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Prenatal ethanol increases ethanol intake throughout adolescence, alters ethanol-mediated aversive learning, and affects μ but not δ or κ opioid receptor mRNA expression

María Carolina Fabio,[1,2] Ana Fabiola Macchione,[1] Michael E. Nizhnikov[3] and Ricardo Marcos Pautassi[1,2]
[1]Instituto de Investigación Médica M. y M. Ferreyra (INIMEC – CONICET-UNC), Friuli 2434, Córdoba, C.P. 5000, Argentina
[2]Facultad de Psicología, Universidad Nacional de Córdoba (UNC), Córdoba, Argentina
[3]Department of Psychology, Southern Connecticut State University, New Haven, CT, USA

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Abstract

Animal models of prenatal ethanol exposure (PEE) have indicated a facilitatory effect of PEE on adolescent ethanol intake, but few studies have assessed the effects of moderate PEE throughout adolescence. The mechanisms underlying this facilitatory effect remain largely unknown. In the present study, we analysed ethanol intake in male and female Wistar rats with or without PEE (2.0 g/kg, gestational days 17–20) from postnatal days 37 to 62. The results revealed greater ethanol consumption in PEE rats than in controls, which persisted throughout adolescence. By the end of testing, ethanol ingestion in PEE rats was nearly 6.0 g/kg. PEE was associated with insensitivity to ethanol-induced aversion. PEE and control rats were further analysed for levels of μ, δ and κ opioid receptor mRNA in the infralimbic cortex, nucleus accumbens shell, and ventral tegmental area. Similar levels of mRNA were observed across most areas and opioid receptors, but μ receptor mRNA in the ventral tegmental area was significantly increased by PEE. Unlike previous studies that assessed the effects of PEE on ethanol intake close to birth, or in only a few sessions during adolescence, the present study observed a facilitatory effect of PEE that lasted throughout adolescence. PEE was associated with insensitivity to the aversive effect of ethanol, and increased levels of μ opioid receptor transcripts. PEE is a prominent vulnerability factor that probably favors the engagement of adolescents in risky trajectories of ethanol use.

Introduction

Exposure to ethanol in the womb promotes problematic ethanol intake (Alati *et al.*, 2006). Animal models of prenatal ethanol exposure (PEE) usually rely on heavy ethanol exposure throughout pregnancy (Nash *et al.*, 1984) or the analysis of ethanol preference over a short period of time during infancy (Díaz-Cenzano *et al.*, 2014; Miranda-Morales *et al.*, 2014) or adulthood (Phillips & Stainbrook, 1976).

Fewer animal studies have used moderate PEE, which is more likely to mimic the human pattern of consumption. To our knowledge, no study has assessed the effects of such moderate exposure to ethanol throughout the course of adolescence in rats [postnatal day (PD)28 to PD60]. Chotro & Arias (2003) observed greater ethanol intake in adolescent rats that were exposed to 1.0 g/kg or 2.0 g/kg ethanol during gestational day (GD)17–GD20. Greater ethanol intake in adolescent rats that are exposed to ethanol prenatally was observed in another study (Fabio *et al.*, 2013), but this study had caveats: the initiation of ethanol intake required significant liquid deprivation, probably leading to stress-induced dehydration, and ethanol intake was measured in 2-h sessions for only 4 days.

The present study analysed ethanol intake after PEE throughout the course of adolescence in Wistar rats. After successfully establishing the reliability of PEE for inducing long-lasting increases in ethanol acceptance, we analysed the mechanisms that underlie this effect. In Experiment 2, we analysed μ opioid receptor (MOR), δ opioid receptor (DOR) and κ opioid receptor (KOR) mRNA with polymerase chain reaction in the prefrontal infralimbic cortex (IL), nucleus accumbens shell (AcbSh) and ventral tegmental area (VTA) – areas involved in ethanol's motivational effects – in adolescent rats that were exposed to ethanol *in utero*. Previously, Nizhnikov *et al.* (2014) reported a reduction in synaptosomal KOR expression in the nucleus accumbens, amygdala and hippocampus in infant rats after PEE. These rats also showed a blunted response to the KOR agonist U62,066E. Another study indicated greater expression of ethanol-induced conditioned place preference, which is mediated by the activation of MORs, in rats with PEE than in control counterparts (Pautassi *et al.*, 2012). On the basis of these studies, we expected to

Correspondence: Ricardo Marcos Pautassi, [1]Instituto de Investigación Médica M. y M. Ferreyra, as above.
E-mail: rpautassi@gmail.com

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observe a decrease in KOR mRNA expression and an increase in MOR mRNA expression following PEE.

Adolescents exposed to ethanol *in utero* show (Fabio *et al.* (2013) a reduction in neural activation in the IL. Alterations in this area are related to an inability to extinguish learned associations (Maroun *et al.*, 2012). We analysed adolescents that were or were not exposed to ethanol *in utero* with regard to their ability to acquire and extinguish an aversive memory (conditioned taste aversion; Experiment 3a). Extinction reflects a decrease in the expression of a conditioned response when the conditioned stimulus (CS) is repeatedly presented without the unconditioned stimulus (US). PEE may also promote ethanol intake by making subjects less sensitive to ethanol's aversive effects, which serves as a natural deterrent to the escalation of ethanol intake (Anderson *et al.*, 2010). This hypothesis was tested in Experiment 3b.

Materials and methods

Subjects

We used 175 Wistar rats, representative of 59 litters. In Experiments 1 and 2, we used 37 adolescents (19 males and 18 females) and 20 adolescents (10 males and 10 females), respectively. These rats were derived from 18 litters {nine treated with ethanol (PEE litters) and nine treated with vehicle (water) during late gestation [prenatal vehicle exposure (PV) litters]}. In Experiment 3, we used 118 male adolescents that were representative of 11 litters (PEE), 15 litters (PV), and 15 litters [untreated during gestation, i.e. prenatally untreated (PUT)]. In each experiment, we assigned no more than one male and one female from a given litter to each conditioning treatment (Zorrilla, 1997). Only males were used in Experiment 3, because of the lack of significant effects of sex in Experiments 1 and 2.

The rats were born and reared in a temperature-controlled vivarium at the Instituto de Investigaciones Médicas M. y M. Ferreyra (INIMEC-CONICET-UNC, Córdoba, Argentina). The colony was kept under a 12-h : 12-h light/dark cycle (lights on at 08:00 h). Female rats were time-mated to provide subjects for this study, and were maintained in standard maternity cages with food and water *ad libitum*. The dams remained undisturbed until the beginning of prenatal treatment on GD17. Births were examined daily, and the day of parturition was considered to be PD0. Weaning was performed on PD21. On PD28, rats from the same litter were housed in same-sex groups of four. All of the experiments complied with the Guide for the Care and Use of Laboratory Animals (National Research Council, 1996), and were approved by the Institutional Animal Care and Use Committee at INIMEC-CONICET-UNC.

Prenatal ethanol treatment (Experiments 1, 2, and 3)

PEE was the same as in a previous report (Fabio *et al.*, 2013). From GD17 to GD20, pregnant dams received one daily intragastric administration of 0.015 mL/g of a 16.8% v/v ethanol solution (vehicle, tap water; ethanol dose, 2.0 g/kg; PEE group) or a similar volume of vehicle (PV group). An additional control group that remained untreated throughout gestation (PUT group) was used in Experiment 3.

