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Prenatal ethanol induces an anxiety phenotype and alters expression of dynorphin & nociceptin/orphanin FQ genes

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ABSTRACT

Animal models have suggested that prenatal ethanol exposure (PEE) alters the κ opioid receptor system. The present study investigated the brain expression of dynorphin and nociceptin/orphanin FQ related genes and assessed anxiety-like behavior in the light-dark box (LDB), shelter-seeking and risk-taking behaviors in the concentric square field (CSF) test, and ethanol-induced locomotion in the open field (OF), in infant or adolescent Wistar rats that were exposed to PEE (0.0 or 2.0 g/kg, intragastrically, gestational days 17–20). We measured brain mRNA levels of prodynorphin (PDYN), κ opioid receptors (KOR), the nociceptin/orphanin FQ opioid peptide precursor prepronociceptin (ppN/OFQ) and nociceptine/orphanin FQ receptors (NOR). Prenatal ethanol exposure upregulated PDYN and KOR mRNA levels in the ventral tegmental area (VTA) in infant and adolescent rats and KOR mRNA levels in the prefrontal cortex in infant rats. The changes in gene expression in the VTA were accompanied by a reduction of DNA methylation at the PDYN gene promoter, and by a reduction of DNA methylation at the KOR gene promoter. The PEE-induced upregulation of PDYN/KOR in the VTA was accompanied by lower NOR gene expression in the VTA, and lower PDYN gene expression in the nucleus accumbens. PEE rats exhibited hypolocomotion in the OF, greater avoidance of the white and brightly lit areas in the LDB and CSF, and greater preference for the sheltered area in the CSF test. These results suggest that PEE upregulates the dynorphin system, resulting in an anxiety-prone phenotype and triggering compensatory responses in the nociceptin/orphanin FQ system. These findings may help elucidate the mechanisms that underlie the effects of PEE and suggest that the dynorphin and nociceptin/orphanin FQ systems may be possible targets for the prevention and treatment of PEE-induced alterations.

1. Introduction

Prenatal ethanol exposure (PEE) is surprisingly prevalent in Western society. A recent study (Lopez et al. 2017) indicated that 75% of pregnant women consume alcohol at least once during gestation. Prenatal ethanol exposure is associated with alcohol-related birth defects and epidemiological studies (Baer et al. 2003a) have shown that even sporadic gestational ethanol exposure can promote alcohol use disorders (AUDs) in offspring.

The reasons why PEE facilitates subsequent AUD are still unclear, but animal models have suggested that PEE alters the function of neurotransmitter systems that are responsible for ethanol's effects. Ethanol induces appetitive and aversive effects, and the net combination of these motivational effects modulates ethanol seeking and intake. Ethanol's appetitive effects are mediated by the μ-opioid receptor-dependent activation of dopaminergic neurons in the ventral tegmental area (VTA) that project to the nucleus accumbens (NAc) and ultimately to the prefrontal cortex (PFC).

Unlike the μ-opioid receptor system, the κ-opioid receptor (KOR) system mediates the acute aversive effects of ethanol and other treatments (including stress exposure) and functions as a figurative brake, reducing the acute reinforcing effects of ethanol. Presynaptic KOR activation in the VTA inhibits dopamine release in the NAc and PFC (Sirohi et al. 2012), via their actions on dopamine terminals (Karkhanis et al. 2017). Rat lines that were selectively bred for high ethanol intake exhibited lower levels of dynorphin (DYN) than their control

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counterparts (Nylander et al. 1994) and the blockade of KOR promotes ethanol-induced conditioned place preference (Pautassi et al. 2012a). Dynorphin is the endogenous ligand of KOR, and is derived from a preprotein that is encoded by the prodynorphin (PDYN) gene. Polymorphisms of PDYN are associated with AUD (Karpyak et al. 2013).

The KOR system undergoes a developmental change during ontogeny (from mediating appetitive motivational effects to mediating aversive motivational effects) during the 1st and 2nd postnatal weeks (Petrov et al. 2006; Varlinskaya et al. 1996). PEE may alter the developmental trajectories of KOR systems that are located in the mesocorticolimbic pathway, resulting in greater ethanol-induced activity and reward and ultimately heightened ethanol intake. This hypothesis fits well with previous studies. After moderate PEE, we observed a decrease in the expression of synaptosomal KOR in the NAc, amygdala, and hippocampus (Nizhnikov et al. 2014) and an increase in ethanol-induced dopaminergic activity in the VTA (Fabio et al. 2015b). In the NAc, KORs are positioned presynaptically on dopaminergic terminals and inhibit the release of dopamine (Karkhanis et al. 2016). Nizhnikov et al. (2014) reported conditioned place aversion that was induced by KOR agonism in control animals but not in animals that were exposed to ethanol in utero. The latter group exhibited no conditioned place aversion after pairings of a texture and administration of a KOR agonist.

Exposure to ethanol (particularly chronic exposure) upregulates KOR expression, resulting in anhedonia, dysphoria, and greater sensitivity to stress (Walker and Koob 2008). This negative affective state promotes relapse or the continuation of ethanol intake through negative reinforcement mechanisms. Upregulation of the KOR system may be involved in promoting the effect of PEE on subsequent ethanol intake. These findings prompted preclinical studies that assessed the potential beneficial effects of KOR antagonism in ethanol-dependent or -experienced rodents. These studies mostly indicated that KOR antagonism had minimal effects on ethanol intake in nondependent and nonstressed subjects but significantly decreased ethanol intake in ethanol-dependent or high-ethanol-consuming animals (Walker and Koob 2008; Walker et al. 2011) or after stress exposure (Wille-Bille et al. 2017).

