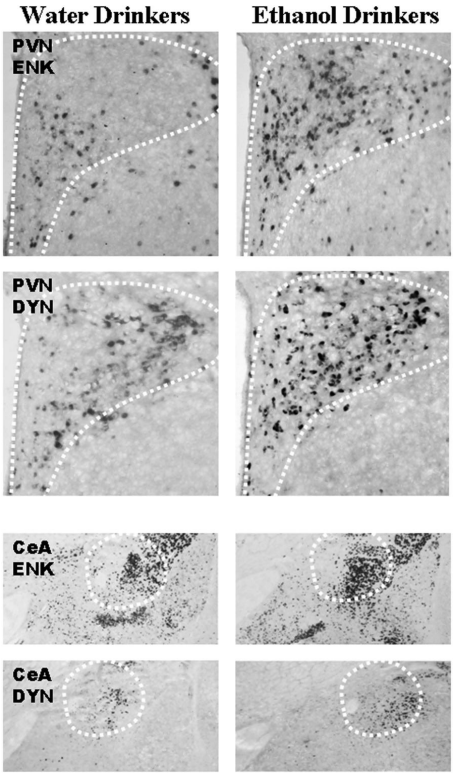

Fig. 4.

Photomicrographs showing digoxigenin-labeled *in situ* hybridization histochemistry analysis of cells expressing enkephalin (ENK) and dynorphin (DYN) mRNA in the PVN (10X magnification) and CeA (4X magnification) of rats drinking water or 3 g/kg/day ethanol.

Table 1

Density of hypothalamic and extra-hypothalamic enkephalin- and dynorphin-expressing cells (cells/μm × 10^{-4}) in rats drinking 3 g/kg/day ethanol (n = 5) vs. water (n = 5), as assessed by digoxigenin-labeled *in situ* hybridization histochemistry.

	Enkephalin		Dynorphin	
	Water	**Ethanol**	**Water**	**Ethanol**
PVN	8.82 ± 0.36	16.54 ± 0.22***	2.65 ± 0.18	3.40 ± 0.06***
NAcSh	37.70 ± 1.44	44.39 ± 0.78***	28.07 ± 1.04	28.25 ± 0.93
NAcC	39.35 ± 0.75	47.67 ± 0.92***	33.39 ± 1.31	33.72 ± 1.31
mPFC	31.13 ± 0.79	46.17 ± 1.84***	3.91 ± 0.19	4.68 ± 0.07**
CeA	22.11 ± 0.95	27.07 ± 1.31**	2.75 ± 0.14	3.78 ± 0.12***

Values are mean ± S.E.M.

See legend to Fig. 2 for abbreviations.

*** $p < 0.001$

** $p < 0.01$ vs. water.