Ethanol intake assessment (Experiment 1)

From PD37 to PD62, the rats underwent a 4-week intermittent access ethanol intake protocol (three sessions per week; 18 h per session). In the first week (PD37, PD39, and PD41), the rats were exposed to two bottles. One bottle contained 5% ethanol (190-proof in tap water, v/v; Porta Hnos, Cordoba, Cordoba, Argentina) with 1% sucrose (Parker Davis, Charlotte, NC, USA), and one bottle contained 1% sucrose. In the second week (PD44, PD46, and PD48), one bottle contained 5% ethanol (mixed with 0.5% sucrose), and the other contained 0.5% sucrose. In the last 2 weeks (PD51, PD53, PD55, PD58, PD60, and PD62), tap water was the vehicle for the ethanol (5%) bottle, and the sole content of the other bottle. The ethanol testing period was limited to 12 sessions, because the aim was to analyse ethanol intake until the end of adolescence, which, in the rat, has been defined as lasting, particularly in males, until approximately PD60 (Spear, 2000). The bottles were weighed to the nearest 0.01 g before and after each session to provide measures of ethanol intake on a gram per kilogram basis, and the percentage selection of ethanol intake was calculated as (ethanol intake/overall liquid intake) $\times$ 100. The readings of the drinking bottles were corrected for leakage. Specifically, leakage was measured in a bottle of water and in a bottle of ethanol located inside an empty home cage, and these values were discounted from the readings of the drinking bottles. Before and after each intake session, the rats were pair-housed in same-sex couples with *ad libitum* access to food and water. During intake sessions, the rats were individually housed in half of their home cage, and separated from their conspecific by a divider made of Plexiglas.

The rationale for using an intermittent access procedure is that every-other-day ethanol-access tests usually result in steeper ethanol intake acquisition curves than continuous access procedures. Simms *et al.* (2008), for instance, employed an intermittent ethanol access procedure to induce high levels of intake of 20% ethanol of 20% v/v in Wistar rats, which are notoriously reluctant to accept ethanol concentrations higher than 6–10% (Ponce *et al.*, 2008).

Microdissection, total RNA isolation, and quantitative polymerase chain reaction (qPCR) assay (Experiment 2)

Male and female adolescent Wistar rats from the PEE and PV groups were killed by decapitation on PD37. The purpose was to determine opioid receptor mRNA expression after ethanol exposure *in utero*. These rats were derived from the same litters that provided subjects for Experiment 1. They were not exposed postnatally to ethanol, and did not undergo additional experimental manipulations. The brains were collected and frozen in dry ice under RNase-free conditions. The brains were immediately stored at -70 °C until relative mRNA determinations of MORs, DORs and KORs were performed by qPCR. Three coronal sections (600 μm for the IL and AcbSh; 500 μm for the VTA) were taken by the use of a special matrix with the aid of a freezing microtome (Ruginsk *et al.*, 2010). According to the Paxinos and Watson (2007) rat brain atlas, IL slices were obtained from bregma 3.72 mm to 2.76 mm, AcbSh slices were obtained from bregma 1.68 mm to 0.96 mm, and VTA slices were obtained from bregma -5.28 mm to -5.40 mm. A tissue micropunch from each slice was transferred to a tube with 250 μL of Trizol (Invitrogen, Waltham, MA, USA). Figure 1 shows the approximate anterior–posterior levels (relative to bregma) where the micropunches were performed. Two samples from the VTA (one from a PV rat and the other from a PEE rat) were lost because of procedural errors.

The homogenates were incubated for 5 min at room temperature, and 50 μL of ice-cold chloroform was added. The samples were shaken for 15 s, and centrifuged at 12 000 ***g*** for 15 min at 4 °C. The upper aqueous phase was transferred to another clean tube, and 125 μL of isopropanol was added. The samples were shaken, stored

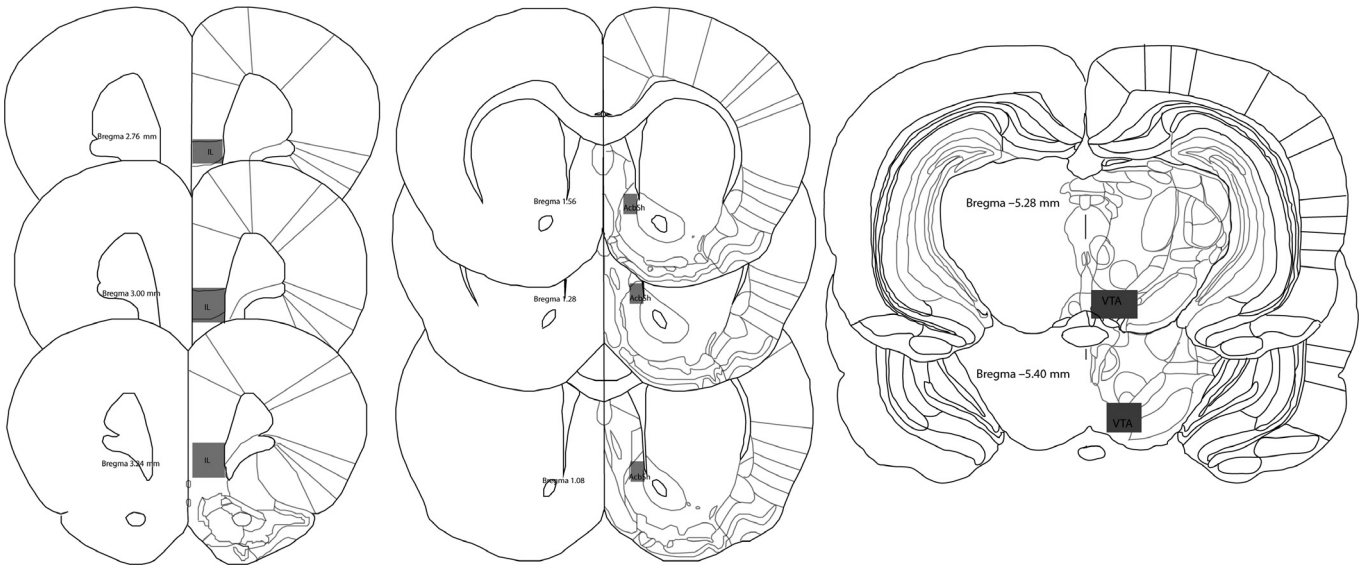

FIG. 1. Diagrams of rat brain sections adapted from the atlas of Paxinos and Watson (2007), representing the anterior–posterior levels (relative to bregma) where micropunches from select brain regions were taken. The location and size of the analysed area for each region is indicated with a gray square. Micropunches were performed bilaterally, and these tissues were used to measure opioid receptor mRNA.

overnight for RNA decantation, and then centrifuged at 12 000 ***g*** for 10 min at 4 °C. The supernatants were discarded. The pellets were washed with 75% ethanol in RNase-free water, and centrifuged at 12 000 ***g*** for 15 min at 4 °C. The supernatants were discarded, and each pellet was suspended in 15 μL of RNase-free water, and solubilised in a hybridisation oven at 60 °C for 10 min. The isolated total RNA was treated with DNase I (Ambion, Waltham, MA, USA) (1 UI) for 20 min at 37 °C, and this was followed by quantification by reading the absorbance at 260 nm and 280 nm with a NanoDrop 2000c/2000 ultraviolet–visible spectrophotometer (Thermo Scientific, Waltham, MA, USA). The integrity and quality of the total RNA extracted were verified in 1.2% agarose gel with 4 μL of ethidium bromide (10 mg/mL). The samples were run on an electrophoresis cube with a power supply (BioRad, Hercules, CA, USA) for 60 min at 90 mV and constant amperage. Bands that corresponded to 18S and 28S ribosomal subunits were visualised with a high-performance ultraviolet transilluminator.

For reverse transcription (RT), 1.5 μg of total RNA was added to a 20-μL reaction mixture containing buffer (5 × Moloney murine leukemia virus reaction buffer; Promega, Madison, WI, USA), ribonuclease inhibitor (Invitrogen; 40 UI), dNTPs (Fermentas, Buenos Aires, Argentina) (1 mM), random hexamers (Biodynamics, Buenos Aires, Argentina) (1 μg), and Moloney murine leukemia virus reverse transcriptase (100 UI; Promega). RT was performed at 37 °C for 60 min followed by 5 min at 95 °C in a Multigene thermocycler (Labnet International, Edison, NJ, USA). cDNA was stored at −20 °C until quantification by qPCR was performed. In each RNA extraction, a reaction tube without RNA was run as a negative control (RT blank).

