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Research report

Prenatal ethanol exposure alters met-enkephalin expression in brain regions related with reinforcement: Possible mechanism for ethanol consumption in offspring

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HIGHLIGHTS

- Prenatal ethanol exposure induces drug intake in offspring.
- Ethanol and opioids during early ontogeny.
- Prenatal ethanol induces changes in Met-enkephalin content in offspring.

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ABSTRACT

The endogenous opioid system is involved in ethanol reinforcement. Ethanol-induced changes in opioidergic transmission have been extensively studied in adult organisms. However, the impact of ethanol exposure at low or moderate doses during early ontogeny has been barely explored. We investigated the effect of prenatal ethanol exposure on alcohol intake and Methionine-enkephalin (Met-enk) content in rat offspring. Met-enk content was assessed in the ventral tegmental area [VTA], nucleus accumbens [NAcc], prefrontal cortex [PFC], substantia nigra [SN], caudate-putamen [CP], amygdala, hypothalamus and hippocampus. Pregnant rats were treated with ethanol (2 g/kg) or water during GDs 17–20. At PDs 14 and 15, preweanlings were evaluated in an intake test (5% and 10% ethanol, or water). Met-enk content in brain regions of infants prenatally exposed to ethanol was quantitated by radioimmunoassay. Ethanol consumption was facilitated by prenatal experience with the drug, particularly in females. Met-enk content in mesocorticolimbic regions – PFC and NAcc – was increased as a consequence of prenatal exposure to ethanol. Conversely, Met-enk levels in the VTA were reduced by prenatal ethanol manipulation. Prenatal ethanol also increased peptide levels in the medial-posterior zone of the CP, and strongly augmented Met-enk content in the hippocampus and hypothalamus. These findings show that prenatal ethanol exposure stimulates consumption of the drug in infant rats, and induces selective changes in Met-enk levels in regions of the mesocorticolimbic and nigrostriatal systems, the hypothalamus and hippocampus. Our results support the role of mesocorticolimbic enkephalins in ethanol reinforcement in offspring, as has been reported in adults.

1. Introduction

Ethanol experiences during early ontogeny – even during prenatal periods – facilitate posterior acceptance and consumption of the drug in these organisms [1–3]. The predisposition to recognize and prefer ethanol as a function of pre- or postnatal exposure has not only been observed in animal models. Human studies suggest

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that maternal intake of ethanol during pregnancy results in neonatal detection of ethanol odor [4]. In addition, alcohol consumption in children has been recently proposed as a serious risk factor that further increases the probability of drinking among these subjects [5].

In animal models, several authors have established that ethanol seeking and intake are modulated by the appetitive and aversive properties of the drug [2]. Preweanling rats have proven valuable for assessing these phenomena. A pattern of high acceptance of ethanol early in ontogeny seems to be associated with the pharmacological effects of the drug, rather than with its orosensory properties [1,2,6–9]. In addition, preweanling rats are sensitive to the locomotor activating effects of ethanol [10,11], suggesting that infants are prone to process the stimulating effects of ethanol rather than its sedative consequences.

The motivational aspects of alcohol in neonates and infant rats have been analyzed using operant approaches [12–17]. These studies strengthen the notion that the developing rat is highly sensitive to the appetitive motivational effects of ethanol, as demonstrated through rapid and robust instrumental learning.

The endogenous opioid system (enkephalins, endorphins and dynorphins) has been shown to play a major role in ethanol reinforcement and drinking behavior. Opioid peptides and ethanol exhibit similar pharmacological properties and behavioral effects. For instance, low doses of ethanol or opioids stimulate locomotor activity through dopaminergic (DAergic) activation in the ventral tegmental area (VTA), while high doses activate DAergic terminals in the nucleus accumbens (NAcc) [18,19]. Activation of the DAergic mesocorticolimbic system by mu (μ) and delta (δ) opioid agonists induces reinforcement, while kappa (κ) opioid receptor activation is associated to dysphoria [20]. These actions are mediated by increases or decreases in DA release from the NAcc, respectively, suggesting that opioid and alcohol reinforcement underlies a common neurobiological mechanism that involves activation of DAergic reward circuits [21].

In adult rats, ethanol increases the release of β-endorphin and Met-enkephalin (Met-enk), particularly during the ascending limb of the blood ethanol curve [22–24]. Ethanol intake is reduced by κ opioid receptor agonists and selective δ and μ receptor antagonists [25–27]. Lower ethanol preference and self-administration have been reported in μ knockout mice [28], in contrast to the greater preference and drug consumption observed in δ knockout animals [29]. The opioid system is also involved in ethanol-induced appetitive conditioning [2,30,31]. Recently, μ but not δ receptors, have been shown to be involved in the psychomotor stimulant effects of ethanol [32–34].

Mu, δ and κ receptors follow different patterns of development, but all are functional by the second postnatal week of life [35]. In this sense, similar to adult rodents [36], ethanol reinforcement and acceptance in preweanling rats seem to be regulated, at least partly, by the opioid system. For example, non-selective opioid antagonists (such as naloxone or naltrexone) co-administered with ethanol during gestation disrupt future increases in appetitive responding towards ethanol [12,37–39]. Furthermore, opioid antagonist administration, prior to conditioning with ethanol, disrupts appetitive reinforcement towards the drug [13]. In newborn and infant rats, μ and κ opioid systems modulate ethanol-mediated appetitive reinforcement [40] through inhibition of positive behaviors, such as the attachment to an artificial nipple [41]. Ethanol intake can also be reduced by non-selective or selective (μ or δ) opioid antagonists during the preweanling period [34,42,43].

Recent research conducted in our laboratory indicates that early in life the opioid system is involved not only in ethanol consumption, but also in operant behavior mediated by the drug (i.e., ethanol seeking and drug consumption). Overall, these results show that a fully functional opioid system is needed to promote ethanol reinforcement during the second postnatal week. Disruption by either a non-selective (i.e., naloxone) or selective opioid antagonist (μ, δ, κ) or agonist (κ) is sufficient for substantial reduction in consummatory and seeking behaviors associated with ethanol reinforcement [13,14].

Changes in opioid neurotransmission are relevant during ethanol intoxication, as well as in the adaptive neural responses induced by the drug. Ethanol-induced changes in opioidergic transmission occur at different levels, such as the expression and release of opioid peptides, as well as ligand binding to opioid receptors (for reviews, [44–46]). We have previously shown that ethanol induces selective changes in enkephalinergic and β-endorphinergic systems in adult rats, particularly in mesocorticolimbic regions involved in the reinforcing aspects of the drug. Acute ethanol administration dose-dependently increases Met-enk release from the NAcc and decreases peptide content in both the NAcc and caudate-putamen (CP) [23]. In contrast, acute ethanol does not modify β-endorphin content in these regions, but decreases peptide levels in the hypothalamus [47]. In addition, ethanol administration differentially alters the binding of selective ligands to μ and δ opioid receptors with different kinetic patterns. Ethanol (2.5 g/kg) significantly reduces ([^{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, but increases binding in the prefrontal cortex (PFC) [48]. The same ethanol dose increases ([^{3}H]-[D-Pen^2,D-Pen^5]-enkephalin) ([^{3}H]-DPDPE) binding to δ receptors in the PFC, NAcc, CP and substantia nigra (SN) [49]. These results indicate that ethanol selectively alters neurotransmission of both opioidergic systems, but has more pronounced effects on δ than on μ receptors. These findings also suggest that opioid receptor down- and up-regulation mechanisms may be involved in these actions. Differential opioid receptor sensitivity in specific brain areas may account for the impact of acute ethanol intoxication [45].