Some of the effects of KOR system activation, in addition to μ- and δ-opioid receptor agonism, are blocked by the nociceptin/orphanin FQ system [hereinafter referred to as N/OFQ when describing the ligand, ppN/OFQ when describing the nociceptin opioid peptide precursor prepronociceptin, and NOR when describing the receptor (Chen et al. 2007)]. There is a high degree of homology between these two systems. Nociceptin/orphanin FQ has high structural homology to DYN-A, and the NOR sequence is closely related to the KOR. However, they appear to have opposite physiological functions in certain physiological paradigms (Reinscheid et al. 1998). Our previous work found selective alterations of gene regulation within the PDYN/KOR and the N/OFQ/NOR systems after postnatal ethanol exposure (D'Addario et al. 2013). We also found that the activation of NOR reduced ethanol's anxiolytic and locomotor-activating effects in infant and adolescent, genetically heterogeneous, rats (Miranda-Morales et al. 2013), but it did not reduce ethanol-induced aversion or ethanol intake (Miranda-Morales and Pautassi 2015). Other studies, however, suggested that NOR may play role in limiting ethanol intake. For instance, a study reported decreased ethanol intake after NOR activation, in rats selectively bred for high ethanol intake (Economidou et al. 2006; Rorick-Kehn et al. 2016).

The present study investigated alterations of the expression of genes that are related to the DYN and N/OFQ systems in the brain, in infant and adolescent rats that were exposed to PEE (2.0 g/kg, i.g.) during late gestation (gestational day [GD] 17–20). Specifically, we assessed the messenger RNA (mRNA) levels of PDYN, KOR, ppN/OFQ, and NOR receptors. We also explored the concomitant behavioral effects of PEE. We analyzed baseline and ethanol-induced motor activity in an open field, anxiety-like behavior in the light-dark box and shelter-seeking and risk-taking behaviors in a modified version of the concentric square field test. The changes in gene expression after PEE may involve epigenetic mechanisms producing long-term changes in both gene expression and behavior. Thus, we also explored DNA methylation patterns – the most stable epigenetic mechanism – at the PDYN gene promoter in the VTA and NAc and KOR gene promoter in PFC and VTA in PEE- and vehicle-exposed rats. DNA methylation consists of the transfer of a methyl group to position 5 of the pyrimidine ring in a cytosine-guanine dinucleotide (CpG), that, at the gene promoter level, ultimately blocks the binding of transcription factors and causes chromatin compaction and gene silencing (Klose and Bird 2006; Pidsley and Mill 2011).

The rationale for assessing these effects during infancy and adolescence was that ethanol drinking is initiated and often escalates during late infancy and adolescence. A previous study indicated that nearly 60% of Argentinean children aged 8–12 sipped or tasted ethanol (Pilatti et al. 2013), with similar findings in the United States (Donovan and Molina 2014). According to a recent study (Pilatti et al. 2017), nearly 80% of Argentinean college students had their first contact with ethanol by age 16; as freshmen, they drank approximately seven drinks on a typical drinking occasion. An early age of ethanol drinking predicts the development of AUD and is significantly influenced by ethanol exposure during gestation (Baer et al. 2003b).

A strength of the present study is the focus on moderate rather than heavy levels of ethanol exposure. More often than not, the animal models of PEE have employed intense ethanol exposure, for instance by exposing dams to 10% ethanol as the sole drinking fluid from gestational day 0 to postnatal day (PD) 7 (Contreras et al. 2017). These studies are valuable to understand consequences of heavy and protracted ethanol exposure in the womb, yet they fell short in terms of modeling the trajectories of alcohol consumption, found in most pregnant women (Lopez et al. 2017).

2. Material and methods

2.1. Experimental design, subjects and prenatal ethanol administration procedures

A 2 (prenatal treatment: ethanol [PEE] vs. prenatal vehicle [PV]) × 2 (age: infancy vs. adolescence) × 2 (sex: male vs. female) factorial design was employed (n = 8/group) in Experiment 1. A 2 (prenatal treatment: PEE vs. PV) × 2 (sex: male vs. female) × 2 (ethanol treatment before OF test: 0.0 or 2.0 g/kg) factorial was employed in Experiment 2 (6–9 animals in each group; please note that data from 2 animals of the male PEE-vehicle group were lost due to technical errors). Sixty-four rats were used (32 males [16 PEE] and 32 females [16 PEE] in Experiment 1, and 59 (27 males [12 PEE] and 32 females [15 PEE]) in Experiment 2. The animals, outbred Wistar rats, were born and reared at Instituto M. M. Ferreyra (Córdoba, Argentina) and were derived from 10 dams treated with ethanol during gestation and from 10 dams treated prenatally with vehicle. The time-mated pregnant dams were administered ethanol from GD17 to GD20, as described previously (Fabio et al. 2015b). Briefly, the dams were given one daily i.g. administration of 0.015 ml/g of a 16.8% *v*/v ethanol solution (vehicle: tap water; ethanol dose: 2.0 g/kg) or a similar volume of vehicle (i.e., tap water; PV group). No more than one male and one female from a given litter were assigned to each group.

All of the procedures were certified by the Institutional Animal Care and Use Committee at INIMEC-CONICET-UNC, complied with the Declaration of Helsinki, the ARRIVE guidelines, and the Guide for the Care and Use of Laboratory Animals as promulgated by the NIH and the EU. Across procedures, experimental blinding was achieved by assigning each animal a code. Group assignment was randomly done using those codes and data sheets and code sheets were kept separately.

2.2. Light-dark box and concentric square field test procedures (Experiment 1)

The male and female rats that were derived from PEE and PV litters were assessed for anxiety-like behavior in the light-dark box on PD14 (infancy) and anxiety-like behavior in the light-dark box and exploratory activity in the concentric square field test (Berardo et al. 2016) on PD30 and PD31 (adolescence; counterbalanced: half of the adolescent rats were tested in the concentric square field test on PD30 and in the light-dark box on PD31, whereas the others underwent the opposite sequence of testing). There was no adaptation to the concentric square field in preweanling animals; therefore, the infants could not be tested in the concentric square field test.