Quantification by qPCR was performed with Power SYBR Green Master Mix (Applied Biosystems, Foster City, CA, USA), oligonucleotides (forward/reverse; 10 pmol) and cDNA (15 ng) in a final reaction volume of 20 μL in duplicate. Real-time amplification reactions were performed with a StepOne Real-Time PCR System (Applied Biosystems). Three pairs of oligonucleotides (Invitrogen) that were specific for each gene of interest were designed with PRIMER-BLAST software by the National Center for Biotechnology Information on the basis of qPCR requirements (see Table 1 for details). The amplification conditions comprised a pre-incubation phase at 95 °C for 15 min, followed by 40 cycles of cDNA amplification phases: 95 °C for 30 s for denaturation, 55 °C for 1 min for annealing, and 72 °C for 30 s for final extension. Relative quantification based on the relative expression of a target gene (MOR, DOR, and KOR) vs. a reference gene [glyceraldehyde-3-phosphate dehydrogenase (GADPH)] was employed (Pfaffl, 2001). qPCR efficiencies were calculated from the given slopes with STEPONE soft-

TABLE 1. Oligonucleotide sequences, number of base pairs, product length and efficiency of each oligonucleotide employed during the PCR conducted in Experiment 2

Target gene	Sequence	Oligonucleotide length	Product length (bp)	Efficiency (%)
GADPH	Forward: TGTGAACGGATTTGGCCGTA	20	124	101
	Reverse: ATGAAGGGGTCGTTGATGGC	20		
MU	Forward: CTGTCTGCCACCCAGTCAAA	20	150	101
	Reverse: TGCAATCTATGGACCCCTGC	20		
DELTA	Forward: TCGTCCGGTACACTAAGCTG	20	124	103
	Reverse: GGCCACGTTTCCATCAGGTA	20		
KAPPA	Forward: CCAAAGTCAGGGAAGATGTGGA	22	165	121
	Reverse: TCAAGCGCAGGATCATCAGG	20		

ware according to the following equation: $E = 10^{-1/\text{slope}}$. The investigated transcripts showed high and optimal qPCR efficiency rates, which were close to 100% in all cases. To confirm the accuracy and reproducibility of qPCR, intra-assay precision was determined in two repeats within each run. Replicas with a coefficient of variation of > 5% were repeated. Inter-assay variation was determined by running a pool sample that was generated from five male vehicle cases, which was used as an internal standard between all runs.

The ratio of a target gene (ΔΔCt) was expressed in a sample relative to a reference sample or calibrator normalised to an endogenous reference gene (*GADPH*). The pool sample was chosen as the calibrator sample. Additionally, a negative control was used in each qPCR run, made with all of the reagents with the exception of cDNA (qPCR blank). In all of the runs, a qPCR blank and an RT blank were included, always in duplicate. Ct values were always > 35 in these blanks.

LiCl-induced and ethanol-induced conditioned taste aversion (CTA) procedures (Experiments 3a and 3b)

The experiment was divided into two phases. In the first phase (Experiment 3a), we analysed extinction patterns of LiCl-induced CTA in rats in the PEE, PV and PUT groups. The hypothesis was that PEE rats would acquire aversive associative learning as compared with their PV and PUT counterparts, but that they would require more trials to extinguish it. This expectation was based on prior work indicating reduced activity in the IL after PEE (Fabio *et al.*, 2013). This area plays a key role in the extinction of associative learning. It should be noted that that the focus was on the level of performance and persistence of the aversive associative learning in rats exposed to ethanol *in utero* vs. their PV and PUT counterparts. LiCl was merely used as a tool to induce a potent aversive memory that would serve as a benchmark for assessing extinction patterns as a function of prenatal treatment.

The second phase of the experiment (Experiment 3b) began 1 week after completion of the extinction tests, and was aimed at assessing sensitivity to the aversive effects of ethanol intoxication in PEE, PUT and PV rats. In Experiment 3b, the control rats that were given only vehicle during LiCl-induced CTA were trained for ethanol-induced CTA. A sequential, within-subjects design was used, in which rats that were representative of each prenatal treatment (PEE, PV, and PUT) that served as controls in the first phase of the experiment (Experiment 3a) were conditioned in the second phase of the experiment (Experiment 3b) to the aversive effects of ethanol. Rats that were given LiCl injections in Experiment 3a were not used in Experiment 3b. To avoid pre-exposure effects, NaCl was used as the CS in Experiment 3b.

A detailed description of both phases of Experiment 3 follows. On PD31 (Experiment 3a), PEE, PV and PUT rats were given 30 min of access to a graduated glass tube that was filled with saccharin (CS, 0.1% w/v; Parker Davis, Buenos Aires, Argentina), and then immediately injected with LiCl [US, 0.0, 0.1, 0.2 and 0.3 M; volume, 1% of body weight (Sigma); vehicle, 0.9% saline]. Five extinction tests (one per day, PD38–PD42), lasting 60 min each, were then conducted. The rats had access to a tube that was filled with 0.1% saccharin in the absence of the US.

After completion of the extinction tests for LiCl-induced CTA, rats that received vehicle injections were housed in pairs for 6 days. Ethanol-induced CTA procedures (Experiment 3b) began on PD42. The rats were then given 30 min of access to a tube that was filled with NaCl (CS, 0.9% saline; Sigma), and then immediately intraperitoneally injected with ethanol (US, 0.0 g/kg or 2.5 g/kg; vehicle, 0.9% saline). A single 60-min test was conducted on PD44. The rats had access to a tube filled with NaCl. To enhance the sample size, 16 new rats were added to Experiment 3b. These rats, derived from PEE, PV and PUT dams, were also exposed to vehicle injections and given saccharin access on PD31, and 60 min of access daily to a saccharin-filled tube on PD33–PD37.

Across conditioning and extinction sessions, the rats were single-housed in a standard cage with access to 50% of their normal ingestion of water. This procedure, which does not result in weight loss and is considerably less severe than those used in Cunningham *et al.* (1992) and Pepino *et al.* (2004), was used to motivate the subjects to approach the CS. The dependent variable was milliliters of CS (saccharin or NaCl) per 100 g of body weight (mL/100 g).

Experimental design and statistical analysis

In Experiment 1, we used a 2 [prenatal treatment: 2.0 g/kg or 0.0 g/kg ethanol (vehicle); PEE and PV groups, respectively] × 2 (sex: male or female groups, respectively) factorial design, with 8–10 subjects in each group (PV male, $n = 9$; PV female, $n = 10$; PEE male, $n = 10$; PEE female, $n = 8$). The dependent variables [body weight, vehicle intake (1.0% or 0.5% sucrose or plain water, expressed as mL/100 g of body weight), overall fluid intake (mL/100 g), and ethanol intake (g/kg and percentage preference)] were examined with separate three-way mixed ANOVAS. Prenatal treatment and sex were

TABLE 2. Body weight (g) of male and female adolescent rats, as a function of prenatal treatment (daily administrations of 0.0 g/kg or 2.0 g/kg ethanol, GD17–GD20) and ethanol intake session (1–12) (values are expressed as mean ± SEM)

Intake session	PV		PEE	
	Male	Female	Male	Female
1	177.10 ± 8.04	156.10 ± 2.89	182.18 ± 4.95	144.50 ± 5.48
2	193.89 ± 10.18	168.70 ± 5.64	196.50 ± 6.32	155.13 ± 5.95
3	206.50 ± 10.60	178.70 ± 4.88	210.36 ± 8.30	160.88 ± 6.01
4	233.00 ± 11.24	192.70 ± 6.20	236.27 ± 10.95	173.63 ± 6.65
5	243.60 ± 13.79	204.40 ± 8.72	252.36 ± 11.56	178.63 ± 6.26
6	259.00 ± 15.03	214.40 ± 9.02	270.09 ± 12.70	187.75 ± 6.97
7	292.80 ± 13.70	220.40 ± 8.31	293.80 ± 16.71	196.88 ± 6.19
8	303.80 ± 14.15	229.80 ± 10.89	299.45 ± 18.16	203.88 ± 7.44
9	319.20 ± 15.67	239.50 ± 11.04	326.82 ± 17.18	209.88 ± 7.03
10	338.70 ± 15.80	247.60 ± 11.31	346.91 ± 19.42	220.13 ± 7.02
11	349.60 ± 16.97	251.80 ± 13.18	345.91 ± 20.99	222.13 ± 7.08
12	359.80 ± 17.20	256.70 ± 13.99	370.64 ± 21.35	229.13 ± 7.09

the between-group factors, and sessions (sessions 1–12) were the repeated measures.