Chronic ethanol exposure selectively affects Met-enk- and β-endorphinergic systems as well. Ethanol (10% v/v, 4 weeks) increases Met-enk content in the adult rat VTA and PFC, but does not change β-endorphin levels in these brain areas [47,50,51]. However, this treatment does not alter ligand binding to μ or δ opioid receptors, suggesting that neuroadaptive changes in enkephalin- and β-endorphin-containing neurons in the mesocorticolimbic system may have occurred along prolonged ethanol exposure.

Even when ethanol-induced molecular changes in opioid systems have been extensively studied in adults, knowledge about the impact of exposure to low or moderate ethanol doses during early ontogeny is scarce in the literature. Therefore, the aim of this work was to investigate the effect of prenatal ethanol exposure on alcohol intake and Met-enk content in offspring. We studied ethanol effects on peptide levels in brain areas involved in reward mechanisms (i.e., mesocorticolimbic system), as well as in other regions in which Met-enk content is commonly high and are sensitive to ethanol actions (i.e., CP, SN, amygdala, hypothalamus and hippocampus) (for reviews, [44,45]). We hypothesized that selective molecular changes occur at the level of the enkephalinergic system when animals are exposed to ethanol during early ontogeny, since previous pharmacological studies from our group show that ethanol rewarding effects, mediated by prenatal ethanol exposure and infantile experiences with the drug, is disrupted when either non-selective or selective opioid antagonists are administered [13,14]. These findings allow us to predict that, as in adult animals, ethanol reinforcement is mediated, at least partially, by the enkephalinergic opioid system.

2. Methods

2.1. Subjects

Female Wistar rats (200–300 g) were maintained on a 12 h light/dark cycle (lights on at 8:00 AM). Standard rat Purina chow and water were available *ad libitum*, except where indicated. Vaginal smears of adult females were microscopically analyzed daily. On the day of proestrus, females were housed during the dark cycle with males (three females per male). Vaginal smears were checked the following morning (10:00–12:00 h), and the presence of sperm was considered the index of fecundity. The day of sperm detection was considered gestational day 0 (GD 0). Females were then individually placed in standard maternity cages filled with wood shavings. The expected length of gestation in this strain is equivalent to 21.5 days [52]. The date of birth was considered postnatal day 0 (PD 0). At PD 1, a maximum of 10 pups per litter were maintained (five males and five females, whenever possible). After handling, each dam and their respective litter remained undisturbed until the beginning of the intake test (PD 14). Animals used in this study were maintained and treated 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 Ethics Committee and Project's Commission approval of the Instituto Nacional de Psiquiatría Ramón de la Fuente.

2.2. Prenatal treatment

Pregnant dams received one daily intragastric (i.g.) administration of ethanol (2.0 g/kg body weight) ($n = 14$) or water ($n = 12$) (control group) on GDs 17–20. The 2.0 g/kg dose was achieved by administering 0.015 ml/g of a 16.8% v/v ethanol solution. The ethanol dose was selected on the basis of previous studies demonstrating fetal chemosensory processing of the drug under similar experimental circumstances, and general lack of deleterious effects of ethanol on several infant morphological and behavioral parameters [53,54]. As a function of prenatal treatment with ethanol or water, two independent prenatal groups were formed: Ethanol-Group (E-Group) and Water-Group (W-Group).

2.3. Infant intake test

Pups from 26 litters were tested. Two hours before the intake test started, pups (PDs 14 and 15) were separated from their dams and placed in holding chambers (15 × 8 × 15 cm) maintained at 30 °C with heating pads. All pups were intraorally cannulated with polyethylene tubing (5 cm length, PE10, Clay Adams, Parsippany, New Jersey, U.S.A.). The intraoral cannulation procedure has been extensively described in previous studies [53–57]. Briefly, a flanged end of the cannula (external diameter 1.2 mm) was shaped by exposure to a heat source. A short dental needle (30GA Monoject, Sherwood Medical, Munchen, Germany) was attached to the non-flanged end and positioned in the middle portion of the internal mucosa of the pup's left cheek. The needle was inserted through the cheek, and the cannula was pulled through the tissue until the flanged end rested in the mouth's mucosa. This cannulation procedure did not last more than 20 s per subject. As demonstrated by previous studies, pups rapidly recover from this minor surgical intervention [58,59]. Pups at this developmental stage are able to control ingestion of fluids delivered via these polyethylene devices [54,60]. This cannula was used to infuse the solutions (0% [distilled water] or 5 and 10% ethanol (v/v)) directly into the oral cavity of the pup. After cannulation and immediately before the beginning of the test, pup's bladders were voided by gentle brushing of the anogenital area to induce miction and defecation; thereafter, pups were weighed. Rats were then placed into individual Plexiglas chambers (15 × 7 × 15 cm) lined with a cotton floor. Intraoral infusions were performed using a 4-syringe infusion pump (APEMA, Argentina) connected to the oral cannula of each pup by a polyethylene catheter (PE50, Clay Adams, Parsippany, New Jersey, U.S.A.). The total volume administered to each pup was equivalent to 5.5% of the subject's body weight and was infused directly into the pup's mouth at a constant rate during 15 min. Pups could either consume or reject the infused solution. In the chambers, pups had 2 min of habituation prior to the 15 min intake evaluation. At the end of this session, each pup's weight was recorded. Consumption was determined by the percentage of body weight gained (%BWG), using the following formula: ([postinfusion weight − preinfusion weight]/preinfusion weight) × 100. The percentage of body weight gained was used as the dependent variable under analysis. The same intake test procedure was performed on PD 15. In order to avoid litter over-representation, no more than two pups from a given litter were included in a particular treatment (one male and one female, whenever possible); the remaining animals were assigned to other studies. Thirty minutes after the intake test, pups were sacrificed by decapitation and the brains and samples of whole blood were immediately removed. Brains were frozen and maintained at −70 °C until the dissection of brain areas. The following structures were dissected according to Ramachandra and Subramanian [61]: VTA, PFC, NAcc, SN, CP, amygdala (Am), hypothalamus (H) and hippocampus (Hp). The anterior-medial (amCP) and medial-posterior (mpCP) areas of the CP were obtained and were separately studied. The VTA was dissected using a micropunch of 1 mm diameter. One (H) or two brain regions (VTA, PFC, NAcc, SN, amCP, mpCP, Am and Hp) were pooled per tube and were further processed. Plasmas were prepared from whole blood samples and were frozen until analysis.

2.4. Met-enk extraction procedure

Peptide extraction was performed as reported previously [62]. Depending on structure size, brain regions were homogenized in different volumes of 1N acetic acid. Tissue samples were centrifuged at 15 000 rpm for 20 min at 4 °C. Supernatants were dried under vacuum in a Speed-Vac concentrator (Savant SC110A-115) and stored at −20 °C until assay. Pellets were resuspended in sterile distilled water and Met-enk content measured by radioimmunoassay (RIA).

