ORIGINAL INVESTIGATION

Ethanol exposure differentially alters pro-enkephalin mRNA expression in regions of the mesocorticolimbic system

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Abstract

Rationale Opioid peptides have been suggested to play a major role in ethanol reinforcement mechanisms and alcohol drinking behaviour. However, in non-selected strains of rodents, it is not known whether opioid biosynthesis is a critical event in these processes.
Objective The aim of this work was to study the effects of a high dose of ethanol (2.5 g/kg body weight) on pro-enkephalin (pro-enk) mRNA expression in brain regions of the mesocorticolimbic system for up to 24 h after drug administration.
Materials and methods Male Wistar rats were administered with ethanol (2.5 g/kg body weight) or distilled water and were killed 30 min, 1, 2, 4, 8 or 24 h after treatment. Coronal brain sections (20 μ) were obtained and pro-enk mRNA expression was studied by in situ hybridization and densitometry.
Results Acute ethanol administration induced a transient decrease and increase in pro-enk mRNA expression in the ventral tegmental area (33.2%) and prefrontal cortex (26.5%) 2 and 4 h after treatment, respectively. In contrast, ethanol induced prolonged increases in pro-enk mRNA expression in the core and shell regions of the nucleus accumbens, with different kinetics. Maximal effects were observed 2 h after ethanol exposure (core, 70.0%; shell, 60.0%).
Conclusions Our results indicate that enkephalin expression in regions of the rat mesocorticolimbic system is differentially altered by acute ethanol treatment and suggest that enkephalins may play a key role in ethanol reinforcement mechanisms.

Introduction

The dopaminergic (DAergic) mesolimbic system plays a critical role in the mechanisms of reinforcement and reward elicited by ethanol, which may lead to the development of addictive behaviour (Koob et al. 1998; Wise and Bozarth 1982). Opioid peptides share several of the behavioural and pharmacological effects produced by ethanol intake. Administration of low doses of ethanol or opioids stimulates locomotor activity through DAergic activation in the ventral tegmental area (VTA), and high doses activate DAergic terminals in the nucleus accumbens (NAcc) (Joyce and Iversen 1979; Kalivas et al. 1983). An important role for opioid peptides has been proposed in alcohol dependence since low doses of mu (μ) receptor agonists increase alcohol consumption and preference (Reid and Hunter 1984; Wild and Reid 1990), and moderate to high doses reduce it (Volpicelli et al. 1991). Mu and delta (δ) selective as well as non-selective opioid receptor antagonists reduce alcohol consumption and preference (Hyytiä and Kiianmaa 2001; Krishnan-Sarin et al. 1995; Reid and Hunter 1984), whereas voluntary ethanol consumption is increased by inhibition of enkephalin degradation (Froehlich et al. 1991). These data suggest that occupation of both μ and δ opioid receptors and activation of the endogenous enkephalinergic and β-endorphinergic systems play a key role in high

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alcohol drinking behaviour. Therefore, opioid peptides may be relevant in the mechanisms of action of ethanol in reward circuits.

Ethanol reinforcement and high alcohol drinking behaviour have been suggested to be partially mediated by a neurobiological mechanism involving the ethanol-induced activation of the endogenous opioid system (Froehlich 1995; Ulm et al. 1995). This activation may in turn enhance the hedonic value and the reinforcing properties of ethanol. Several studies indicate that ethanol may alter opioid transmission at different levels, including the processing, release and/or receptor binding of opioid peptides. For instance, ethanol has been reported to increase the release of β-endorphin (Marinelli et al. 2003) and Met-enkephalin (Met-enk) (Marinelli et al. 2005) from the NAcc. The in vivo administration of a high dose of ethanol decreases [^{3}H] [D-Ala^2,MePhe^4,Gly-ol^5]-enkephalin ([^{3}H]-DAMGO) binding to μ receptors in the VTA and the shell region of the NAcc (NAccSh) and increases ligand binding in the prefrontal cortex (PFC), with different kinetics (Méndez et al. 2001). In contrast, the same treatment increases [^{3}H] [2-D-penicillamine, 5-D-penicillamine]-enkephalin ([^{3}H]-DPDPE) binding to δ receptors in these regions 2 h after drug administration (Méndez et al. 2004). These findings suggest that ethanol may differentially alter enkephalinergic and β-endorphinergic transmission and that these ethanol-induced changes may be region-specific.