In Experiment 2, the rats were distributed into four groups with four or five rats per group as a function of prenatal treatment (PEE or PV) and sex (male or female). The dependent variable ($\Delta\Delta$Ct) was the relative expression of the gene of interest (i.e. for MOR, DOR, and KOR) in each brain structure (IL, AcbSh, and VTA) as a function of the calibrator (normalised to *GADPH*), which was analysed with separate factorial ANOVAS (prenatal treatment × sex).

The expression of LiCl-induced (Experiment 3a) and ethanol-induced (Experiment 3b) CTA was analysed as a function of prenatal treatment [2.0 g/kg or 0.0 g/kg ethanol (vehicle) or untreated during pregnancy; PEE, PV, and PUT groups, respectively] and treatment during conditioning (Experiment 3a, saccharin intake paired with 0.0, 0.1, 0.2 or 0.3 M LiCl; Experiment 3b, NaCl intake paired with 0.0 g/kg or 2.5 g/kg ethanol). In both experiments, the rats were randomly distributed in the different conditioning groups, and CS consumption during conditioning was analysed with a one-way ANOVA, with prenatal treatment as the comparative factor. Saccharin or NaCl consumption during the 60-min tests was analysed with independent ANOVAS, with prenatal treatment and conditioning as between-groups factors and extinction session (1, 2, 3, 4, and 5) in Experiment 3a as the repeated measure. In Experiment 3a, the PUT groups that were treated with 0.0, 0.1, 0.2 and 0.3 M LiCl had 12, nine, seven and eight subjects, respectively. The PEE and PV groups were composed of eight subjects, with the exception of the PEE 0.3 M and PV 0.0 M groups, which had seven and 11 subjects, respectively. In Experiment 3b, the PUT groups that were treated with 0.0 g/kg and 2.5 g/kg ethanol had nine and 11 subjects, respectively. The PV groups that were treated with 0.0 g/kg and 2.5 g/kg ethanol had seven and eight subjects, respectively, and the PEE groups had 10 subjects each.

In Experiment 3b, NaCl consumption during the 60-min test was analysed with a three-way mixed ANOVA, with prenatal treatment and conditioning as between factors and extinction session (1, 2, 3, 4, and 5) as the repeated measure. The PUT groups that were treated with 0.0, 0.1, 0.2 and 0.3 M LiCl had 12, nine, seven and eight subjects, respectively. The PEE and PV groups were composed of eight subjects, with the exception of the PEE 0.3 M and PV 0.0 M groups, which had seven and 11 subjects, respectively.

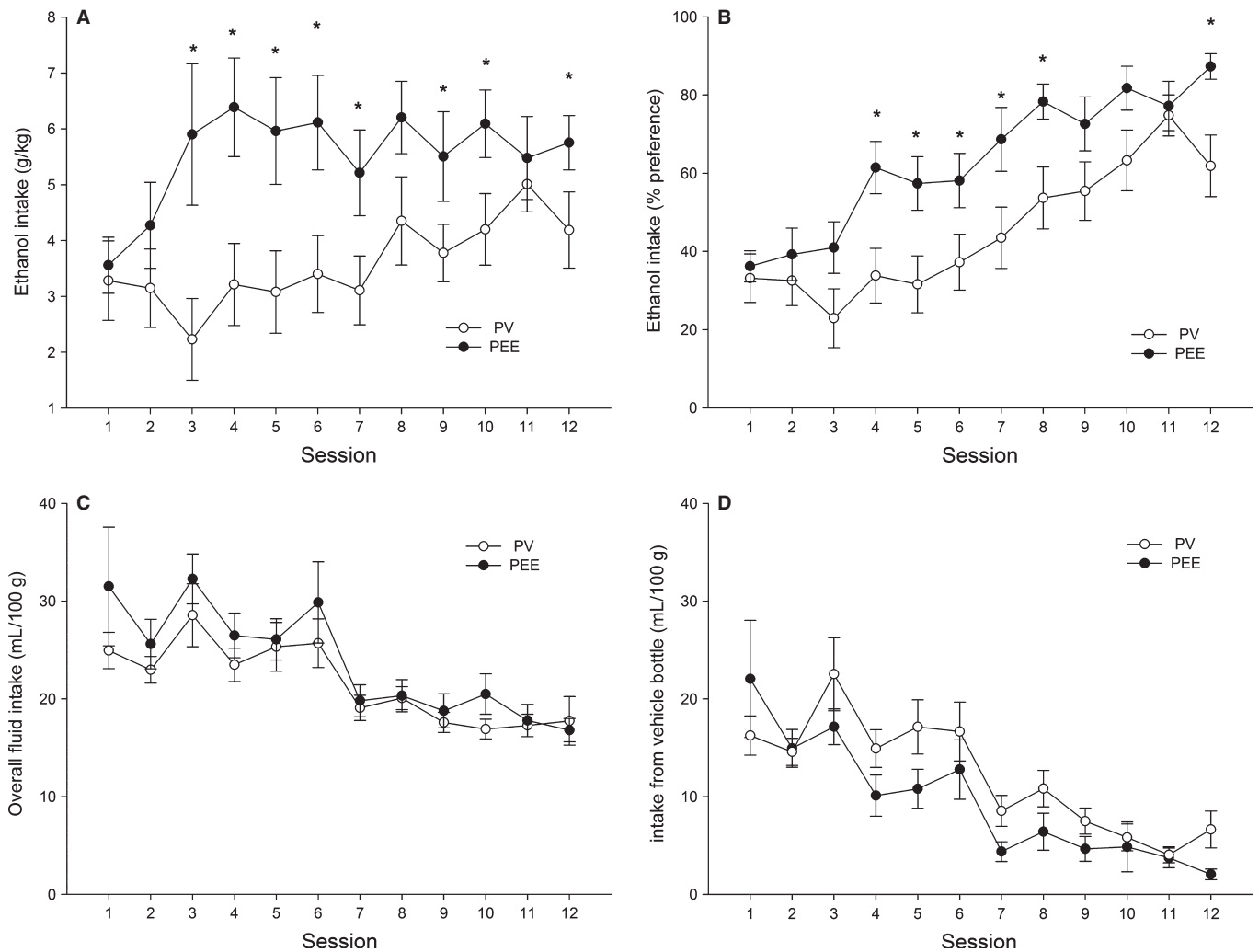

FIG. 2. Ethanol intake (g/kg and percentage preference) (A and B), overall fluid intake (mL/100 g of body weight) (C) and vehicle intake (mL/100 g of body weight) (D) in male and female adolescent rats as a function of prenatal treatment (daily administration of 0.0 g/kg or 2.0 g/kg ethanol, GD17–GD20; PV and PEE groups, respectively) and ethanol intake session (1–12). The asterisk indicates a significant difference between PEE and PV rats in a given session. For visualisation purposes, the data were collapsed across males and females. ANOVA indicated that sex did not significantly interact with prenatal treatment. The data are expressed as mean ± SEM.

Pairwise comparisons were used to analyse the loci of significant main effects and interactions ($\alpha = 0.05$). The Tukey test was used to determine significant main effects or interactions of the between factors. The significant interactions that involved between × within factors were further scrutinised by the use of orthogonal planned comparisons. This statistical approach was chosen because there is no unambiguous choice of adequate error terms for *post hoc* comparisons that involve between × within factors (Winer *et al.*, 1991). The software STATISTICA 8.0 (StatSoft, Tulsa, OK, USA) was used.

Results

Experiment 1

The repeated-measures ANOVA of body weight indicated significant main effects of sex ($F_{1,32} = 54.50$, $P = 0.00$) and day of assessment ($F_{11,352} = 239.67$, $P = 0.00$), and a significant sex × day interaction ($F_{11,352} = 34.28$, $P = 0.00$). Pairwise comparisons indicated that males had significantly greater body weight than females, and this difference significantly increased as a function of testing. Prenatal treatment did not show significant main effects or interactions. Body weights are shown in Table 2.