The light-dark box apparatus consisted of a white compartment (24.5 cm × 25 cm × 25 cm, 400 lx illumination) and a black compartment (17.5 cm × 25 cm × 25 cm, 0 lx illumination) that were separated by a divider with an opening at floor level. The test lasted 5 min and commenced when the animal was placed in one corner of the white compartment, facing away from the black compartment. The following variables were measured: latency (in seconds) to first exit the white compartment, time spent (in seconds) in the white compartment, number of transfers between compartments and frequency of peering out (i.e., while in the dark compartment, the rat stretches the upper body and introduces its head into the bright area). Also, we virtually divided the white and black compartments into 9 and 6 quadrants, respectively and registered the frequency of transfers between these quadrants. The total number of transfers between the quadrants was considered an index of overall locomotor activity.

The concentric square field apparatus [48 cm × 48 cm; description in (Berardo et al. 2016)] had a central open field square (serving as the starting area) that was connected to other areas by several passages (P). One of these areas was a dark, enclosed area that normally evokes shelter-seeking behavior (SHEL), whereas the other areas evoke exploration, risk assessment, and risk taking. A challenge (CHA) area is inaccessible by regular horizontal locomotion and can only be accessed by performing a risk-taking behavior (i.e., jump through an elevated hole). The front section of the maze had a brightly lit runway, separated from the outside by transparent plastic, and a ramp (RAMP; 12 cm × 10 cm, 20° incline) that was connected to an elevated bridge (BRIDGE; 30 cm × 10 cm) that was made from wire mesh. The test lasted 20 min. The lighting conditions were the following: SHEL (0 lx), P and CHA (20–30 lx), and RAMP and BRIDGE (600–650 lx). The time spent in and frequency of entries into each area were recorded by a trained experimenter using JWatcher 0.9. Unlike other tests that measure anxiety-like and risk-taking behavior, the concentric square field test is more naturalistic and allows a concurrent and graded set of exploratory activities, instead of imposing a single or binary behavioral option (Karlsson and Roman 2016).

2.3. Microdissection, RNA isolation, and quantitative polymerase chain reaction (Experiment 1)

One day after the behavioral tests, 32 rats (four males and four females from each prenatal treatment and age group, randomly selected) were sacrificed by decapitation. The brains were immediately collected under RNase-free conditions, frozen in dry ice, and transferred to a − 70°C freezer until procedures performed to measure the relative levels of PDYN, KOR, ppN/OFQ, and NOR. The brains were placed on an ice-cold plate, and brain regions were dissected through the coronal plane. The slices began at 3.20 mm anterior to bregma (PFC), 1.68 mm anterior to bregma (NAc), and − 5.28 mm anterior to bregma (VTA; Fig. 1). Total RNA was extracted using TRIzol Reagent (Thermo Scientific, Waltham, MA, USA), according to the manufacturer's instructions. The integrity of RNA was confirmed by electrophoresis using 1% agarose gel and the concentration was measured spectrophotometrically. One µg of total RNA was converted to cDNA using the

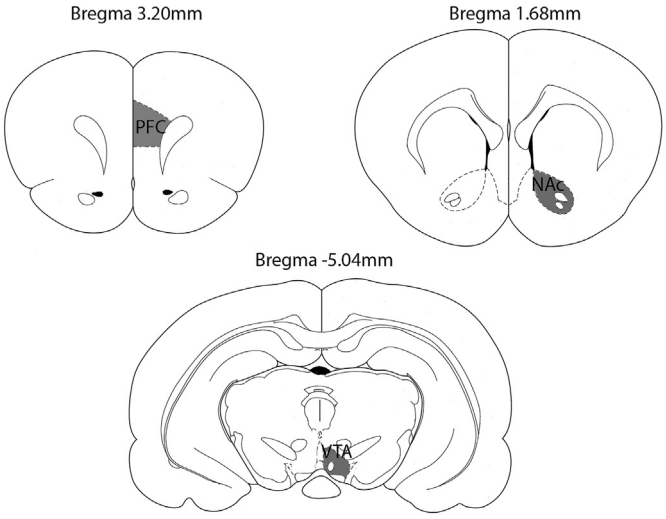

Fig. 1. Diagrams of rat brain sections, representing the anterior–posterior levels (relative to bregma) where the select brain regions were taken. The location of the analyzed area for each region is indicated by the shaded area. These tissues were used to measure opioid mRNA levels.

RevertAid H Minus First Strand cDNA Synthesis kit (Thermo Scientific, Waltham, MA, USA) in presence of random hexamer and oligo $(dT)_{18}$ primers. The relative abundance of mRNA of the genes of interest was measured by real-time quantitative polymerase chain reaction (RT-qPCR), using SensiMix SYBR Low-ROX Kit (Bioline Reagents, London, UK), on a DNA Engine Opticon 2 Continuous Fluorescence Detection System (MJ Research, Waltham, MA, USA). The relative amount of each mRNA species was defined as the threshold cycle number (Ct), in which the fluorescence was above background levels in the early log phase of product accumulation in the PCR. The β-actin and glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) genes were used to normalize the gene expression of the target genes using the ΔΔCt method and converted to the relative expression ratio ($2^{-\Delta\Delta Ct}$) for the statistical analysis. The primers used for PCR amplification are reported in Table 1.

2.4. Analysis of DNA methylation by methylation-specific primer real-time polymerase chain reaction (Experiment 1)

Genomic DNA was extracted from PFC, VTA and NAc samples using TRI Reagent solution (Thermo Scientific). These samples were representative of the 32 rats (four males and four females from each prenatal treatment and age group) in which we measured the relative levels of PDYN, KOR, ppN/OFQ, and NOR. Methylation of PDYN was measured in the VTA and NAc, of infants only; whereas methylation of KOR was measured in the PFC and VTA, of infants and adolescents. The rationale for measuring only certain brain areas in infants or

Table 1
Primers employed during the quantitative polymerase chain reaction.