2.5. Met-enk radioimmunoassay

[^{125}I]-Met-enk was obtained by the chloramine T method as described previously [63–65], with some modifications. Briefly, 2 μg of Met-enk (Bachem) were labeled using 400 μCi of Na[^{125}I] (103 mCi/ml, Perkin-Elmer) and 4 μg chloramine T (Sigma). The reaction was stopped 20 s later with 20 μg sodium metabisulphite (Sigma). [^{125}I]-Met-enk was separated from free Na[^{125}I] by gel filtration on Sephadex G-10 (Sigma) in 0.05 M sodium phosphate buffer containing 0.01% bovine serum albumin (BSA) (Sigma, RIA grade). [^{125}I]-Met-enk was preserved at −20 °C until assay.

Met-enk RIA was performed as previously reported [66,67], with some modifications. The Met-enk antibody used in this study was kindly provided by Dr. M. Asai (Instituto Nacional de Psiquiatría Ramón de la Fuente) and was a rabbit anti-rat serum with 100 and 2.90% cross-reactivity with the oxidized and non-oxidized forms of Met-enk, respectively [66,68]. This antiserum has been previously characterized and shown to have a low cross-reactivity with other opioid peptides (0.01% with Leu-enkephalin, 0.76% with Met-enk-Arg, and <0.01% with Leu-enk-Arg, Met-enk-Arg-Phe, Met-enk-Arg-Gly-Leu, dynorphin 1-8, α-endorphin, β-endorphin and γ-endorphin) [67]. RIA buffer was made of 50 mM sodium phosphate, pH 7.4, containing 140 mM NaCl, 0.01% BSA (Sigma, RIA

grade) and 0.02% NaN_3 (Sigma). The reaction mixture consisted of 100 μl of Met-enk antiserum in RIA buffer (1/1000), 50 μl of [^{125}I]-Met-enk diluted in RIA buffer (7000 cpm/tube) and 100 μl of Met-enk standards (Bachem) or tissue sample extracts. Before RIA, Met-enk standards and tissue samples were oxidized with 30% H_2O_2 (1/250) overnight. The mixture (300 μl final volume) was incubated for 24 h at 4 °C and the assay was stopped by the addition of cold absolute ethanol (1 ml). The bound and free fractions of labeled material were separated by centrifugation at 4000 rpm for 30 min at 4 °C. Bound radioactivity in the pellets was measured in a gamma emission counter (Perkin-Elmer, Wallac Wizard 1470). The sensitivity of the assay was 70 pg and the IC_{50} 650 pg. The intra- and inter-assay coefficients of variation were 4.8% and 8.2%, respectively.

2.6. Protein determination

Proteins in homogenates from brain areas were measured according to Lowry et al. [69].

2.7. Determination of blood alcohol concentration

Blood alcohol concentrations (BACs) were measured in plasma according to Poklis and Mackell [70], with some minor modifications. Briefly, alcohol in plasma samples was oxidized to acetaldehyde by alcohol dehydrogenase (ADH), with the simultaneous reduction of nicotinamide adenine dinucleotide (NAD) to NADH. The consequent increase in absorbance at 340 nm was measured in a spectrophotometer (PG Instruments T70).

2.8. Statistical analyses

Data obtained during the intake test were analyzed with a $2 \times 3 \times 2 \times 2$ mixed analysis of variance (ANOVA). Prenatal treatment (E-Group or W-Group), solution intraorally infused (water, 5% ethanol or 10% ethanol) and gender (female or male) represented the between-subjects factor, whereas the day of evaluation (PD 14 and PD 15) was the within-subjects factor. Significant main effects or interactions were further analyzed with Tukey-HSD *post hoc* comparisons. Results were considered to be significant at $p < 0.05$.

Met-enk content and BAC data were analyzed using a $2 \times 3 \times 2$ factorial ANOVA, defined by prenatal treatment (E-Group or W-Group), solution intraorally infused (water, 5% ethanol or 10% ethanol) and gender (female or male). Significant differences between groups were determined by the *post hoc* Tukey-HSD test. In some experiments, comparisons were performed by the Student's t test. Results were considered to be significant at $p < 0.05$.

3. Results

3.1. Maternal body weight gain during late gestation, litter size and infant body weight

The percentage of body weight gain of dams across gestational days was calculated using the following formula: ([maternal body weight at GD 20 − maternal body weight at GD 17]/maternal body weight at GD 17) × 100. One-way ANOVA showed that prenatal treatment had no significant effect on this maternal weight index. Litter size also failed to be affected by prenatal manipulations during late gestation. Pup's body weight, measured minutes before evaluation of consumption at PD 14, was analyzed as a function of prenatal manipulation. This dependent variable did not showed significant differences explained by prenatal history of pups. Taken together, these results suggest that prenatal manipulations had no gross teratological effects, consistent with previous reports [1,53,71,72].

3.2. Infant intake test

Statistical analysis of consumption scores during PDs 14–15 began with a four-way mixed ANOVA (prenatal treatment [E-Group, W-Group] × gender [male, female] × solution infused [water, 5% ethanol or 10% ethanol] × evaluation day [PD 14 or PD 15]). The interaction between the four factors under analysis, failed to show significant effects. The triple interaction established by prenatal manipulation, solution infused and gender of subjects resulted significant ($F_{(2,112)} = 5.04$, $p < 0.01$). In addition, the two-way interaction between prenatal treatment and solution infused also achieved significance ($F_{(2,112)} = 3.17$, $p < 0.05$). This analysis also revealed a significant main effect of solution infused ($F_{(2,112)} = 9.89$, $p < 0.01$). To better understand the significant triple interaction between prenatal manipulation, solution infused and gender, separated follow-ups ANOVAs defined by prenatal treatment, solution infused (between factors) and PDs 14 and 15 (repeated measures) were run for females and males. For female pups, the triple interaction failed to achieve significant effects. Nevertheless, and of major importance, the two-way interaction between prenatal treatment and solution infused did achieve significance ($F_{(2,52)} = 7.76$, $p < 0.01$). The ANOVA also showed a significant main effect of solution infused ($F_{(2,52)} = 4.66$, $p < 0.025$). Tukey-HSD *post hoc* test indicated that those female pups prenatally treated with ethanol consumed significantly higher levels of a 5% ethanol solution than those evaluated in terms of 10% ethanol or water intake (Fig. 1A). In addition, ethanol-treated females significantly increased consumption scores of a 5% ethanol solution in comparison with their own control (water-treated females). The triple interaction analysis of consumption scores in male pups, was not significant (Fig. 1B). However, the two-way interaction between solution and day of evaluation threw a significant effect in males ($F_{(2,60)} = 3.62$, $p < 0.05$). Prenatal treatment and solution infused also resulted in significant main effects ($F_{(1,60)} = 4.23$, $p < 0.05$ and $F_{(2,60)} = 5.29$, $p < 0.01$, respectively). These data suggest that male pups prenatally exposed to ethanol expressed higher levels of consumption, independently of the solution infused. Additionally, Tukey-HSD *post hoc* comparisons showed that consumption – as a function of ethanol concentration and day of test – was only significantly higher in those male pups evaluated with a 5% ethanol solution than male pups that ingested the 0% solution, particularly at PD 14. The interaction between these factors (solution infused × day of evaluation) failed to be significant in females pups (Fig. 2A and B).

In summary, the most relevant results described here suggest that, under the present experimental conditions, the profile of ethanol intake is different across gender. In female pups, the predisposition to consume ethanol during infancy was more evident in those subjects prenatally exposed to the drug. This effect was not evident in male subjects (Fig. 1A and B). Nevertheless, male pups effectively showed increases in ethanol consumption when experiencing a 5% ethanol concentration regardless of prenatal treatment, particularly during the first day of intake evaluation (Fig. 2A and B).