The ANOVAS of absolute ethanol intake (g/kg) and percentage ethanol preference indicated significant main effects of prenatal treatment (g/kg, $F_{1,33} = 6.56$, $P = 0.02$; percentage preference, $F_{1,33} = 6.03$, $P = 0.02$) and day of assessment (g/kg, $F_{11,363} = 2.83$, $P = 0.00$; percentage preference, $F_{11,363} = 23.30$, $P = 0.00$), and a significant prenatal treatment × day of assessment interaction (g/kg, $F_{11,363} = 2.11$, $P = 0.02$; percentage preference, $F_{11,363} = 1.86$, $P = 0.04$). Subsequent pairwise comparisons indicated significantly greater ethanol intake (g/kg) in the PEE group than in the PV group during sessions 3, 4, 5, 6, 7, 9, 10, and 12. Similarly, the percentage ethanol preference was greater in the PEE group than in the PV group during sessions 4, 5, 6, 7, 8, and 12.

Sex did not show a significant main effect on ethanol intake (g/kg) ($F_{1,33} = 1.289$, $P = 0.27$), and the prenatal treatment × sex ($F_{1,33} = 0.23$, $P = 0.63$), sex × day of assessment ($F_{11,363} = 1.50$, $P = 0.12$) and prenatal treatment × sex × day of assessment ($F_{11,363} = 0.77$, $P = 0.66$) interactions did not reach significance. Moreover, sex did not show a significant main effect on percentage ethanol preference ($F_{1,33} = 0.39$, $P = 0.53$), and the prenatal treatment × sex ($F_{1,33} = 0.20$, $P = 0.66$) and prenatal treatment × sex × day of assessment ($F_{11,363} = 1.50$, $P = 0.82$, $P = 0.61$) interactions did not reach significance. The ANOVA for percentage ethanol preference indicated a significant sex × day of assessment interaction ($F_{11,363} = 3.68$, $P < 0.00$). Subsequent *post hoc* comparisons indicated that percentage ethanol preference in session 2 was lower in males than in females, whereas males showed greater percentage ethanol preference during intake sessions 10 and 11. Figure 2 shows the significant prenatal treatment × day of assessment interaction in terms of grams per kilogram of body weight and percentage preference (upper panels), and vehicle and overall liquid intake (lower panels). Descriptive data [mean ± standard error of the mean (SEM)] for ethanol intake (g/kg and percentage preference) as a function of sex and session are shown in Table 3.

Vehicle intake and overall fluid intake (mL/100 g) were unaffected by prenatal treatment and sex. Both ANOVAS indicated only a significant main effect of day of assessment ($F_{11,385} = 17.51$, $P = 0.00$, and $F_{11,385} = 13.19$, $P = 0.00$, respectively). Planned comparisons indicated significantly greater vehicle intake during intake sessions 1–6 than during intake sessions 7–12. Absolute

TABLE 3. Ethanol intake (g/kg and percentage preference), vehicle intake (mL/100 g of body weight) and overall fluid intake (mL/100 g) of male and female adolescent rats, as a function of prenatal ethanol treatment (0.0 g/kg or 2.0 g/kg, GD17–GD20; PV and PEE groups, respectively) and ethanol intake session (1–12) (values are expressed as mean ± SEM)

	PV						PEE					
	Ethanol intake (g/kg)		Ethanol intake (% preference)		Vehicle intake (mL/100 g)		Overall intake (mL/100 g)		Ethanol intake (g/kg)		Ethanol intake (% preference)		Vehicle intake (mL/100 g)		Overall intake (mL/100 g)	
Intake session	Male	Femal	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female
1	2.77 ± 0.88	3.75 ± 1.11	34.02 ± 10.5	32.35 ± 7.63	23.62 ± 3.92	29.40 ± 4.76	21.26 ± 1.90	28.26 ± 2.75	3.37 ± 0.58	3.79 ± 0.91	36.46 ± 6.23	35.87 ± 4.95	46.39 ± 20.36	25.81 ± 5.44	34.78 ± 10.57	27.39 ± 4.20
2	2.10 ± 0.79	4.09 ± 1.08	24.50 ± 8.97	39.74 ± 8.87	29.27 ± 3.44	22.60 ± 2.79	21.95 ± 2.20	23.89 ± 1.76	3.41 ± 1.00	5.36 ± 1.16	28.42 ± 7.27	52.74 ± 10.66	34.67 ± 3.80	16.53 ± 3.69	26.11 ± 3.43	24.98 ± 4.01
3	1.12 ± 0.55	3.23 ± 1.25	18.19 ± 10.53	27.15 ± 11.05	34.23 ± 5.35	48.77 ± 11.26	20.75 ± 2.42	35.59 ± 4.80	6.02 ± 2.02	5.75 ± 1.47	41.02 ± 9.67	40.91 ± 9.24	35.87 ± 6.1	27.65 ± 3.54	32.43 ± 3.86	32.08 ± 3.42
4	2.02 ± 0.81	4.29 ± 1.12	28.06 ± 10.93	38.95 ± 9.20	30.96 ± 4.89	30.43 ± 5.38	19.75 ± 1.69	26.84 ± 2.46	5.31 ± 0.98	7.73 ± 1.50	55.88 ± 9.03	68.34 ± 9.67	24.91 ± 6.22	14.12 ± 3.40	25.13 ± 2.88	28.19 ± 3.80
5	2.48 ± 0.91	3.62 ± 1.16	32.45 ± 10.63	30.76 ± 10.55	29.23 ± 4.41	44.05 ± 9.29	19.58 ± 1.72	30.50 ± 3.87	4.94 ± 0.94	7.24 ± 1.78	56.81 ± 7.52	58.07 ± 12.86	25.38 ± 5.61	19.26 ± 5.10	23.44 ± 2.63	29.39 ± 3.22
6	2.68 ± 0.75	4.06 ± 1.12	38.32 ± 10.27	36.24 ± 10.46	27.82 ± 4.79	45.89 ± 10.09	18.97 ± 2.13	31.75 ± 3.35	6.73 ± 0.94	5.34 ± 1.21	65.61 ± 9.78	48.73 ± 9.35	24.59 ± 8.9	29.23 ± 6.89	29.83 ± 6.63	29.91 ± 4.86
7	2.46 ± 0.68	3.70 ± 1.00	43.49 ± 11.70	43.47 ± 11.06	25.11 ± 5.78	28.56 ± 6.62	15.68 ± 1.16	22.14 ± 1.74	5.76 ± 0.77	4.53 ± 1.47	83.35 ± 7.39	50.28 ± 13.81	10.45 ± 5.85	19.69 ± 5.61	18.04 ± 1.63	22.02 ± 3.03
8	3.85 ± 1.11	4.81 ± 1.15	54.05 ± 11.40	53.34 ± 11.54	17.93 ± 6.19	23.45 ± 5.94	17.53 ± 1.70	22.37 ± 1.33	5.81 ± 0.64	6.70 ± 1.26	84.03 ± 3.62	71.18 ± 8.71	8.91 ± 2.72	14.26 ± 4.15	17.66 ± 1.60	23.66 ± 2.79
9	3.57 ± 0.56	3.97 ± 0.87	64.86 ± 9.52	46.91 ± 11.12	16.26 ± 4.86	23.14 ± 4.64	15.21 ± 1.19	19.72 ± 1.35	5.90 ± 0.96	5.01 ± 1.41	83.72 ± 4.6	58.66 ± 13.29	9.18 ± 3.26	15.00 ± 4.68	17.71 ± 2.24	20.09 ± 2.82
10	4.23 ± 0.59	4.17 ± 1.13	74.80 ± 9.19	52.90 ± 11.71	11.80 ± 4.22	20.00 ± 5.29	14.94 ± 0.63	18.69 ± 1.68	5.75 ± 0.47	6.52 ± 1.27	89.13 ± 2.47	72.51 ± 11.81	6.28 ± 1.50	20.52 ± 13.56	16.49 ± 1.12	25.48 ± 3.88
11	4.74 ± 0.27	5.26 ± 0.93	82.29 ± 2.66	68.02 ± 9.37	9.62 ± 1.88	13.53 ± 3.72	15.72 ± 1.16	18.68 ± 1.87	5.58 ± 0.84	5.36 ± 1.37	83.91 ± 5.39	68.80 ± 12.39	9.78 ± 4.00	11.44 ± 4.43	16.97 ± 2.10	18.82 ± 2.76
12	2.93 ± 0.75	5.32 ± 1.01	56.90 ± 13.96	66.34 ± 8.76	19.54 ± 6.23	18.37 ± 6.98	14.05 ± 1.04	21.07 ± 4.46	5.14 ± 0.6	6.51 ± 0.75	91.48 ± 3.14	82.06 ± 5.97	3.99 ± 1.39	7.95 ± 2.56	14.18 ± 1.33	20.08 ± 1.47