Gene		Sense 5′ - 3′
GAPDH	Sense	AGACAGCCGCATCTTCTTGT
	Antisense	CTTGCCGTGGGTAGAGTCAT
β-Actin	Sense	AGATCAAGATCATTGCTCCTCCT
	Antisense	ACGCAGCTCAGTAACAGTCC
PDYN	Sense	CCTGTCCTTGTGTTCCCTGT
	Antisense	AGAGGCAGTCAGGGTGAGAA
KOR	Sense	AGCTCTTGGTTCCCCAACTG
	Antisense	CACCACAGAGTAGACAGCGG
ppN/OFQ	Sense	TGCAGCACCTGAAGAGAATG
	Antisense	CAACTTCCGGGCTGACTTC
NOR	Sense	AGCTTCTGAAGAGGCTGTGT
	Antisense	GACCTCCCAGTATGGAGCAG

Table 2
Primers employed during the analysis of DNA methylation by methylation-specific primer real-time polymerase chain reaction.

Gene	Primer (5′ – 3′)	Sequence*	Genomic location (Rnor_6.0)
PDYN	Fwd: atgtggttatttggtgagtatttg Rev-biot: acaaacctactataacattacttttctaaa Seq: gttatttggtgagtatttggta	ctgtagcattgcttttctgaggatacggtgaaacaagcggcaatgtctgtgacg	crh 3: 122208312–122,208,370
KOR	Fwd: gagttttttttagttttggaaggtataag Rev-biot: aaaaccccaaactcacct Seq: agttttggaaggtataagtt	acatcagggacgtggacccatcgaggctgaacagctaccccggagccgaagtgGTGACCTGGAAAA	crh 5:13760292–13,760,358

* Bold text = CpG sites analyzed; Lowercase letters = 5′ upstream sequence; Uppercase letters = untranslated region (exon 1).

adolescents obeyed to the significant differences found the in gene expression assays. Generally speaking, PEE infants exhibited the greatest differences in PDYN gene expression at VTA and NAc, whereas PDYN gene expression was not affected by PEE at PFC. On the other hand, significant differences in KOR gene expression were observed at VTA and PFC, in adolescents and infants, respectively.

Methylation status of promoter regions was determined using pyrosequencing of bisulfite converted DNA. Details of pyrosequencing assays used, including primers and sequences are provided in Table 2. After DNA extraction, 0.5 μg of DNA from each sample was treated with bisulfite, using a DNA methylation kit (Zymo Research, Orange, CA, USA). Bisulfite-treated DNA was amplified by PyroMark PCR Kit (Qiagen) in accordance with the manufacturer's protocol. PCR conditions were as follows: 95 °C for 15 min, followed by 45 cycles of 94 °C for 30 s, 56 °C for 30 s, 72 °C for 30s and, finally, 72 °C for 10 min. Methylation analysis was conducted using the PyroMark Q24 (Qiagen). The level of methylation was analyzed using PyroMark Q24 software (Qiagen), which calculates the methylation percentage for each CpG site, allowing quantitative comparisons (Pucci et al. 2016). Three and five cytosine-guanine dinucleotide (CpG) sites were considered, within the PDYN and KOR gene promoter, respectively.

2.5. Measurement of baseline and ethanol-induced motor activity in the OF (Experiment 2)

Motor activity was assessed at PD30 (adolescence), following procedures routinely used in our lab (Miranda-Morales and Pautassi 2015). Briefly, the animals were given 0.0 or 2.5 g/kg ethanol and, five minutes later, they were placed into dimly-lit (60 cm × 60 cm × 60 cm, ~50 lx) Plexiglas chambers. The floor of the chambers had photocell beams that created a matrix of cells. Beam breaks were recorded and sent for further processing to an activity monitoring system (ITCOMM, Córdoba, Argentina) that provided a measure of horizontal distance travelled (cm).

2.6. Data analysis

Analysis of variance (ANOVA) indicated mostly no significant main effects of sex or significant interactions that involved sex across the variables of interest. Females exhibited a longer latency to enter the light compartment of the light-dark box, but this effect occurred independently of age and prenatal treatment. Therefore, descriptive and inferential analyses were performed by collapsing the data across sex.

The dependent variables in the concentric square field test and light-dark box were analyzed using independent *t*-tests or separate factorial ANOVAs (prenatal treatment × age). Similar ANOVAs were used to analyze the relative expression ($2^{-\Delta\Delta Ct}$) of the genes of interest (i.e., PDYN, KOR, ppN/OFQ and NOR) in each brain region (VTA, NAc, and PFC) as a function of the calibrator (i.e., normalised to β-actin and GADPH). Distance travelled (cm) in the open field was analyzed via a repeated measure ANOVA [comparative factors between groups: prenatal treatment and ethanol treatment before OF; within-factor: minute of evaluation (1–15)]. Thirty-seven data points that were randomly distributed across brain regions, genes, and conditions could not be calculated because of technical problems. Similarly, two data points of distance travelled were lost due to equipment malfunction. These data were not replaced. DNA methylation at the PDYN promoter in PEE and PV samples of infant rats was analyzed using the two-tailed Student's *t*-test, whereas DNA methylation at the KOR promoter was analyzed using factorial ANOVAs (prenatal treatment × age). These analyses were conducted individually for each CpG site.

The locus of significant main effects and significant interactions in the ANOVAs was analyzed using Tukey's post hoc test or planned comparisons. The partial eta squared (η^2p) is presented as a measure of the effect size in the ANOVAs, and the α level was set at $p \leq .05$. Descriptive data are presented as means ± SEM.

3. Results

3.1. Light-dark box (Fig. 2) and concentric square field test (Table 3)

Prenatal treatment affected the latency to exit the white compartment in infant rats but not in adolescent rats (Fig. 2A). The ANOVA revealed significant main effects of age and prenatal treatment ($F_{1,49} = 130.15$, $p < 0.001$, $\eta^2p = 0.73$, and $F_{1,49} = 11.42$, $p < 0.005$, $\eta^2p = 0.19$, respectively) and a significant age × prenatal treatment interaction ($F_{1,49} = 9.63$, $p < 0.005$, $\eta^2p = 0.16$). The post hoc tests revealed a significantly shorter latency to escape from the white compartment in PEE infants than in PV infants. It should be noted that adolescent rats, regardless of the prenatal treatment, exited the white compartment in approximately 4 s. This raises the possibility that, in adolescents, the lack of a significant effect of PEE on the latency to exit the white compartment resulted from a floor effect.