The role of enkephalins and β-endorphin in ethanol reinforcement mechanisms and alcohol drinking behaviour has not been extensively studied. Most of the studies regarding enkephalin and β-endorphin biosynthesis in response to ethanol exposure have been performed in inbred strains of rodents that consume high amounts of alcohol and exhibit a high preference for the drug. For instance, pro-opiomelanocortin (POMC) mRNA levels in the pituitary and hypothalamus of alcohol-preferring inbred rodent strains have been shown to be higher than that in the corresponding non-preferring strains (de Waele et al. 1992; Gianoulakis et al. 1992), whereas β-endorphin content is variable depending on the brain region (Gianoulakis et al. 1992). Moreover, acute exposure to ethanol induces a higher increase in POMC mRNA content in the anterior and intermediate lobes of the pituitary in alcohol-preferring (P) than that in alcohol non-preferring rats (NP) (Froehlich 1995). On the other hand, pro-enkephalin (pro-enk) mRNA levels and Met-enk content in the midbrain of alcohol-preferring mice are lower than that in non-preferring animals (Ng et al. 1996). A high alcohol intake in alcohol-preferring mice induces a higher expression of enkephalins in the midbrain and striatum in comparison with naive animals (Ng et al. 1996). These findings suggest that endogenous opioid systems in alcohol-preferring strains of rodents exhibit an increased sensitivity to ethanol,

which may be associated with an enhanced vulnerability towards high alcohol drinking behaviour.

Although the studies performed in alcohol-preferring rodents have contributed to identifying neurotransmitter and neuromodulator systems involved in ethanol mechanisms of action in brain, little is known about these effects in non-selected animals. Moreover, it is not known whether opioid biosynthesis is a critical event related to the reinforcing properties of ethanol and in maintaining a high alcohol drinking behaviour. We have previously shown that the in vivo intragastric administration of a single acute dose of ethanol (2.5 g/kg) increases pro-enk mRNA levels in the NAcc, the frontal cortex and the paraventricular nucleus of the hypothalamus of Wistar rats, whereas the drug exhibits biphasic effects in the dentate gyrus and CA1, CA2 and CA3 regions of the hippocampus (de Gortari et al. 2000). The intraperitoneal (IP) administration of the same dose of ethanol has been reported to increase Met-enk and β-endorphin content in the striatum of Sprague–Dawley rats, whereas Met-enk content is reduced in chronic ethanol-treated rats (Schulz et al. 1980; Seizinger et al. 1983). These findings indicate that acute and chronic ethanol exposure affects opioidergic transmission in non-selected rodents and suggest that enkephalin biosynthesis could represent a key event in ethanol reinforcement and in the neuroadaptive responses to the drug. The aim of this work was to study the effects of a high dose of ethanol (2.5 g/kg) on pro-enk mRNA expression in brain regions of the reward circuits (i.e. the VTA, the PFC, the core region of the NAcc (NAccC) and the NAccSh) for up to 24 h after drug administration. In the present study, we further explored the time-course effects of a high dose of ethanol on pro-enk mRNA expression in the rat mesocorticolimbic system in relation to a previous study of our group in which we examined three time points (de Gortari et al. 2000). Moreover, in order to perform a more detailed analysis, we studied ethanol effects in subregions of the NAcc (i.e. core and shell regions) and of the frontal cortex (i.e. the PFC). Finally, ethanol time-course studies on pro-enk mRNA expression in the VTA have not been previously assessed.

Materials and methods

Animals and treatment

All experiments were conducted in accordance with the National Institute of Health *Guide for the Care and Use of Laboratory Animals* (NIH Publications No. 80-23, revised 1996), as well as with the project's commission approval of the Instituto Nacional de Psiquiatría Ramón de la Fuente.