3.3. Met-enkephalin content in brain areas of PD 15 pups

After the preweanling intake test, Met-enk concentration (pg/mg protein) was measured in several brain areas of DP 15 pups. Three-way ANOVA was performed, considering prenatal treatment (ethanol or water), solution intake (0-, 5- or 10% ethanol) and gender as sources of variation. Statistical analysis revealed a significant main effect of prenatal treatment ($F_{(1,27)} = 0.171$, $p < 0.004$), as well as a significant interaction between prenatal treatment, intake and gender in the VTA ($F_{(2,27)} = 8.334$, $p < 0.002$). However, no

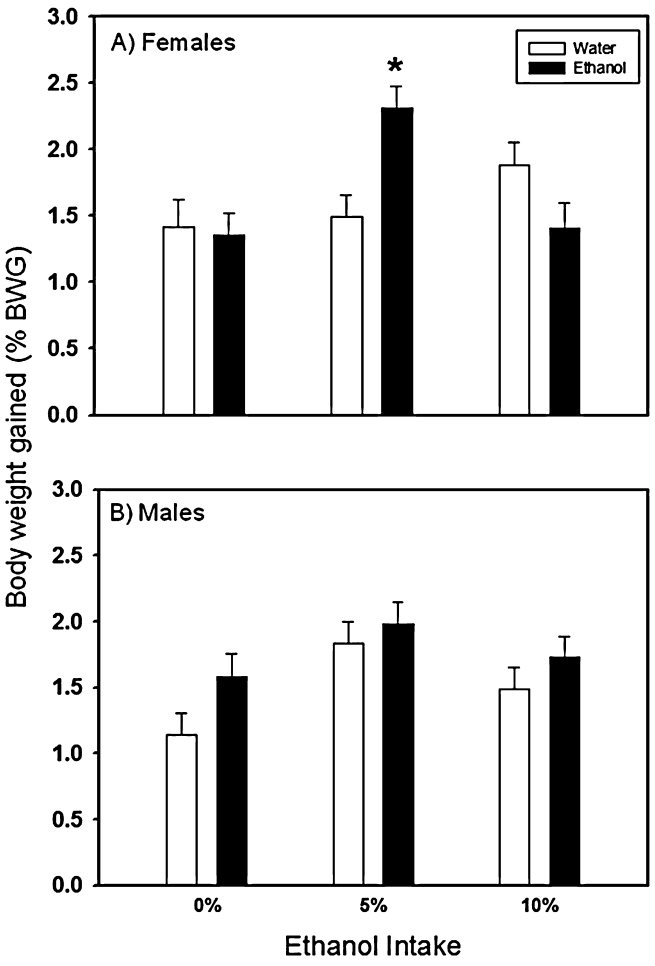

Fig. 1. Percentage of body weight gain in pups as a function of prenatal treatment, ethanol intake and gender. Wistar rat dams were daily administered with ethanol (2 g/kg body weight, i.g.) on GDs 17–20. Preweanling's intake test was performed on PDs 14 and 15. Data show the percentage of body weight gain (%BWG) in females (A) and males (B) and represent the mean ± SEM of 7–11 (water pretreatment) or 8–11 (ethanol pretreatment) in females. In males, 10–11 (water pretreatment) or 10–13 (ethanol pretreatment) animals were used. $^{*}p < 0.001$, versus their own control (prenatal water treatment) and ethanol-treated pups evaluated in terms of 0- and 10% ethanol solution.

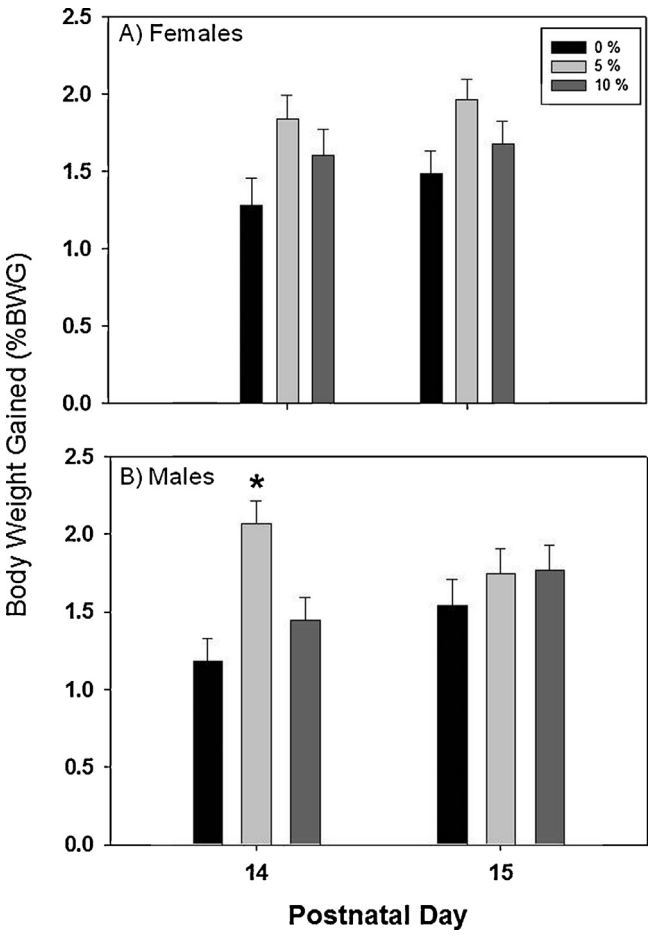

Fig. 2. Percentage of body weight gain as a function of ethanol intake (0-, 5 and 10% ethanol), gender and day of evaluation (PDs 14 and 15). Wistar rat dams were daily administered with ethanol (2 g/kg body weight, i.g.) on GDs 17–20. Preweanling's intake test was performed on PDs 14 and 15. Data show the percentage of body weight gain (%BWG) in females (A) and males (B) and represent the mean ± SEM of 18–22 females and 21–23 male pups. $^{*}p < 0.05$, versus their own 0% ethanol intake control at PD 14.

significant triple interactions were found in the other brain structures analyzed in this study. When gender was added as a source of variation, Met-enk concentration was only affected by this factor in the VTA. Since the VTA is a very small region (particularly in infant subjects) and Met-enk content is very low, several samples were at the limit of sensitivity of the assay. Thus, a considerable number of samples were discarded and could not be used in the analysis. Statistical effects of either main factors or interactions derived from this analysis could be attributed to a Type I error, more than a consistent effect. For this reason, and since no significant differences were found between males and females in the other brain regions studied in this work, the effects of prenatal treatment and intake on Met-enk concentrations were analyzed by two-way ANOVA.