The time spent in the white compartment (Fig. 2B) was significantly less in PEE rats than in PV rats, regardless of their age (significant main effect of prenatal treatment: $F_{1,49} = 16.66$, $p < 0.001$, $\eta^2p = 0.25$) and significantly less in adolescent rats than in infant rats (significant main effect of age: $F_{1,49} = 28.61$, $p < .001$, $\eta^2p = 0.37$; to avoid clutter in the figure this effect is not indicated in Fig.2B via asterisks or other signs). The ANOVA of the frequency of transfers between compartments revealed significant main effects of age and prenatal treatment ($F_{1,49} = 13.\ 45$, $p < 0.001$, $\eta^2p = 0.22$, and $F_{1,49} = 7.41$, $p < 0.01$, $\eta^2p = 0.13$, respectively) and a significant age × prenatal treatment interaction ($F_{1,49} = 4.\ 69$, $p < .05$, $\eta^2p = 0.09$). The ANOVA of the frequency of transfers between quadrants revealed a significant main effect of age ($F_{1,49} = 10.\ 66$, $p < .005$, $\eta^2p = 0.18$) and a significant age × prenatal treatment interaction ($F_{1,49} = 4.\ 80$, $p < .05$, $\eta^2p = 0.09$). The frequency of transfers between compartments (Fig. 2C) and the frequency of transfers between quadrants (Fig. 2D) were similar in PEE and PV infants but significantly lower in PEE adolescents than in PV adolescents. Peering out was significantly higher in adolescent rats than in infant rats but similar across prenatal treatments (data not shown).

The analysis of the behavioral data in the concentric square field test revealed that PEE rats exhibited a significant, two-fold, increase in the time spent in the dark and enclosed SHEL area compared with PV controls ($t_{14} = 2.64$, $p < 0.05$) and significantly fewer entries into the brightly lit RAMP area ($t_{14} = -2.06$, $p < 0.05$). The time spent in and