intake of ethanol (i.e. mL) during the 12 sessions was analysed as a function of prenatal treatment, sex, and day of assessment. The ANOVA and subsequent planned comparisons indicated more milliliters of ethanol ingested by males than by females in intake sessions 9, 10, and 11 (significant sex × day interaction; $F_{11,308} = 2.50$, $P = 0.01$), and more milliliters of ethanol ingested by PEE rats than by their PV counterparts in intake sessions 3, 4, 5, 6, 7, 10, and 12 (significant prenatal treatment × day interaction; $F_{11,308} = 2.39$, $P = 0.01$).

Experiment 2

The ANOVAS of the relative mRNA expression (ΔΔCt) of MOR, DOR and KOR in the IL and AcbSh indicated the absence of significant main effects of prenatal treatment and sex. The interaction between these factors also lacked significance across these brain areas and type of receptor. Similarly, the ANOVAS of the ΔΔCt of DOR and KOR in the VTA indicated no significant main effects or sex × prenatal treatment interactions. There seemed to be higher DOR mRNA expression in the VTA in PEE males, but the prenatal treatment × sex interaction was not significant. PEE rats showed significantly greater relative MOR mRNA expression (ΔΔCt) in the VTA ($F_{1,16} = 6.86$, $P = 0.02$). These results are shown in Fig. 3. The ANOVAS for each brain area showed that the levels of the housekeeping gene mRNA were not altered by prenatal treatment or sex.

Experiment 3

The ANOVAS indicated similar saccharin and NaCl consumption across prenatal treatments during conditioning. The amounts consumed by PUT, PV and PEE rats were 6.35 ± 0.36 mL, 5.32 ± 0.42 mL and 6.27 ± 0.43 mL in Experiment 3a, and 5.05 ± 0.60 mL, 4.16 ± 0.49 mL and 4.19 ± 0.34 mL in Experiment 3b.

Experiment 3a

The ANOVA for the extinction tests indicated significant main effects of LiCl dose ($F_{3,90} = 6.68$, $P = 0.00$) and extinction session ($F_{4,360} = 18.06$, $P = 0.00$), and a significant LiCl dose × extinction session interaction ($F_{12,360} = 4.90$, $P = 0.00$). The prenatal treatment × extinction session interaction was also significant ($F_{8,360} = 2.31$, $P = 0.02$). Planned comparisons indicated that PEE rats drank more of the fluid than their control counterparts, regardless

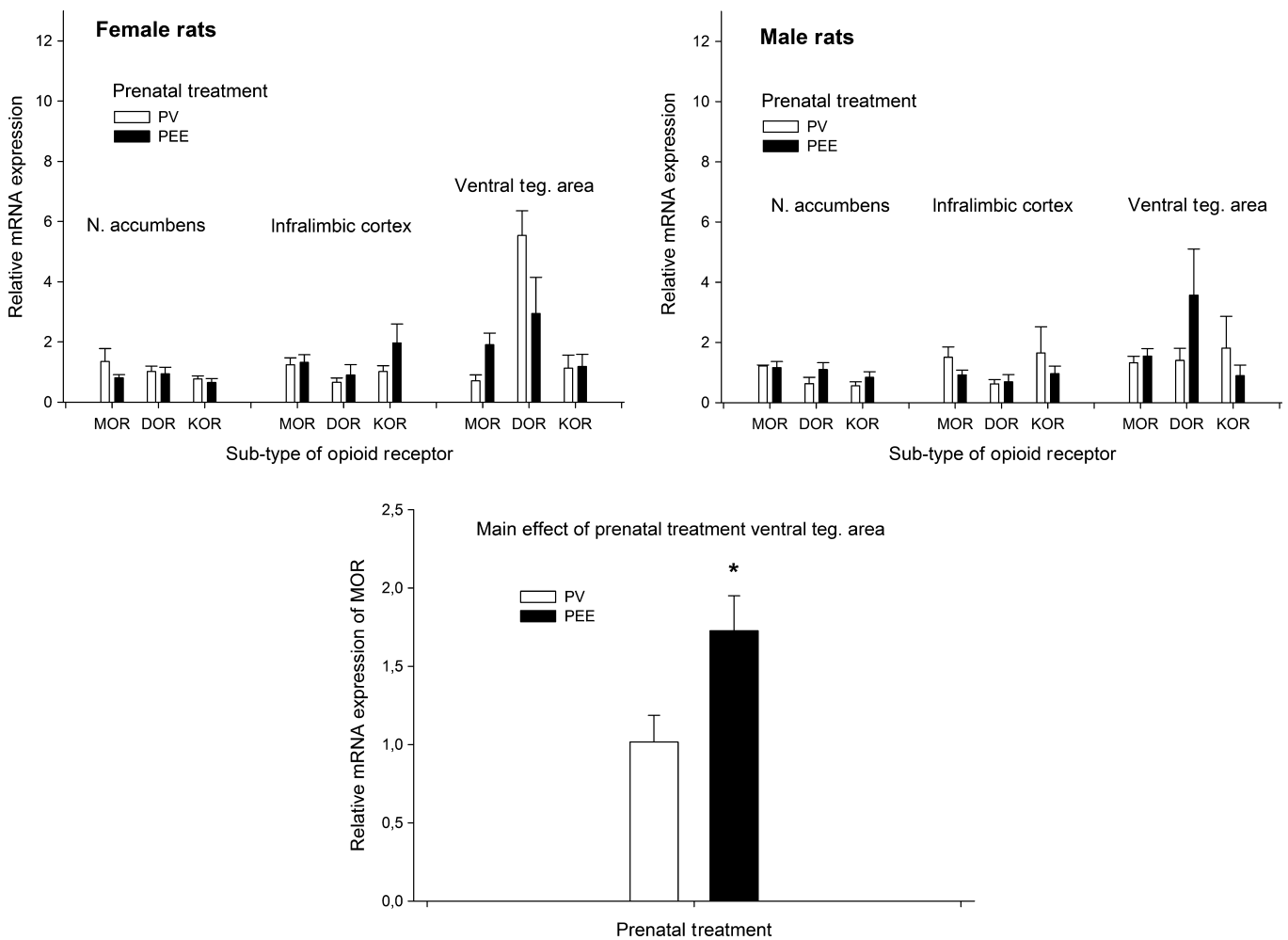

FIG. 3. Relative mRNA expression (ΔΔCt) of opioid receptors. The upper panels depict relative mRNA expression between the genes that encode MOR, DOR, and KOR and the housekeeping gene (*GADPH*) in adolescent male and female rats as a function of prenatal treatment (daily administration of 0.0 g/kg or 2.0 g/kg ethanol, GD17–GD20; PV and PEE groups, respectively) and site of measurement (AcbSh, IL, and VTA). The lower panel depicts the significant main effect of prenatal treatment on MOR mRNA expression in the VTA. The asterisk indicates greater MOR mRNA expression in PEE rats than in PV rats. The data are expressed as mean ± SEM.

of LiCl treatment, particularly during the first two extinction tests. Planned comparisons also indicated that rats given pairings of 0.1, 0.2 or 0.3 M LiCl and saccharin showed less CS consumption than rats given only vehicle (i.e. 0.0 M) during extinction sessions 1, 2, and 3. These differences, which are indicative of LiCl-induced CTA, were no longer evident during the subsequent testing days. Moreover, during the first testing day, rats given 0.3 M LiCl showed significantly less saccharin intake than their counterparts given 0.1 M LiCl.