3.3.1. *Effect of prenatal ethanol treatment and intake on Met-enk content in regions of the mesocorticolimbic system*

Statistical analyses of Met-enk concentration data showed significant main effects of prenatal treatment in the VTA ($F_{(1,33)} = 5.699$, $p < 0.023$), PFC ($F_{(1,53)} = 5.509$, $p < 0.023$) and NAcc ($F_{(1,60)} = 8.416$, $p < 0.005$), although no significant effects of prenatal treatment × intake interactions were found. Analysis of data from the PFC revealed that intake effects were close to the limit of significance ($F_{(1,53)} = 3.102$, $p = 0.053$). Prenatal ethanol treatment significantly decreased (45.4%) Met-enk levels in the VTA of 0% ethanol intake group of pups, as shown by Student's t test ($t = 2.754$, $p < 0.019$) (Fig. 3A). In contrast, prenatal ethanol treatment increased Met-enk content by 67.7% in the PFC and 78.3% in the NAcc. These effects were observed in 0- ($t = -2.373$, $p < 0.029$) and 10% ($t = -2.175$, $p < 0.042$) ethanol intake groups of pups, respectively (Fig. 3B and C). In the NAcc, a 100% increase in peptide content was observed in pups from the 5% ethanol intake group, although this effect was at the limit of significance ($t = -2.048$, $p = 0.054$) (Fig. 3C).

3.3.2. *Effect of prenatal ethanol treatment and intake on Met-enk content in regions of the nigrostriatal pathway*

Two-way ANOVA of Met-enk concentration data in brain areas of the nigrostriatal pathway revealed no significant interactions between prenatal treatment and intake in the SN, amCP and mpCP. No significant main effects of prenatal treatment or intake were observed in the SN and amCP (Fig. 4A and B). However, significant main effects of prenatal treatment were found in the mpCP ($F_{(1,65)} = 4.715$, $p < 0.034$) (Fig. 4C). As shown by Student's t test, in this zone of the CP, prenatal ethanol treatment increased Met-enk levels by 57.9% and 53.5% in 0- ($t = -2.009$, $p < 0.029$) and 5% ($t = -1.854$, $p < 0.038$) ethanol intake groups of pups, respectively (Fig. 4C).

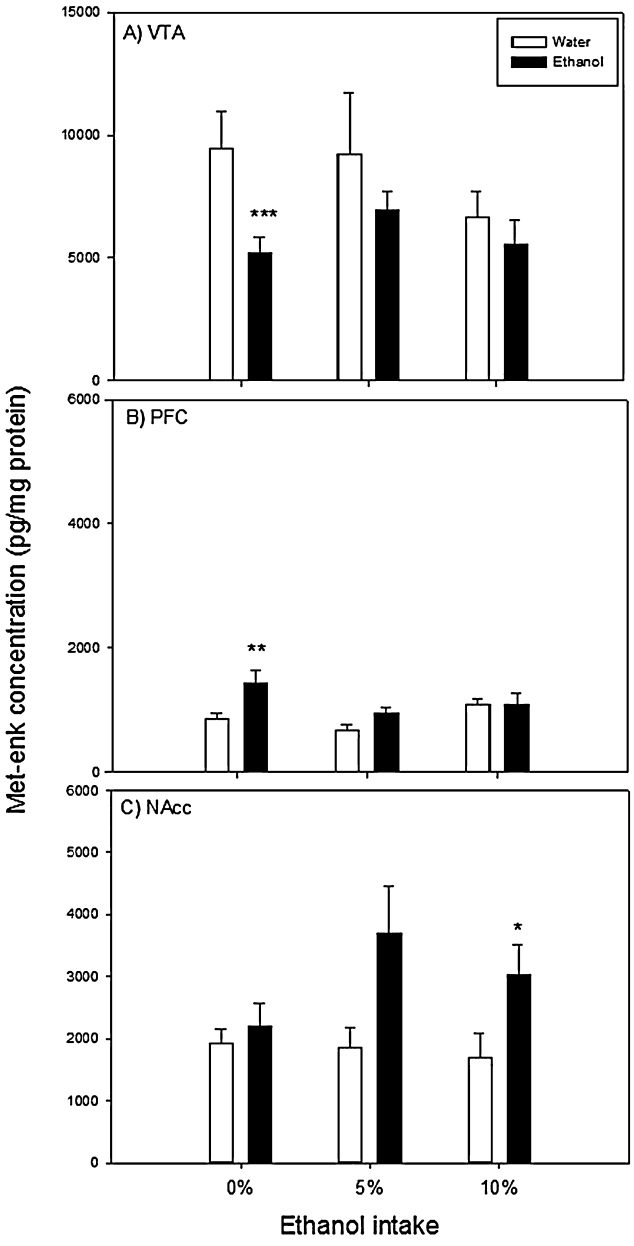

Fig. 3. Effect of prenatal ethanol treatment on Met-enk concentration in regions of the mesocorticolimbic system. Animal treatment and preweanling's intake test were performed as described in Fig. 1. Thirty min after evaluation of consumption (0-, 5- or 10% ethanol solution), pups were sacrificed and their brains removed and frozen. Met-enk concentration was quantitated in several brain areas by RIA. Met-enk concentration values (pg/mg protein) are shown for the VTA (A), PFC (B) and NAcc (C). Data are the mean ± SEM of 6 (prenatal water) or 7 (prenatal ethanol) pups in the VTA, 8–11 (prenatal water) or 9–11 (prenatal ethanol) in the PFC, and 10–11 (prenatal water) or 11–12 (prenatal ethanol) in the NAcc. ***$p < 0.020$, **$p < 0.030$, and *$p < 0.050$, versus their own prenatal water control groups.

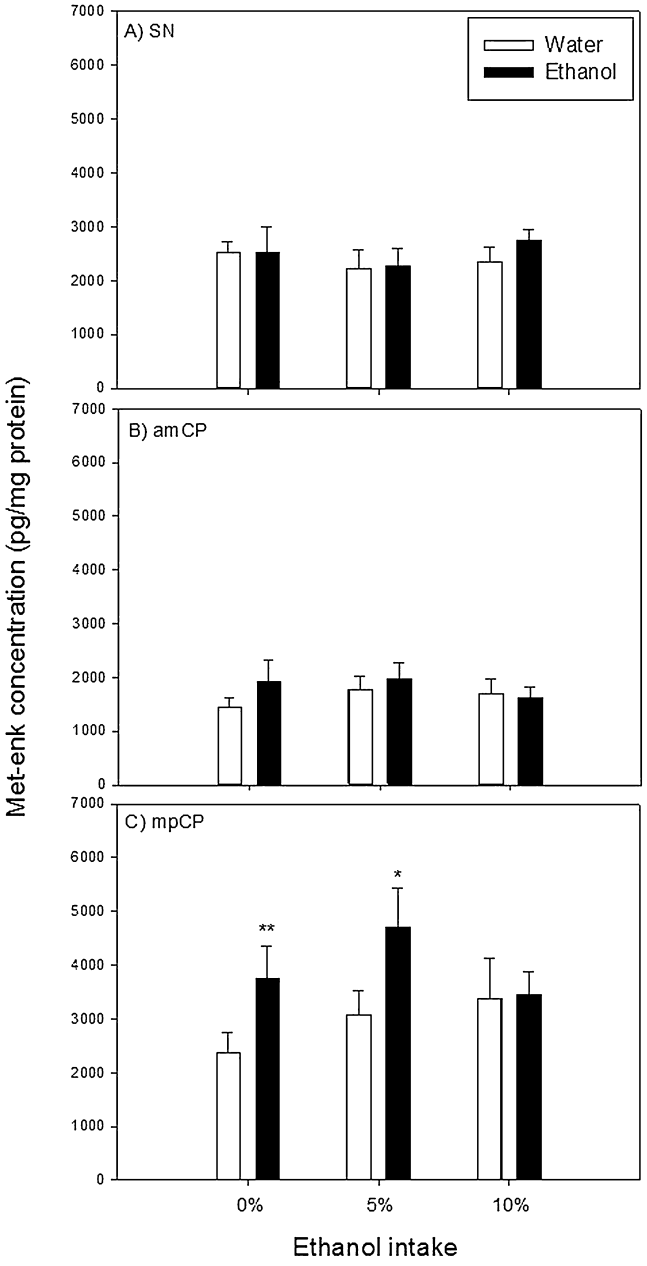

Fig. 4. Effect of prenatal ethanol treatment on Met-enk concentration in regions of the nigrostriatal pathway. Animal treatment, preweanling's intake test and Met-enk quantitation were performed as described in Fig. 3. Met-enk concentration values (pg/mg protein) are shown for the SN (A), amCP (B) and mpCP (C). Data are the mean ± SEM of 5–6 pups in the SN, and 10–12 (prenatal water) or 10–13 (prenatal ethanol) in the amCP and mpCP. **$p < 0.030$ and *$p < 0.040$ versus their own prenatal water control groups.