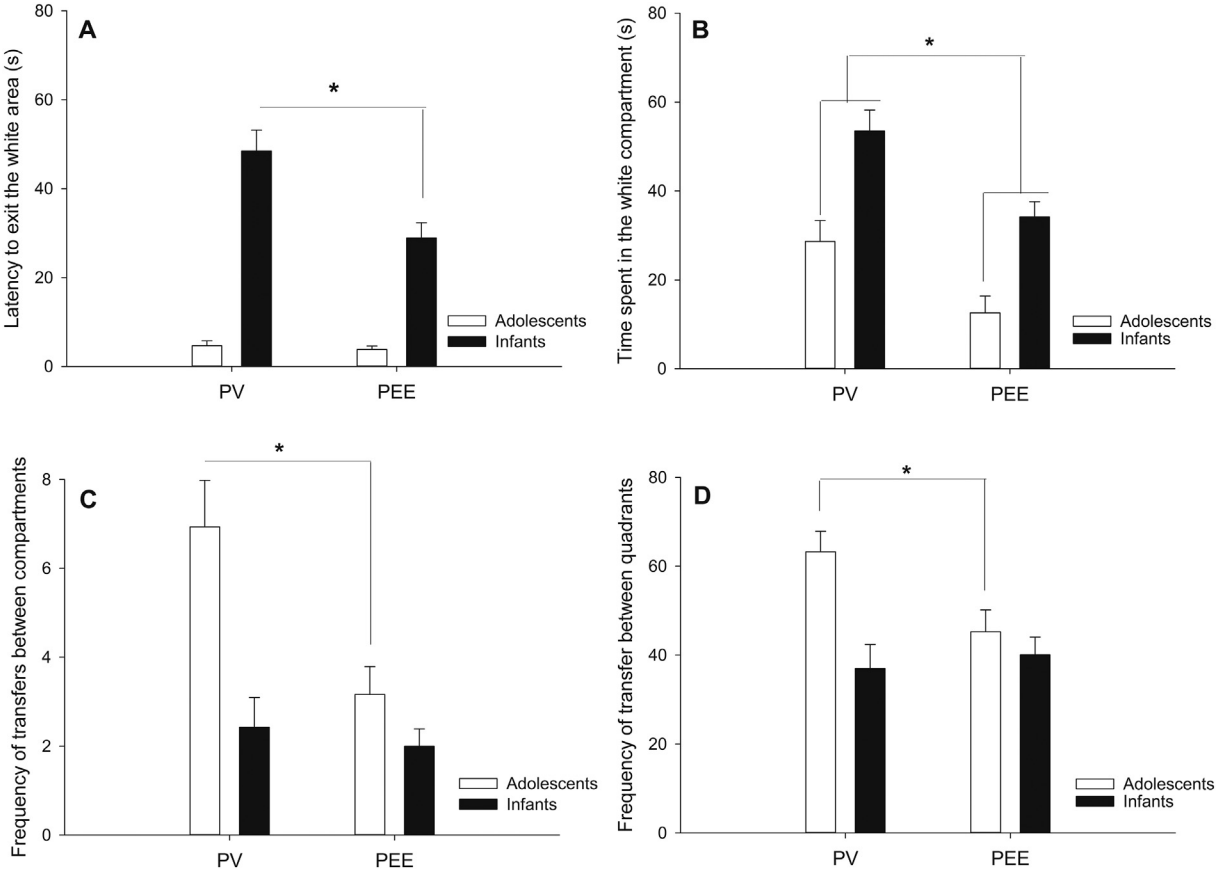

Fig. 2. Prenatal ethanol exposure increases anxiety responses in a light-dark box (LDB) test. Latency to exit and Time spent in the white area (s, Panels A and B, respectively); and frequency of transfers between compartments or quadrants (Panels C and D, respectively), in male and female infant and adolescent rats as a function of prenatal treatment (daily administration of 0.0 g/kg or 2.0 g/kg ethanol, gestational days 17–20; PV and PEE groups, respectively). For visualization purposes, the data were collapsed across males and females. ANOVA indicated that sex did not significantly interact with prenatal treatment. All the variables were analyzed via factorial ANOVAs (prenatal treatment × age), and the locus of significant main effects and significant interactions in the ANOVAs was analyzed using Tukey's post hoc test. The asterisk (*) sign in panel A indicates a significantly shorter latency to escape from the white compartment in PEE infants than in PV infants ($p < .001$). The asterisk sign in panel B indicates that time spent in the white compartment was significantly less in PEE rats than in PV rats, regardless of their age ($p < .001$). The asterisk sign in panels C and D indicates that the frequency of transfers between compartments and the frequency of transfers between quadrants were significantly lower in PEE adolescents than in PV adolescents ($p < 0.05$). The data are expressed as mean mean ± SEM.

frequency of entries into the passages that connected the different areas, and the total number of entries into all areas (i.e., a measure of overall locomotor activity [OLA]) were significantly less in PEE rats than in PV controls ($t_{14} = -3.16$, $p < 0.005$, $t_{14} = 2.61$, $p < 0.05$, and $t_{14} = -2.54$, $p < 0.05$, respectively).

3.2. Gene expression scores

3.2.1. PDYN/KOR systems (Figs. 3 and 4, respectively)

In the VTA, the gene expression study revealed a significant increase in the levels of PDYN (Fig. 3A) in infant PEE rats but not in adolescent

Table 3
Behavioral activity in the concentric square field apparatus.

	Time spent in each compartment (s)			Frequency of entries		
	PV	PEE	α	PV	PEE	α
OF	264.61 ± 23.38	305.20 ± 17.06	n.s.	42.29 ± 2.75	36.36 ± 1.97	n.s.
P	526.30 ± 23.51	411.55 ± 27.65	≤**0.005**	64.00 ± 3.72	51.79 ± 2.84	≤**0.05**
SHEL	143.66 ± 20.20	290.92 ± 52.09	≤**0.05**	11.50 ± 1.59	10.86 ± 0.70	n.s.
CHA	34.16 ± 22.50	1.71 ± 1.71	n.s.	1.14 ± 0.70	0.07 ± 0.07	n.s.
RAMP	169.35 ± 39.00	130.44 ± 28.11	n.s.	13.50 ± 1.49	9.07 ± 1.55	≤**0.05**
BRIDGE	61.08 ± 21.37	57.40 ± 18.88	n.s.	3.86 ± 1.27	2.36 ± 0.64	n.s.
OLA	–	–		136.29 ± 8.31	110.50 ± 5.80	≤**0.05**

Time spent and frequency of entries in each section of the concentric square field maze [OF = open field, P = passages, SHEL = a dark, enclosed area that evokes shelter-seeking behavior, CHA = an area that can only be accessed by performing a risk-taking behavior, and RAMP = an illuminated and inclined surface that leads to the BRIDGE (an elevated structure made of a wire mesh)], in male and female Wistar rats tested at postnatal day 30 or 31. OLA indicates the overall level of activity (i.e., total number of entries to all maze sections). The data are expressed as mean ± SEM and shown as a function of prenatal treatment (daily administration of 0.0 g/kg or 2.0 g/kg ethanol. Gestational days 17–20; PV and PEE groups. respectively). The alpha (α) column indicates the *p* value associated with a T student test conducted between PV and PEE groups, for each variable (n.s.: non-significant, bold numbers indicate a significant difference between PV and PEE groups, in a given variable). Refer to the text for a full account of statistical analysis. For visualization purposes, the data were collapsed across males and females.