Animal treatment was performed as previously reported (Méndez et al. 2001, 2004). Briefly, adult male Wistar rats

(250–300 g), fed ad libitum (Purina Chow) and maintained in a 12 h light–dark cycle, received a single acute dose of ethanol (2.5 g/kg body weight) (experimental group) or distilled water (control group) by oral administration with an intragastric cannula. The ethanol solution was prepared by diluting 2.5 g ethanol in distilled water to a final volume of 5 ml. Animals were habituated to the cannula by one daily oral administration of distilled water (5 ml/kg body weight) for the previous 7 days in order to diminish stress. Rats were killed by decapitation 30 min, 1, 2, 4, 8 or 24 h after administration, and their brains were immediately removed and frozen. Coronal brain sections (20 μ) were obtained in a cryostat and maintained at −70°C until assayed by in situ hybridization. All brain sections were obtained from animals used in previous studies (Méndez et al. 2001, 2004) as part of a large research project on the effects of ethanol on opioid peptide systems.

In situ hybridization

In situ hybridization was performed as previously described (de Gortari et al. 2000). Brain sections mounted in gelatin-coated slides were fixed by immersion in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 20 min, rinsed three times with PBS (5 min each) and treated with 0.25% acetic anhydride in 0.1 M triethanolamine, 4× standard saline citrate (SSC) buffer (pH 8.0) for 10 min. After dehydration in increasing concentrations of alcohol, slices were delipidated in chloroform for 20 min and air-dried. All solutions were made with autoclaved 0.05% diethylpyrocarbonate-treated water.

Pro-enk probe used was a 130- to 145-fragment of the rat pro-enk cDNA (Howells et al. 1984), which was labelled at the 3′end using α [^{35}S] dATP (1,000 Ci/mmol, Amersham) and desoxynucleotidyl transferase (25 U/μl, Roche Applied Science) to a specific activity of 3.18×10^8 cpm/μg. Sections were preincubated for 1 h at 42°C and then incubated overnight in hybridization buffer containing 4× SSC, 50% deionized formamide, 2.5× Denhard's solution (100×=0.5% Ficoll, 0.5% polyvinyl pyrrolidone, 0.5% bovine albumin), 100 μg/ml sheared single-stranded salmon sperm DNA (Sigma), 10% dextran sulphate, 10 mM dithiothreitol (DTT), 100 mM Na phosphate buffer (pH 7.4), 250 μg/ml yeast tRNA (Sigma) and ^{35}S-labelled oligonucleotide probe to get 250,000 cpm per section. After incubation, sections were washed during 30 min in 4× SSC and 0.02% sodium dodecyl sulphate (SDS) at 42°C, followed by 30 min in 2× SSC and 0.02% SDS at 42°C, 30 min in 1× SSC and 0.02% SDS at 42°C and 10 min in 0.5× SSC at room temperature. Sections were dehydrated in increasing concentrations of alcohol and 95% alcohol containing 0.3 M ammonium acetate and air-dried. Slices were exposed to βmax Hyperfilm (Amersham)

for 4 weeks at room temperature in the dark. Non-specific pro-enk hybridization was tested with an excess of 100 times the oligonucleotide concentration, and the signal was subtracted from total hybridization. Autoradiograms were quantitated by densitometry using an image analysis system (Image-Pro Plus Software, Media Cybernetics). Brain structures were identified according to Paxinos and Watson (1998). Five to 15 density measurements (5–8 for the PFC and the VTA and 10–15 for the NAcc) from five different sections were collected per brain structure and were averaged for each animal.

Statistical analysis

Data are expressed as units of optical density and represent the mean±SEM of six animals in each group. Statistics were performed by two-way analysis of variance (ANOVA) considering treatment and time after administration as the main sources of variation. Significant differences between groups were investigated by the post hoc Tukey honest significant difference (HSD) test. Each experimental group had its own control administered with distilled water and was killed at the same time as the group receiving ethanol. Analyses performed between the various control groups showed no significant differences at any time in each brain structure studied. Results were considered to be significant at $p < 0.05$.

Results

Pro-enk mRNA levels in the VTA, the PFC and the NAcc of control animals were similar to previously reported data (Harlan et al. 1987; Yoshikawa et al. 1984). Highest pro-enk mRNA expression was observed in the NAcc, followed by the PFC and then the VTA (Fig. 1a–c). Ethanol exposure differentially affected pro-enk mRNA expression (Fig. 1e–g). Non-specific hybridization is shown at the level of the NAcc for control (Fig. 1d) and ethanol-treated (Fig. 1h) animals.