3.3.3. *Effect of prenatal ethanol treatment and intake on Met-enk content in the amygdala, hypothalamus and hippocampus*

Statistical analyses of Met-enk concentration data revealed no significant interactions between prenatal treatment and intake in the amygdala, hypothalamus and hippocampus. Significant main effects of prenatal treatment were detected in the hypothalamus ($F_{(1,69)} = 22.553$, $p < 0.001$) and hippocampus ($F_{(1,69)} = 26.258$, $p < 0.001$), but not in the amygdala (Fig. 5A). As shown by Student's t test, prenatal ethanol treatment significantly increased Met-enk levels by 76.6-, 56.4- and 80.7% in the hypothalamus of 0- ($t = -3.399$, $p < 0.001$), 5- ($t = -1.962$, $p < 0.031$) and 10% ($t = -3.226$, $p < 0.002$) intake groups of pups (Fig. 5B). Similarly, prenatal ethanol exposure increased peptide levels by 85.2-, 98.7- and 127.2% in the hippocampus of 0- ($t = -2.500$, $p < 0.010$), 5- ($t = -3.515$, $p < 0.001$) and 10% ($t = -3.099$, $p < 0.003$) intake groups (Fig. 5C).

3.4. *Blood alcohol concentration in DP 15 pups*

Analysis of BAC data showed a significant main effect of ethanol intake ($F_{(2,118)} = 70.985$, $p < 0.001$). No significant interactions were found between prenatal treatment and intake. As expected, BACs were significantly increased in 5- and 10% intake groups of pups in comparison with their 0% intake controls (Table 1).

Table 1
Blood alcohol concentrations (BAC) in PD15 pups as a function of prenatal treatment and ethanol intake. Animal treatment and preweanling's intake test were performed as described in Fig. 1. BAC in PD 15 pups was determined in plasma by an ADH assay.

Prenatal treatment	BAC (mg/l)		
	0% ethanol intake	5% ethanol intake	10% ethanol intake
Water	5.85 ± 1.13 (18)	269.25 ± 27.34* (17)	435.21 ± 53.14* (17)
Ethanol	6.20 ± 0.81 (24)	299.16 ± 40.56* (24)	406.97 ± 45.42* (24)

* $p < 0.001$ versus their 0% intake controls. Significant differences were also found between 5- and 10% intake groups in both prenatal water ($p < 0.005$) and prenatal ethanol ($p < 0.042$) pups.

3.5. Discussion

The results obtained in this work show that, during early ethanol exposure, the organism can learn about ethanol effects and modify its latter responsiveness to the drug as a function of these experiences. Specifically, we assessed the consumption of ethanol in infant rats as a function of prenatal drug exposure. Ethanol consumption, particularly at a low concentration (5%), was facilitated by prenatal experience with the drug, as previously reported by us and other groups [12,37,38,54]. Prenatal modulation of ethanol intake in infancy was particularly evident in ethanol-treated female pups. In males, prenatal exposure to ethanol promoted infantile consumption of all the infused solutions. An interesting data derived from these results is that infantile intake of a lower ethanol concentration (5%) was increased in male pups at PD 14. These findings are consistent with previous reports showing infantile predisposition to consume ethanol, even in pups with no prior experiences with the drug [73,74]. Even when gender differences in ethanol consumption have not been extensively documented in preweanling animals, recent studies suggest gender effects upon intake of palatable substances (such as saccharin) when pups are under the effects of 0.5 g/kg ethanol [75]. An aspect that remains to be elucidated in these works is the possible differences elicited by prenatal and/or postnatal manipulations in comparison with basal levels of response in naïve animals to, either ethanol consumption during infancy or Met-enk content in brain regions under analysis. A previous study showed that prenatal stress from handling did modify sensitivity to ethanol's postabsorptive consequences when comparing with an untreated control in terms of the dose yielding effective reinforcing effects. It appears that pups whose dams were handled and given i.g. intubation during late gestation require a somewhat higher alcohol blood concentration than prenatally naïve subjects for ethanol reinforcement [76]. The possible causal and/or modulating effects of prenatal stress upon subsequent ethanol responsiveness remain a matter of debate (for a review, see [6]) and deserve further research.

Several studies conducted in rodents have clearly shown that ethanol exposure during gestation induces increased intake of ethanol after birth. This effect has been consistently found when the drug is administered during the last days of pregnancy (GDs 17–20) [38,54,77], either with low and moderate ethanol doses (1–2 g/kg), or after exposure to a relatively high dose (3 g/kg), which induces taste aversion in adult rats [78]. The increased effect on ethanol intake has also been shown to be accompanied by an enhanced palatability of ethanol flavor [37,79]. The ethanol effect in pregnant rats was blocked by administration of naloxone (a non-selective opioid receptor antagonist), suggesting that the enhanced acceptance of ethanol is mediated by the endogenous opioid system [12,37,38,78]. In addition, the reinforcing attributes of ethanol (assessed through a self-administration paradigm) also seem to be mediated by the opioid system in early ontogeny [12–14]. The pharmacological effects of ethanol have also been shown to be modified by prenatal treatment with selective μ and κ opioid receptor antagonists. Mu opioid receptor blockade completely reverses the effects on intake and palatability, while kappa receptor antagonism partially reduces palatability [80]. So far, the involvement of the opioid system in modulation of ethanol reinforcing effects during gestation has been demonstrated employing behavioral and/or pharmacological approaches. However, the impact of these early experiences with moderate ethanol doses upon molecular changes in neurobiological systems remains to be elucidated. Thus, we investigated the ethanol-induced changes in Met-enk concentrations in several offspring brain areas as a consequence of prenatal and infantile exposure to moderate drug doses.