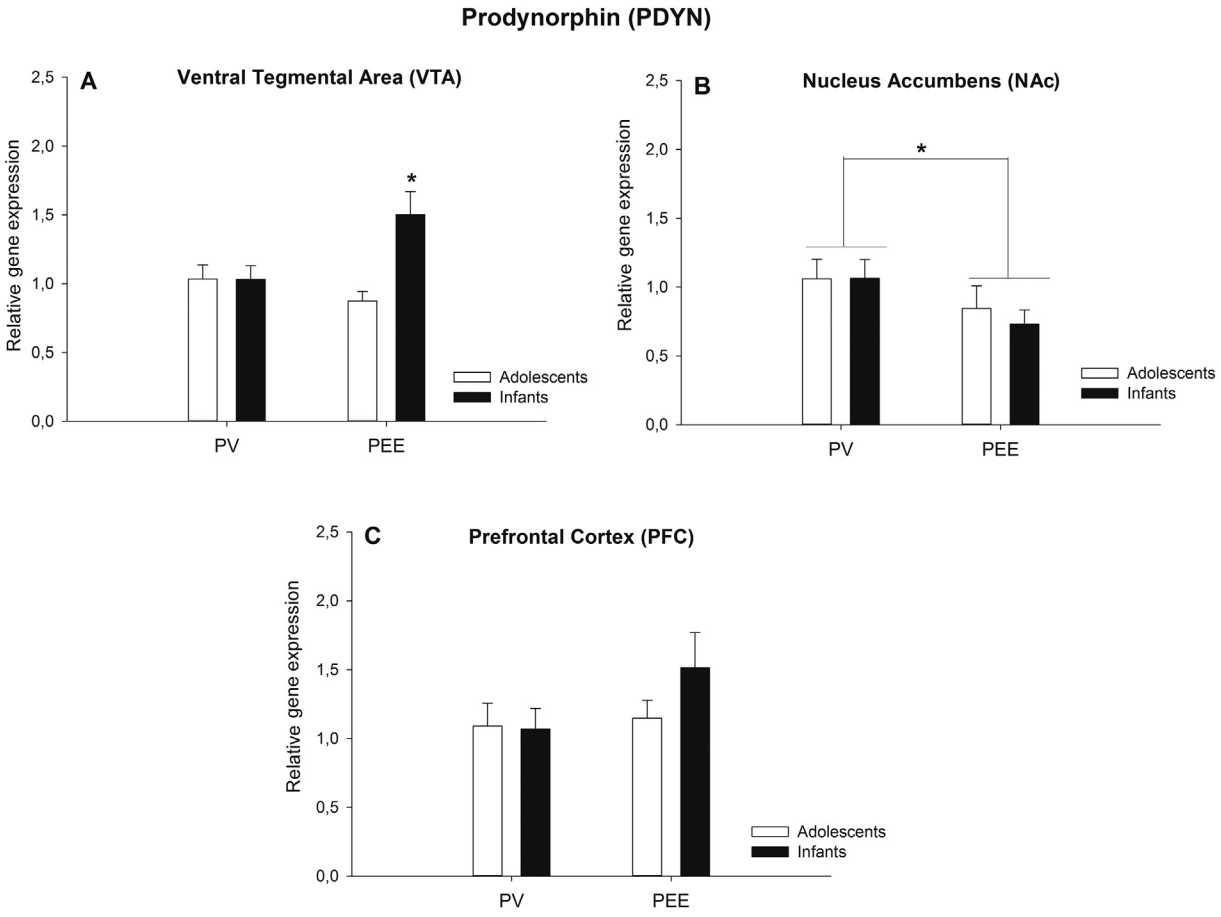

Fig. 3. Effects of prenatal ethanol exposure on mRNA levels of prodynorphin (PDYN) in the ventral tegmental area (VTA, Panel A), nucleus accumbens (NAc, Panel B) and prefrontal cortex (PFC, Panel C), in male and female infant and adolescent rats as a function of prenatal treatment (daily administration of 0.0 g/kg or 2.0 g/kg ethanol, gestational days 17–20; PV and PEE groups, respectively). For visualization purposes, the data were collapsed across males and females. An ANOVA indicated that sex did not significantly interact with prenatal treatment. All the mRNA measures were analyzed via factorial ANOVAs (prenatal treatment × age), and the locus of significant main effects and significant interactions in the ANOVAs was analyzed using Tukey's post hoc test. The asterisk (*) sign in panel A indicates that infant PEE rats had higher levels of PDYN mRNA at VTA than any of the other groups ($p < 0.05$). The asterisk sign in panel B indicates that the levels of the PDYN mRNA transcript in the NAc were significantly lower in PEE than in PV rats ($p < 0.05$), regardless age. The data are expressed as relative expression ($2^{-\Delta\Delta Ct}$) of the gene of interest as a function of the calibrator (i.e., normalised to β-Actin and GADPH), and described as mean ± SEM.

PEE rats (significant main effect of age: $F_{1,27} = 6.90$, $p < .05$, $\eta^2p = 0.20$; significant age × prenatal treatment interaction: $F_{1,49} = 6.98$, $p < 0.05$, $\eta^2p = 0.21$, respectively). The post hoc tests confirmed that infant PEE rats had higher levels of PDYN mRNA than any of the other groups. The levels of the PDYN mRNA transcript in the NAc (Fig. 3B) significantly decreased after PEE in both infants and adolescents (main effect of prenatal treatment: $F_{1,25} = 4.75$, $p < 0.05$, $\eta^2p = 0.16$), whereas the PDYN mRNA transcript in the PFC was not significantly affected by age or PEE (Fig. 3C).

The ANOVA of KOR mRNA transcript levels in the VTA (Fig. 4A) indicated a significant main effect of prenatal treatment ($F_{1,25} = 4.43$, $p < 0.05$, $\eta^2p = 0.15$), in which PEE resulted in higher relative levels compared with PV. Visual inspection of the data in Fig. 4A suggested that this facilitatory effect was greater in adolescents than in infants, and the age × prenatal treatment interaction revealed a trend toward an interaction ($F_{1,25} = 2.80$, $p < 0.10$, $\eta^2p = 0.10$). Therefore, we conducted planned comparisons between PEE and PV rats, one for each age. These analyses indicated higher levels of KOR mRNA in the VTA in PEE adolescents compared with PV adolescents ($F_{1,25} = 6.92$, $p < 0.05$) but not in PEE infants compared with PV infants. KOR mRNA levels in the NAc (Fig. 4B) were unaffected by prenatal treatment or age, whereas KOR mRNA transcript levels in the PFC (Fig. 4C) were significantly higher in PEE infants than in any of the other groups. The ANOVA of KOR mRNA transcript levels in the PFC revealed significant main effects of age and prenatal treatment ($F_{1,27} = 5.62$, $p < 0.05$, $\eta^2p = 0.17$, and $F_{1,27} = 15.07$, $p < 0.001$, $\eta^2p = 0.36$, respectively) and a significant age × prenatal treatment interaction ($F_{1,27} = 5.35$, $p < 0.05$, $\eta^2p = 0.17$).

3.2.2. ppN/OFQ and NOR systems (Figs. 5 and 6, respectively)

In the NAc (Fig. 5B), ppN/OFQ mRNA transcript levels were significantly higher in PEE rats than in PV rats ($F_{1,25} = 2.80$, $p < 0.10$, $\eta^2p = 0.10$), and this effect was similar in both age groups. The ANOVA indicated that ppN/OFQ levels in the VTA (Fig. 5A) and PFC (Fig. 5C) were unaffected by prenatal treatment or age, with no interaction between these factors.

As shown in Fig. 6A, prenatal ethanol exposure significantly decreased NOR mRNA levels in the VTA ($F_{1,25} = 12.04$, $p < .005$, $\eta^2p = 0.33$) and tended to increase NOR mRNA levels in the PFC (Fig. 6C). In the PFC, the main effect of prenatal treatment only approached significance ($F_{1,27} = 3.27$, $p < 0.08$, $\eta^2p = 0.11$). These effects were similar in infant and adolescent rats. NOR mRNA levels in the NAc (Fig. 6B) were unaffected by age or prenatal treatment, with no interaction between factors.