Statistical analysis of pro-enk mRNA data revealed significant main effects of time after administration in the VTA [$F(5,60)=5.879$, $p<0.0001$], as well as significant main effects of treatment in the PFC [$F(1,60)=5.723$, $p<0.020$]. In addition, significant main effects of time after administration and treatment were found in the NAaccC [$F(5,60)=16.689$, $p<0.0001$ and $F(1,60)=42.837$, $p<0.0001$, respectively] and the NAccSh [$F(5,60)=9.671$, $p<0.0001$ and $F(1,60)=19.931$, $p<0.0001$, respectively]. On the other hand, significant time administration × treatment interactions were also revealed by two-way ANOVA in the VTA [$F(5,60)=4.199$, $p<0.003$], the PFC [$F(5,60)=4.243$, $p<0.002$], the NAaccC [$F(5,60)=18.944$, $p<0.0001$] and

Fig. 1 Pro-enk mRNA expression in the rat prefrontal cortex, nucleus accumbens and ventral tegmental area of control and ethanol-treated animals. Male Wistar rats received a single acute dose of ethanol (2.5 g/kg body weight) or distilled water and were killed 30 min, 1, 2, 4, 8 or 24 h after treatment, and their brains were removed. Pro-enk mRNA expression was studied by in situ hybridization on fresh frozen tissue sections, and non-specific pro-enk hybridization was determined using a 100-fold excess of pro-enk probe. Hybridization signal is shown in control (**a**–**d**) and ethanol-treated (**e**–**h**) animals, 2 h (**b**, **c**, **f**, **g**) or 4 h (**a**, **e**) after administration. Photographs show the hybridization signal in the prefrontal cortex (*PFC*) (**a**, **e**), the core and shell regions of the nucleus accumbens (*NAccC* and *NAccSh*) (**b**, **d**, **f**, **h**) and the ventral tegmental area (*VTA*) (**c**, **g**). Non-specific pro-enk hybridization signal is shown for the nucleus accumbens (*NAcc*) in control (**d**) and ethanol-treated (**h**) animals. *c* core region of the NAcc, *sh* shell region of the NAcc
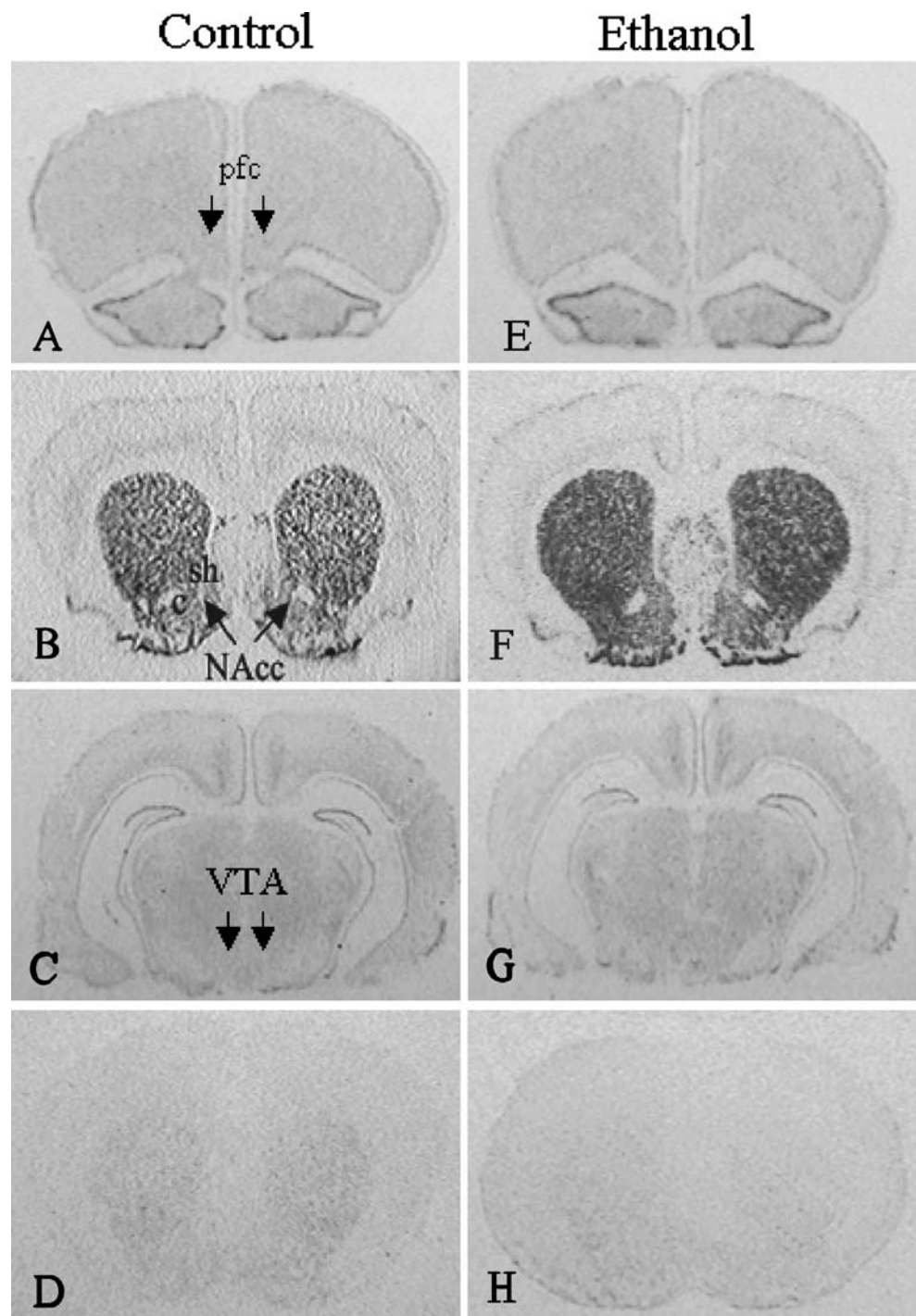
the NAccSh [$F(5,60)=10.132$, $p<0.0001$], indicating that the time after administration effects were only observed in animals that received ethanol.