In contrast to what we expected, our results show that prenatal ethanol exposure selectively alters Met-enk content, regardless of the ethanol concentration infused during infancy. Ethanol treatment during gestation significantly increased Met-enk concentration in the PFC, NAcc, mpCP, hypothalamus and hippocampus, but decreased peptide levels in the VTA. The most prominent alterations induced by ethanol were observed in the NAcc, the hypothalamus and the hippocampus. Two-way factorial ANOVA used to analyze the levels of Met-enk in different brain regions, failed to shed statistical interactions between prenatal treatment and infused solution at PDs 14–15. However, a descriptive analysis of the data suggests that Met-enk levels among prenatal ethanol and water groups is not uniform, if considering the condition of ethanol solution infused during childhood. This speculative approach suggested that in the hypothalamus and hippocampus the changes seem to be global, since there was an increase in Met-enk levels no matter what fluid was infused. In contrast, in other brain regions it was suggested that differences in Met-enk levels might be, at least partially, due to the fluid delivered during intake. For example, the 0% group in the PFC seems to differ from the 5% and 10% prenatal ethanol animals. The fact that differences between ethanol and controls are found for some delivered fluids in some regions (for instance, VTA ethanol decrease for 0% infusion but not for ethanol of any concentration), but not in others, also argues for this point. In the case of Met-enk levels attained in the NAcc, it seems that changes were more pronounced when ethanol-treated pups consumed 5% or 10% ethanol solution. In the case of the mpCP, the changes seem to be more markedly expressed in 0% and 5% ethanol. Overall, these results seem to indicate that prenatal ethanol exposure modulate Met-enk content unevenly as a function of a subsequent experience with the drug during the second postnatal week. The ethanol-induced changes in Met-enk content observed in this study could be due to alterations in Pro-enk mRNA expression and/or processing of the precursor. In addition, changes in peptide levels could be attributed to alterations in Pro-enk mRNA stability. Peptide release could also contribute to the observed effects.

A small number of studies have addressed the impact of prenatal ethanol exposure upon opioid peptide levels in specific brain regions. Consistent with our results, ethanol exposure of Sprague–Dawley dams has been shown to increase mRNA Pro-enk levels in the NAcc of 5-, 12-, and 19- [81] or 15-day old pups [82]. This effect is particularly evident in the core of the NAcc [82]. In addition, prenatal (GD 1–22) and postnatal (PD 2–10) ethanol

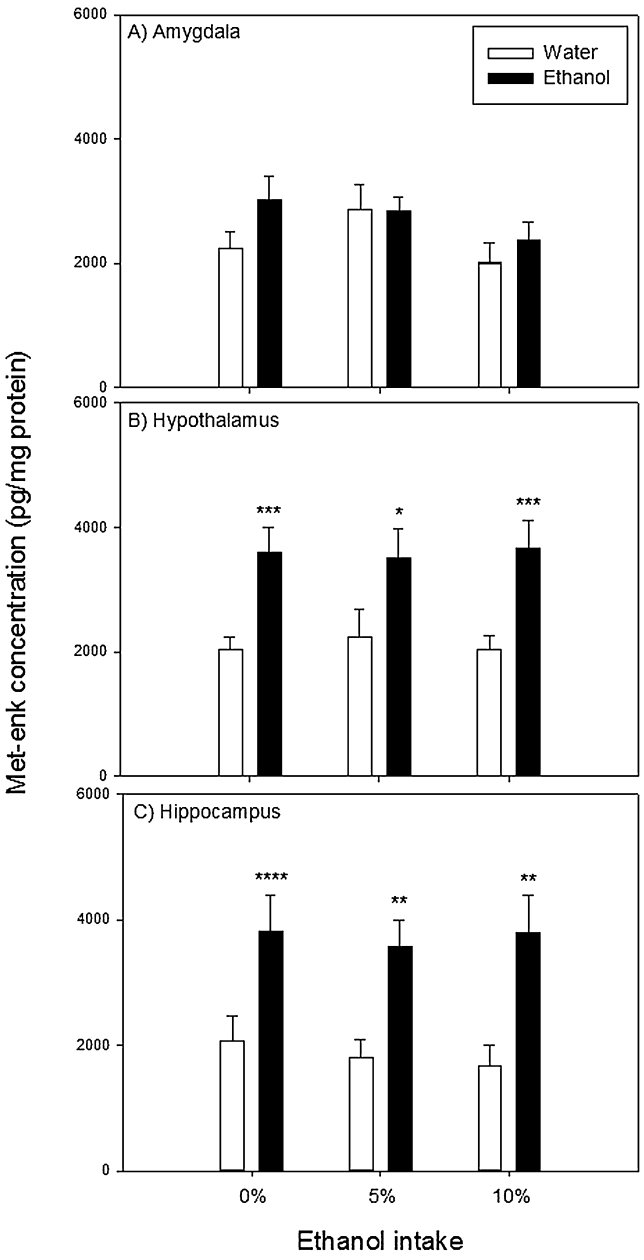

Fig. 5. Effect of prenatal ethanol treatment on Met-enk concentration in the amygdala, hypothalamus and hippocampus. Animal treatment, preweanling's intake test and Met-enk quantitation were performed as described in Fig. 3. Met-enk concentration values (pg/mg protein) are shown for the amygdala (A), hypothalamus (B) and hippocampus (C). Data are the mean ± SEM of 8–10 (prenatal water) or 11–13 (prenatal ethanol) pups in the amygdala, and 12 (prenatal water) or 13 (prenatal ethanol) in the hypothalamus and hippocampus. $^{****}p < 0.0001$, $^{***}p < 0.005$, $^{**}p < 0.010$ and $^{*}p < 0.050$ versus their own prenatal water control groups.
Animal treatment and preweanling's intake test were performed as described in Fig. 1. BAC in DP 15 pups was determined in plasma by an ADH assay. Data are the mean ± SEM of the number of animals indicated in parentheses. $^{*}p < 0.001$ versus their 0% intake controls. Significant differences were also found between 5- and 10% intake groups in both prenatal water ($p < 0.005$) and prenatal ethanol ($p < 0.042$) pups.

exposure increases Met-enk levels in the NAcc of Long-Evans pups, particularly in females, although peptide content was not modified in the PFC and VTA [83]. These findings indicate that the impact of pre- and postnatal ethanol exposure in offspring may depend on several factors, including the alcohol dose, the duration of drug exposure, the rat strain and the developmental and hormonal/sexual maturation stage.

Ethanol, under different experimental conditions, modifies opioidergic transmission in adult rodents and humans (for reviews, [20,36,44–46]). Studies from our group show that both acute and chronic ethanol treatment alters enkephalin- and β-endorphinergic systems in adult rats. These effects occurr at different levels of transmission and are region-specific and dose-dependent. For instance, a single ethanol dose (2.5 g/kg) decreases Met-enk concentration in the NAcc and increases peptide release from this area in Wistar rats [23]. The same ethanol dose induces a sustained increase in Pro-enk mRNA levels in the NAcc [84]. In addition, [^{3}H]-DAMGO and [^{3}H]-DPDPE binding are selectively altered by acute ethanol in the VTA, NAcc and PFC [48,49], suggesting that activation of μ and δ receptors may participate in the reinforcing actions of ethanol. On the other hand, chronic ethanol exposure increases Pro-enk mRNA levels in the medial PFC, the VTA and the NAcc (core and shell) of Sprague–Dawley rats [82], as well as Met-enk content in the VTA and PFC of Wistar animals, with no change in the NAcc [50,51]. These findings suggest that during a prolonged exposure to ethanol, neuroadaptive changes may occur in mesocortical and accumbal enkephalin-containing neurons.