The magnitude of LiCl-induced CTA was statistically similar across the different prenatal treatment conditions. Figure 4 shows saccharin intake during extinction sessions as a function of LiCl and prenatal treatment condition.

Experiment 3b

The ANOVA for NaCl consumption during testing (mL/100 g) indicated a significant main effect of ethanol dose during conditioning ($F_{1,49} = 8.36$, $P = 0.00$) and a significant prenatal treatment × ethanol dose during conditioning interaction ($F_{2,49} = 3.20$, $P = 0.04$). *Post hoc* comparisons indicated significantly lower NaCl intake by rats exposed to vehicle or untreated during gestation (PV and PUT prenatal groups, respectively) after pairings of this CS and ethanol administration than by controls given NaCl–vehicle pairings. This significant difference, which is indicative of ethanol-induced CTA, was not observed in PEE rats, which showed similar NaCl ingestion during testing, regardless of pairing of this CS with the pharmacological consequences of ethanol. These results are shown in Fig. 5.

Discussion

The present study showed that moderate PEE induced a significant facilitatory effect on ethanol intake that lasted throughout most of adolescence. During the course of most of the testing, PEE rats ingested nearly 6.0 g/kg ethanol per day and showed 60–80% ethanol preference, which was twice as much as controls. Intriguingly, ethanol intake in these genetically heterogeneous adolescent Wistar rats was similar to the ethanol intake shown by adolescent rats that were genetically selected for high ethanol consumption through

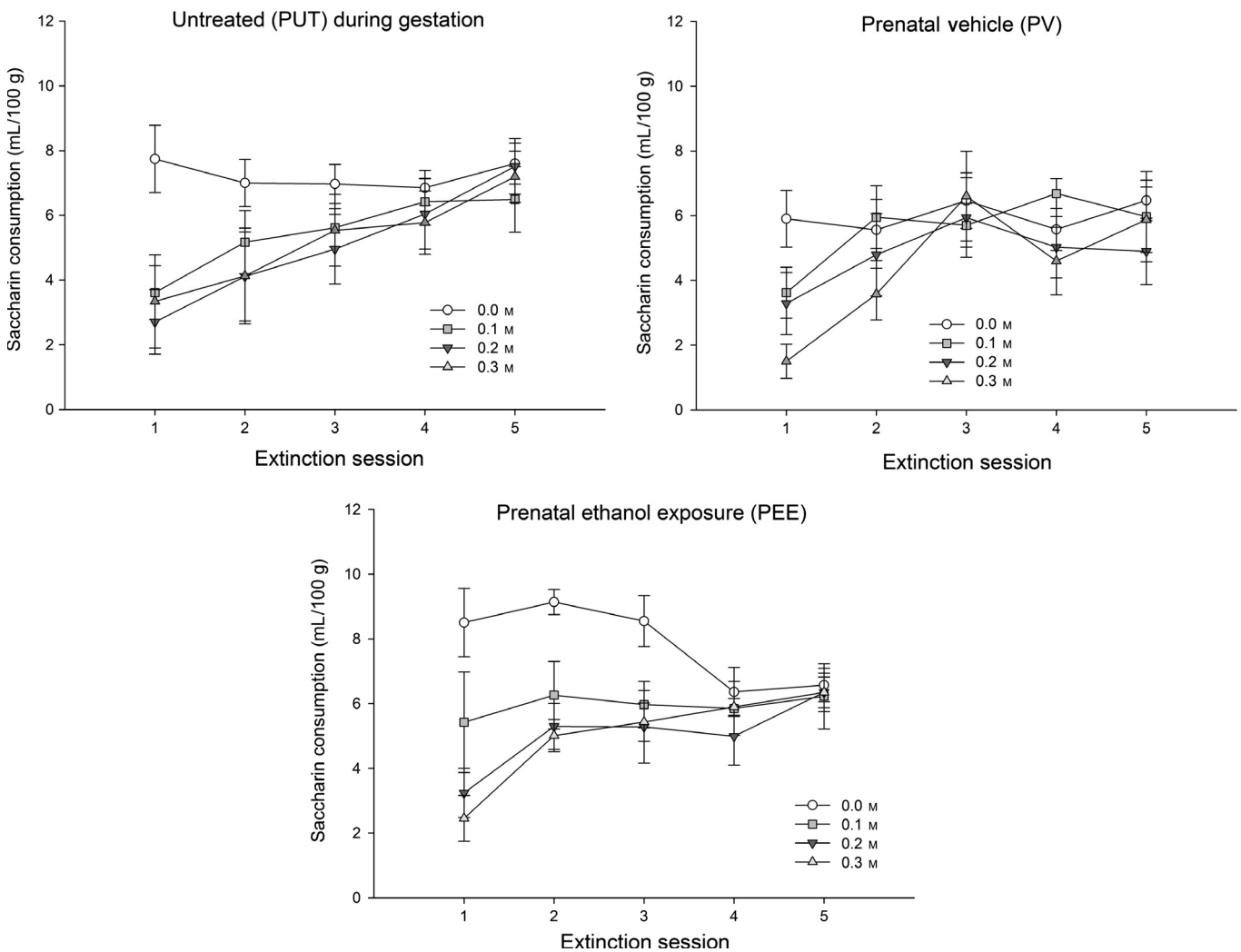

FIG. 4. Saccharin consumption (mL/100 g) in adolescent male rats as a function of conditioning treatment (saccharin intake paired with intraperitoneal administration of 0.0, 0.1, 0.2 and 0.3 M LiCl), prenatal treatment (untreated during gestation or daily administration of 0.0 g/kg or 2.0 g/kg ethanol on GD17–GD20; PUT, PV, and PEE groups, respectively), and extinction session (five sessions of 60 min each). The statistical analysis revealed a significant difference between LiCl-treated groups (0.1, 0.2 and 0.3 M) and the 0.0 M control group in extinction sessions 1, 2, and 3. This difference, which is indicative of LiCl-induced conditioned aversion, was similar in the PUT, PV and PEE groups. See the text for a detailed account of the results. The data are expressed as mean ± SEM.

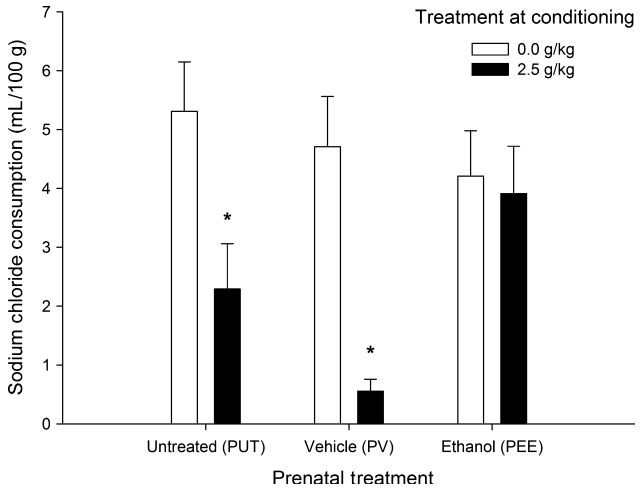

FIG. 5. NaCl consumption (mL/100 g) in adolescent male rats as a function of conditioning treatment (NaCl paired with intraperitoneal administration of ethanol, 0.0 g/kg or 2.5 g/kg) and prenatal treatment (daily administration of 0.0 g/kg or 2.0 g/kg ethanol on GD17–GD20 or untreated during gestation; PV, PEE, and PUT groups, respectively). The asterisk indicates a significant difference between a group treated with ethanol during conditioning and the corresponding control group with the same prenatal treatment. The data are expressed as mean ± SEM.

cross-breeding over several generations. A previous study (Bell *et al.*, 2006) assessed ethanol intake in ethanol-preferring adolescent and adult rats for 4 weeks, beginning on PD30. Adolescents had higher overall ethanol intake than adults, and showed average ethanol consumption levels between 5.5 and 7.5 g/kg/day (Bell *et al.*, 2006), which are similar to those shown by PEE adolescent rats in the present study.