The administration of a single acute dose of ethanol (2.5 g/kg body weight) significantly decreased pro-enk mRNA expression by 33.2% in the VTA 2 h after drug exposure (Fig. 2a). Thereafter, pro-enk mRNA levels were recovered and were similar to those observed in control animals. In contrast, pro-enk mRNA expression was significantly increased by ethanol in the PFC (Fig. 2b) and the NAcc (Fig. 3). In the PFC, ethanol produced a 26.5% increase in pro-enk mRNA levels 4 h after treatment, which were recovered thereafter (Fig. 2b). A sustained increase in pro-enk mRNA expression was induced by ethanol in both the NAaccC and the NAccSh. In the NAccC, ethanol produced increases of 26.9, 70.0, 21.2 and
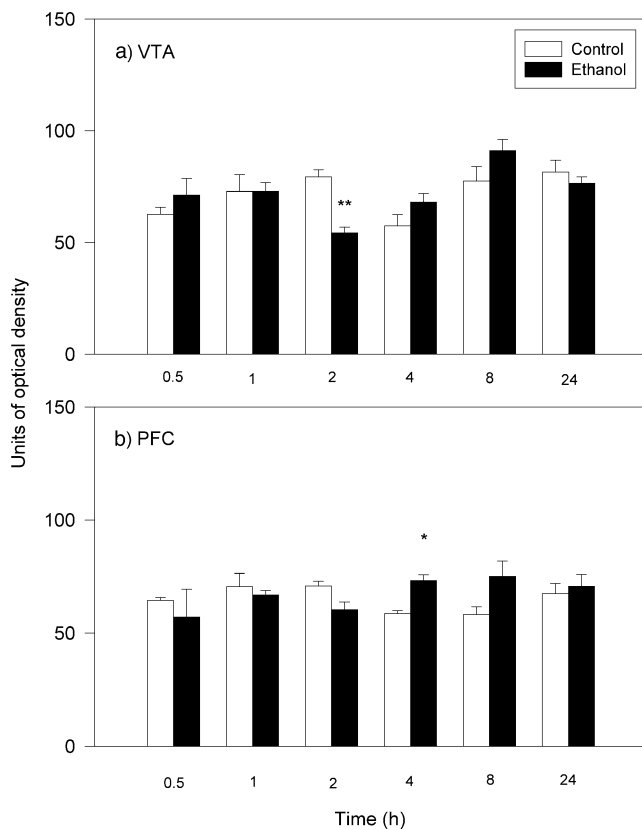
Fig. 2 Effect of acute ethanol treatment on pro-enk mRNA expression in the rat ventral tegmental area and prefrontal cortex. Animal treatment and in situ hybridization were performed as in Fig. 1. Quantitation of the hybridization signal was performed by densitometry and is shown for the ventral tegmental area (*VTA*) (**a**) and the prefrontal cortex (*PFC*) (**b**). Data are expressed as the mean± SEM of six animals. *$p<0.05$ vs control; **$p<0.02$ vs control. Significant differences were also observed between ethanol-treated animals in the VTA: 30 min vs 2 h ($p<0.050$), 1 h vs 2 h ($p<0.037$), 4 h vs 2 h ($p<0.036$), 8 h vs 2 h ($p<0.0001$) and 24 h vs 2 h ($p<0.024$)