Our results suggest that the exposure to ethanol during a short period of late gestation with a moderate dose of the drug is enough to promote changes in terms of Met-enk levels in particular regions of the mesocorticolimbic system. Moreover, these findings suggest that, as in adult rats, changes in enkephalinergic transmission may be involved in the reinforcing properties of ethanol in offspring. If so, it is possible that ethanol, through activation of μ and δ receptors, may facilitate DAergic transmission at different levels in the mesocorticolimbic system (μ receptors at the VTA; μ and δ receptors in the NAcc), as previously suggested [85–87]. Knowledge of other aspects of opioidergic transmission in the mesocorticolimbic system would contribute to understand the mechanisms of ethanol actions in prenatally exposed offspring. Another important issue that deserves further research is the lack of effects mediated by infantile experiences with ethanol.

In the nigrostriatal pathway, Met-enk levels were only affected in the mpCP by prenatal ethanol exposure. Druse et al. reported an increase in Pro-enk mRNA levels in the striatum of Sprague–Dawley pups prenatally treated with ethanol [81]. In adult Wistar rats, we have previously shown that acute ethanol (2.5 g/kg) decreases Met-enk content in the CP [23]. However, other authors found increased peptide content in the striatum of adult Sprague–Dawley rats [88,89]. Additionally, acute ethanol transiently decreases Pro-enk mRNA levels in the SN, but increases mRNA expression in the CP (amCP and mpCP) [90,91], suggesting a compensatory effect for the observed reduction in peptide content. Moreover, ethanol (2.5 g/kg) decreases [^{3}H]-DAMGO binding and increases [^{3}H]-DPDPE binding in the SN *pars reticulata* (SNr) of adult Wistar rats. The same treatment does not affect [^{3}H]-DAMGO binding in the CP, but increases [^{3}H]-DPDPE binding in this brain area [49,92]. These findings suggest that ethanol differentially modulates the DAergic activity in this pathway through selective activation of μ and δ receptors.

The role of nigrostriatal opioid peptides and receptors in ethanol's actions is not clearly understood. Ethanol and opioids increase the firing rate of DAergic neurons in the SN *pars compacta* (SNc) [93,94], as well as DA synthesis, release and metabolism in the striatum [95–97]. These actions may be exerted by opioid receptors located at distinct levels in the SN and striatum [98,99]. Although the nigrostriatal pathway has not been involved in ethanol reward circuits, some studies suggest that activation of DAergic transmission in this pathway could be critical in determining brain sensitivity to ethanol, thus contributing to alcohol addiction [100]. In fact, some studies suggest that brain sensitivity to ethanol is an important indicator of the liability for ethanol addiction, since a low level of response to the drug in humans has been associated with a greater likelihood of future alcoholism [101]. In rodents, ethanol

and pentobarbital induce narcosis and this effect can be evaluated as a reduction in drug-induced sleep time. This response has been interpreted as a decreased sensitivity (increased resistance) of the brain to the narcotic effects of these drugs. The number of striatal DAergic receptors is increased and correlates with an enhanced brain resistance to ethanol-induced narcosis, suggesting that postsynaptic DAergic receptors may be relevant in ethanol sensitivity [100,102]. Other studies suggest that numerous neurotransmitters and/or neuromodulators, such as opioid peptides, may also participate in brain sensitivity to ethanol [103]. For instance, β-endorphin has been shown to increase ethanol sensitivity to the anesthesic effects of the drug in mice [104]. Mice strains with higher sensitivity to the hypothermic effects of acute ethanol administration are more sensitive to drugs affecting opioid systems [103] and show differences in μ opioid receptor densities [105]. Microinjection of a selective δ receptor antagonist into discrete rat brain regions blocks the ethanol-induced hypothermia and sedation [106]. Overall, these findings support a central role of opioid peptides and receptors in ethanol-induced narcosis.

Our results show that a short prenatal exposure to a moderate dose of ethanol promoted changes in Met-enk content in the mpCP. The mechanisms underlying the observed effect remain to be determined, as well as the lack of effects on Met-enk content mediated by infantile experiences with ethanol. Whether ethanol sensitivity mechanisms are already established in 15-day-old pups is unknown and remains to be investigated.

Prenatal manipulations with ethanol strongly increased Met-enk levels in the hypothalamus and hippocampus. In the amygdala, the impact of a fetal experience with the drug was not evident, although other authors found that peptide concentration in the central nucleus of the amygdala (CeA) is decreased by prenatal ethanol exposure [83]. These results may reflect differences in experimental conditions and rat strains.

The effect of prenatal ethanol exposure on enkephalin expression in the hippocampus and hypothalamus of offspring has not been extensively studied. In adult rats, ethanol (2.5 g/kg) increases Met-enk content in the hypothalamus of Sprague–Dawley animals [88,89], but has no effect in the hippocampus [89]. Ethanol also increases Pro-enk mRNA levels in the paraventricular nucleus of the hypothalamus (PVN) and mammilary bodies of Wistar rats, and reduces expression in the arcuate nucleus [107]. The same ethanol dose produces biphasic effects in Pro-enk mRNA expression in the hippocampus, particularly at the level of the dentate gyrus, CA1 and CA3 [107]. On the other hand, chronic ethanol decreases Met-enk content in the hypothalamus [89], but increases mRNA expression in the PVN [108], suggesting ethanol-induced compensatory effects. In contrast to acute effects, chronic ethanol does not alter Met-enk content [89] or expression in the hippocampus [108]. In the present study, prenatal ethanol exposure promoted prominent increases in Met-enk content in the hippocampus of PD 15 pups. The role of these alterations in hippocampal function remains to be established.

Ethanol exposure during gestation (GD 9–21; 1 or 3 g/kg/day) stimulates Pro-enk mRNA expression in the PVN during infancy (PD 15 pups) [82], consistent with the increased enkephalin levels observed in offspring prenatally exposed to a higher ethanol (4.5 g/kg) [81,83]. Similar increases in mRNA levels of orexigenic peptides were observed in the PVN (galanin) and in the perifornical lateral hypothalamus (PFLH) (orexin). The fact that the effects of prenatal ethanol exposure persist in PD 15 offspring indicates that ethanol, during gestation, affects *in utero* development of these peptidergic systems and induces long-lasting neuronal changes. These results suggest that hypothalamic orexigenic peptides are involved in the increased ethanol consumption observed in prenatal ethanol-exposed offspring [82]. Other drugs of abuse exhibit similar effects. For instance, nicotine exposure during gestation increases Pro-enk mRNA expression and density of enkephalin-containing neurons in the PVN and CeA, as well as orexin mRNA levels in the PFLH [109]. As ethanol-exposed animals, nicotine effects persisted until puberty, even in the absence of the drug [82,109]. Therefore, these findings suggest that enkephalin- and orexin-containing neurons in the hypothalamus exhibit a remarkable sensitivity to ethanol and nicotine effects during gestation. Changes in these peptidergic systems could be involved in the mechanisms leading to high drug consumption during infancy or adolescence.

In conclusion, we have shown that prenatal exposure to a moderate ethanol dose stimulates acceptance and consumption of the drug in infant rats, particularly in female pups. In addition to consummatory responses, prenatal ethanol exposure induced selective changes in Met-enk content in regions of the mesocorticolimbic and nigrostriatal systems, as well as in the hypothalamus and hippocampus. As in adult rats, our results suggest that changes in mesocorticolimbic enkephalin neurons in 15-day-old offspring prenatally exposed to ethanol could contribute to the reinforcing actions of the drug. The role of enkephalinergic transmission in ethanol consummatory responses in offspring, as well as the possible differences elicited by prenatal and/or postnatal manipulations in comparison with basal levels of response in naïve animals should be further investigated.

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