The intermittent access protocol induced a gradual and steady increase in ethanol acceptance, even in controls, which reached ~4.0 g/kg in their last intake sessions. It could be argued that the effect of PEE on voluntary ethanol intake was transient. Unlike controls, which gradually increase their ethanol intake, the PEE group reached peak intake levels in session 3, and their intake did not significantly change for the rest of the study. This, however, may be attributable to a ceiling effect in terms of ethanol intake, rather than disappearance of the facilitative effect of prenatal ethanol. Moreover, PEE rats continued to show significantly greater ethanol intake than controls during the last phase of testing (i.e. sessions 7, 9, and 10), and during the final test (i.e. session 12) PEE rats drank ~2 g/kg and consumed ~25% more ethanol than control counterparts.

The difference in ethanol intake between the PEE and PV groups was more evident in the early sessions, when ethanol was mixed with sucrose. This raises the question of whether PPE rats are more prone to drink sucrose or any other palatable solution without ethanol. Dominguez *et al.* (1998) employed our PEE protocol (i.e. 2.0 g/kg, GD17–GD20), and measured postnatal intake of different tastants, including ethanol and a configuration (sucrose + quinine) that mimics the psychophysical properties of ethanol. They found that water intake, sucrose intake and quinine intake were not affected by the prenatal condition of the subjects. However, PEE increased both ethanol and sucrose + quinine intake as compared with vehicle controls. Moreover, in Experiment 3 of the present study, the intake of saccharin or NaCl during conditioning was similar in the PEE, PV and PUT groups. The implications are that *in utero* ethanol exposure selectively affects postnatal acceptance of ethanol or solutions that share certain sensory characteristics with this drug.

Wistar rats have been notorious for their reluctance to accept ethanol. A previous study utilising this strain (Fabio *et al.*, 2014) imposed substantial liquid deprivation as a means to induce ethanol acceptance. The present intake protocol removed the confounding effect of dehydration-induced stress, and allowed repeated measurements of ethanol intake during adolescence in the absence of extensive social deprivation. As already noted, one limitation was the slightly sweetened water that was used as the vehicle during the first 2 weeks. The concentrations of sucrose, however, were much lower than those of the widely used sucrose-fading technique that is used in adult subjects (Loos *et al.*, 2013), and the differences between the PEE and PV groups persisted after fading was complete. Moreover, ingestion from the vehicle bottle and overall fluid intake were unaffected by PEE.

Several studies have indicated that the endogenous opioid system probably mediates ethanol-induced reinforcement and the effects of PEE. Administration of MOR antagonists during gestation inhibits the preference for ethanol's odor (Youngentob *et al.*, 2012) and the enhancement of ethanol intake and palatability (Arias & Chotro, 2005) that is observed after PEE. Moreover, a recent study reported enhanced ethanol intake in preweanling rats that were exposed to 1.0 g/kg ethanol on GD17–GD20, which was associated with altered behavioral reactivity to KOR antagonists and reduced synaptosomal KOR expression in the nucleus accumbens, amygdala, and hippocampus (Nizhnikov *et al.*, 2014). On the basis of these studies, our expectation was a reduction in relative KOR mRNA expression and perhaps greater MOR mRNA expression in PEE adolescents. This expectation was only partially met. Similar levels of KOR and DOR mRNA expression were found in the IL, AcbSh, and VTA. This suggests that mechanisms other than alterations in the KOR system may contribute to the greater propensity to ingest ethanol during adolescence after PEE. Prenatal ethanol may also triggers a cascade of events whereby transient alterations in KORs during early ontogeny alter other neurobehavioral domains or transmitter systems later in life.

Experiment 2 also indicated that PEE enhanced relative MOR mRNA expression in the VTA. This result is consistent with studies reporting that prenatal MOR blockade inhibited the augmenting effect of PEE on subsequent ethanol intake and preference (Chotro & Arias, 2003; Diaz-Cenzano & Chotro, 2010). Moreover, rats that are selectively bred for high ethanol intake show a higher density of MORs in the limbic system than rats that are selected for low ethanol preference (de Waele *et al.*, 1995). Acute postnatal ethanol administration (2.5 g/kg, single dose) upregulated MOR levels in the prefrontal cortex (Mendez *et al.*, 2001). Another study reported higher levels of MOR binding in ethanol-dependent subjects than in control subjects (Weerts *et al.*, 2011), and ethanol self-administration was significantly lower in MOR knockout mice than in wild-type controls (Becker *et al.*, 2002).

PEE adolescents have been shown to have lower neural activation in the IL (Fabio *et al.*, 2013). This brain area is involved in the extinction of associative learning. Specifically, functional impairment of the IL makes animals more resistant to extinction (Millan *et al.*, 2011; Burgos-Robles *et al.*, 2013). A potential deficit in extinction processes after PEE is relevant, because previous studies (Abate *et al.*, 2001) have indicated that PEE induces associative learning in the fetus. The pairing between the odor of ethanol and the rewarding effects of ethanol endows the odor of the drug with positive incentive value, and postnatal exposure to the taste or odor of ethanol may trigger ethanol seeking and consumption (Spear & Molina, 2005). This association serves as a vulnerability factor for problematic ethanol consumption, but its impact should be amenable

to extinction. Alterations in the function of the IL may further increase the magnitude of ethanol-seeking behavior after PEE, because the appetitive memory of ethanol's odor and taste that is acquired *in utero* would be resistant to extinction. As a first step towards analysing this hypothesis, Experiment 3a assessed extinction processes in PEE rats trained in a CTA task. The focus of this experiment was not whether or not LiCl would able to induce an aversion, but instead the persistence of the aversive memory across prenatal treatments. The use of LiCl was intended to allow the induction of an associative memory and the analysis of extinction patterns of that memory as a function of prenatal treatment. The adolescents showed significant but transient CTA, which was extinguished after three exposures to the CS in the absence of the US. Our hypothesis, however, was not corroborated. The pattern of extinction of the LiCl-mediated conditioned aversion was similar in PEE, PV and PUT adolescents.

Ethanol also has potent aversive effects that support the acquisition of aversive associative learning. Once an ethanol-mediated aversion is encoded, retarded extinction (as observed in PEE rats in Experiment 3a) could work as a protective factor against excessive ethanol consumption. Within this framework, persistence of the aversive memory would deter animals from subsequent ethanol seeking. The results of Experiment 3b, however, refuted this possibility, and indicated that PEE animals may be at greater risk for excessive ethanol consumption because of their blunted response to the aversive effects of ethanol. PEE appeared to induce tolerance to the malaise-inducing effects of this drug, or perhaps resulted in a US pre-exposure effect, similar to the one found in adolescent rats with prior exposure to 3.0 g/kg ethanol (Acevedo *et al.*, 2013). The effect occurs when the acquisition of a conditioned response is blocked after prior experience with the US (Revillo *et al.*, 2013).

In summary, PEE during late gestation increased later ethanol intake. Unlike previous studies that assessed this effect close to birth (Nizhnikov *et al.*, 2014), in only a few sessions during adolescence (Chotro & Arias, 2003), or under conditions of stress (Fabio *et al.*, 2013), the present study observed a facilitatory effect of PEE throughout adolescence that resulted in absolute levels of ethanol intake that were similar to those observed in rats that are genetically selected for ethanol preference. This study also explored several possible mechanisms that may underlie the augmenting effect of PEE on ethanol intake. PEE was associated with insensitivity to the aversive effects of ethanol, and alterations in MOR mRNA expression in the VTA. Adolescence is a developmental stage during which ethanol consumption usually begins and escalates. These results indicate that PEE is a prominent vulnerability factor that probably favors the engagement of subjects in risky trajectories of ethanol abuse.

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Abbreviations

ΔΔCt, ratio of a target gene; AcbSh, nucleus accumbens shell; CS, conditioned stimulus; CTA, conditioned taste aversion; DOR, δ opioid receptor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GD, gestational day; IL, prefrontal infralimbic cortex; KOR, κ opioid receptor; MOR, μ opioid receptor; PD, postnatal day; PEE, prenatal ethanol exposure; PUT, prenatally untreated; PV, prenatal vehicle exposure; qPCR, quantitative polymerase chain reaction; RT, reverse transcription; SEM, standard error of the mean; US, unconditioned stimulus; VTA, ventral tegmental area.

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