23.6% (1, 2, 4 and 8 h post-treatment) (Fig. 3a), whereas in the NAccSh, increases of 60.0, 30.2 and 25.0% were observed 2, 4 and 8 h after drug exposure (Fig. 3b).

Discussion

Endogenous opioid systems have been suggested to play a major role in alcohol preference and high alcohol drinking behaviour. Intrinsic differences in opioid peptide and precursor mRNA content in regions of the mesocorticolimbic system may be linked to alcohol preference and consumption patterns. For instance, alcohol-preferring Fawn-Hooded (FH) rats have been shown to exhibit lower pro-enk mRNA levels in the NAcc than alcohol-non-preferring Wistar-Kyoto (WKY) rats (Cowen et al. 1998).
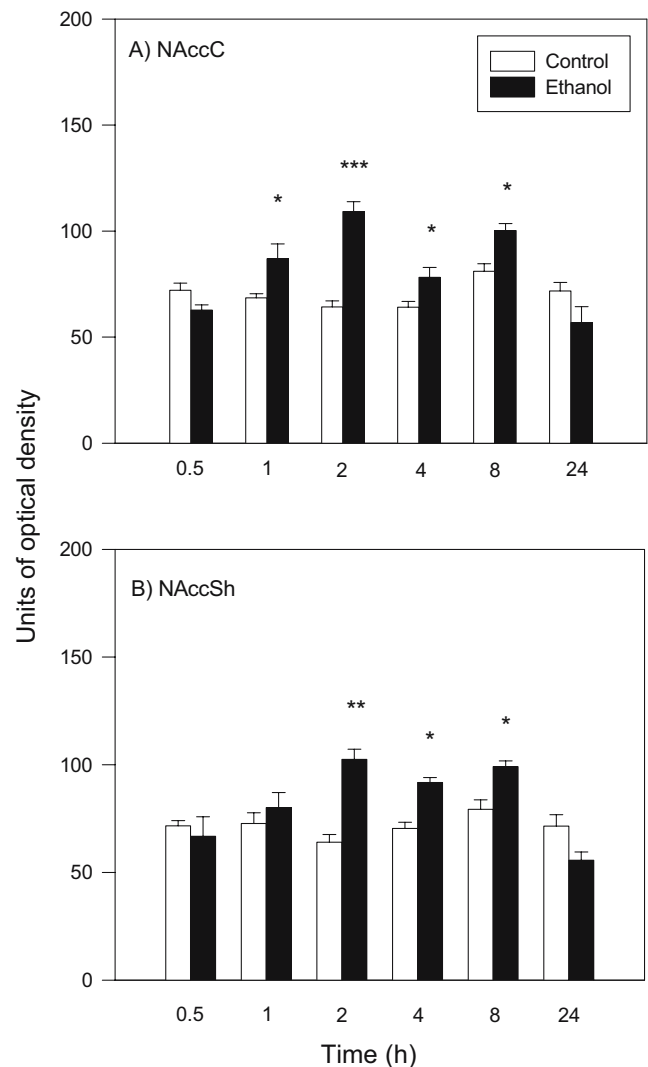

Fig. 3 Effect of acute ethanol treatment on pro-enk mRNA expression in the rat nucleus accumbens. Animal treatment and in situ hybridization were performed as in Fig. 1. Quantitation of the hybridization signal was performed by densitometry and is shown for the core (*NAccC*) (**a**) and shell (*NAccSh*) (**b**) regions of the nucleus accumbens. Data are expressed as the mean±SEM of six animals. *$p<0.05$ vs controls; **$p<0.001$ vs control; ***$p<0.0001$ vs control. Significant differences were also observed between ethanol-treated animals in both regions of the NAcc. NAccC: 30 min vs 1 h ($p<0.001$), 30 min vs 2 h ($p<0.0001$), 30 min vs 4 h ($p<0.001$), 30 min vs 8 h ($p<0.0001$), 1 h vs 2 h ($p<0.003$), 1 h vs 24<h ($p<0.0001$), 2 h vs 4 h ($p<0.012$), 2 h vs 24 h ($p<0.0001$), 4 h vs 24 h ($p<0.0001$) and 8 h vs 24 h ($p<0.0001$). NAccSh: 30 min vs 2 h ($p<0.0001$), 30 min vs 4 h ($p<0.008$), 30 min vs 8 h ($p<0.0001$), 1 h vs 2 h ($p<0.028$), 1 h vs 24 h ($p<0.011$), 2 h vs 24 h ($p<0.0001$), 4 h vs 24 h ($p<0.0001$) and 8 h vs 24 h ($p<0.0001$)

Other studies have reported similar pro-enk mRNA levels in the NAcc and the VTA of Alko alcohol-preferring (AA) and Alko non-alcohol-preferring (ANA) rats, whereas higher pro-enk mRNA content is present in the PFC of AA vs ANA rats (Marinelli et al. 2000). In addition, Met-enkephalin-Arg[6]-Phe[7] and Leu-enk-Arg[6] content is lower in the NAcc and the VTA of AA rats compared with that in

ANA rats (Nylander et al. 1994). These data suggest that enkephalin expression may be differentially involved in alcohol preference and alcohol-seeking behaviour in distinct rat strains.

Several studies indicate that an enhanced vulnerability towards high alcohol drinking behaviour in alcohol-preferring strains of rodents may be associated with an increased sensitivity of the endogenous opioid system to ethanol (de Waele et al. 1992; Froehlich 1995; Gianoulakis et al. 1992; Li et al. 1998; Ng et al. 1996). Nevertheless, the contribution of opioidergic systems to ethanol's actions in non-selected rodents remains unknown. In this study, we have shown that a high dose of ethanol produces a transitory decrease and increase in pro-enk mRNA expression in the VTA and PFC of Wistar rats 2 and 4 h after drug administration, respectively. In contrast, the same ethanol treatment produced a prolonged and sustained increase in the expression of this mRNA in the NAcc. Ethanol effects followed different kinetics in the NAccC and NAccSh, with the highest increases 2 h after drug exposure. The maximal response to ethanol found in this study occurred in the NAcc, in comparison with the moderate responses elicited by the drug in the VTA and the PFC. These findings indicate that ethanol induces different responses on pro-enk-containing neurones in regions of the mesocortical and meso-accumbens pathways: a reduction in pro-enk mRNA levels in the VTA, which contains the DAergic somata, and an increase in the PFC and the NAcc, which contain the DAergic terminal projections of these pathways. Therefore, our results suggest that ethanol may regulate pro-enk mRNA expression by different mechanisms in regions of the reward circuits. Pro-enk mRNA expression in the NAccC and NAccSh of P but not NP rats is also increased by an ethanol dose of 2.5 g/kg 1 h after treatment (Li et al. 1998). These findings suggest that non-selected Wistar rats, as well as P rats, may exhibit an enhanced responsiveness of the enkephalinergic system to ethanol that could be linked to high alcohol drinking behaviour.

Opioidergic transmission in response to ethanol treatment may be regulated at different levels. The observed effects in this study may reflect ethanol-induced changes in mRNA transcription and/or mRNA stability (i.e. altered mRNA degradation), as previously reported (Valles et al. 1997). The transient decrease in pro-enk mRNA expression found in the VTA could reflect a reduction in enkephalin biosynthesis in response to acute ethanol exposure. In contrast, the increased pro-enk mRNA expression observed in the NAcc and PFC could indicate that enkephalin biosynthesis is also increased, particularly in the NAcc, where ethanol effects were prolonged and sustained. Increases in Met-enk content in brain regions of Sprague–Dawley rats in response to a high dose of ethanol (2.5 g/kg) have been reported (Schulz et al. 1980; Seizinger et al.

1983), although the NAcc was not examined in these studies. Unfortunately, data on the acute effects of ethanol on enkephalinergic systems are scarce. In contrast, most of the studies have focused on the chronic effects of the drug. For instance, pro-enk mRNA levels in the NAcc have been reported to decrease in FH rats (Cowen and Lawrence 2001) or to remain unchanged in Sprague–Dawley rats (Mathieu-Kia and Besson 1998) in response to chronic ethanol exposure. The same treatment increases Met-enkephalin-Arg^6-Phe^7 content in the NAcc of AA but not ANA rats (Nylander et al. 1994), although no effect has also been reported in AA rats (Ploj et al. 2002). Pro-enk mRNA expression in cortical areas of FH rats (Cowen and Lawrence 2001) is not affected by chronic ethanol, and Met-enk-Arg^6-Phe^7 content in the VTA of AA rats is reduced by the drug (Ploj et al. 2002). These findings indicate that transitory changes in enkephalinergic systems in response to acute ethanol treatment, as well as the neuroadaptive changes induced by the prolonged exposure to the drug, may be relevant in the reinforcement mechanisms elicited by ethanol and in the development of a high alcohol drinking behaviour. Therefore, enkephalin biosynthesis is probably one of the events in opioidergic transmission largely affected by ethanol. If so, one may speculate that ethanol-induced increases in enkephalin expression reflect changes in intracellular enkephalin pools available for release. This hypothesis remains to be confirmed.

Biochemical studies have revealed that ethanol reinforcement mechanisms involve activation of the DAergic mesolimbic system, which may contribute to the development of addictive behaviour (Koob et al. 1998; Wise and Bozarth 1982). Ethanol stimulates the firing rate of mesolimbic DA neurones in the VTA, which leads to increases in the extracellular concentrations of DA in the NAcc (Gessa et al. 1985). In addition, acute doses of ethanol stimulate DA release from the NAcc (Di Chiara and Imperato 1985). The ethanol-induced DA release is critical in mediating the reinforcing properties of ethanol. However, several studies indicate that this effect is mediated by opioid peptides since opioid receptor antagonists decrease the ethanol-induced DA release (Acquas et al. 1993; Benjamin et al. 1993). These actions may involve the ethanol-induced release of opioid peptides, as suggested by various authors (Di Chiara et al. 1996; Méndez et al. 2001). Accordingly, the ethanol-induced release of β-endorphin and Met-enk from the NAcc has been demonstrated (Marinelli et al. 2003, 2005; Méndez et al. in preparation). The ethanol-induced changes in pro-enk mRNA expression observed in this study correlate with high ethanol concentrations in plasma (Li et al. 1998; Méndez et al. 2001) and NAcc (Yim et al. 2000), as well as with the acute tolerance to ethanol-induced DA release in the NAcc developed after

a single acute dose of ethanol (Yim et al. 2000). Therefore, our results are consistent with the reported positive reinforcing actions of ethanol in the mesocorticolimbic system (Di Chiara et al. 1996; Koob et al. 1998; Yim et al. 2000) and support a role for enkephalins in these mechanisms.

In conclusion, we have shown that a single acute dose of ethanol transiently alters pro-enk mRNA expression in the mesocorticolimbic system of Wistar rats with different kinetic patterns. A transitory decrease and increase in pro-enk mRNA levels were observed in the VTA and PFC, respectively, whereas a prolonged and sustained increase took place in the NAcc. Ethanol-induced changes in enkephalin expression may play a key role in the reinforcing properties of the drug. Further research is needed in order to elucidate the mechanisms involved in ethanol's actions on enkephalinergic neurones.

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