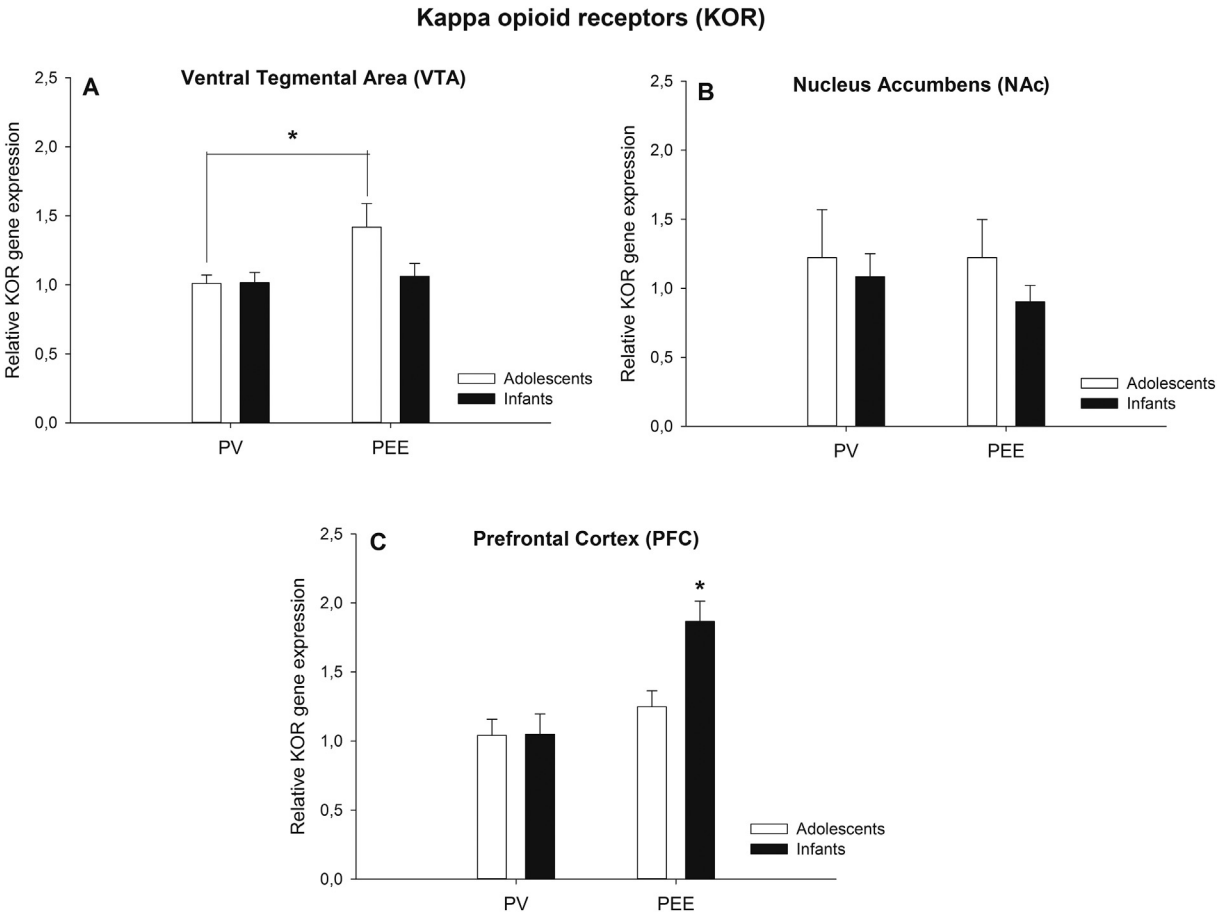

Fig. 4. Effects of prenatal ethanol exposure on mRNA levels of kappa opioid receptors (KOR) in the ventral tegmental area (VTA, Panel A), nucleus accumbens (NAc, Panel B) and prefrontal cortex (PFC, Panel C), in male and female infant and adolescent rats as a function of prenatal treatment (daily administration of 0.0 g/kg or 2.0 g/kg ethanol, gestational days 17–20; PV and PEE groups, respectively). For visualization purposes, the data were collapsed across males and females. An ANOVA indicated that sex did not significantly interact with prenatal treatment. All the mRNA measures were analyzed via factorial ANOVAs (prenatal treatment × age), and the locus of significant main effects and significant interactions in the ANOVAs was analyzed using Tukey's post hoc test or planned comparisons. The asterisk (*) sign in panel A indicates that KOR mRNA transcript levels in the VTA were significantly higher in PEE adolescents compared with PV adolescents ($p < .05$). The asterisk in panel C indicates that KOR mRNA transcript levels in the PFC were significantly higher in PEE infants than in any of the other groups ($p < .05$). The data are expressed as relative expression ($2^{-\Delta\Delta Ct}$) of the gene of interest as a function of the calibrator (i.e., normalised to β-Actin and *Gapdh*), and described as mean ± SEM.

3.3. DNA methylation scores

DNA methylation was measured, in infant rats, at the PDYN gene promoter and was not significantly different between PEE rats and controls, at any of the CpG sites measured in the NAc (site 1: 81.15 ± 1.30 vs. 84.48 ± 0.86; site 2: 91.59 ± 0.85 vs. 98.39 ± 0.53; site 3: 68.27 ± 2.99 vs. 82.46 ± 2.10). In the VTA, there was a significant effect of prenatal treatment at CpG site 3 ($t_{14} = -2.13$, $p = 0.05$), in which PEE rats exhibited a reduction of methylation compared with controls (69.57 ± 2.59 vs. 88.33 ± 1.33, respectively). No significant differences were found in the other two CpG sites measured at VTA (site 1: 82.18 ± 2.00 vs. 88.72 ± 0.85; site 2: 98.63 ± 0.45 vs. 98.57 ± 0.27).

DNA methylation of the KOR gene promoter was measured, in infant and adolescent rats, at the PFC and VTA. The ANOVAs for methylation scores at PFC indicated no significant effect of prenatal treatment or age nor a significant interaction between these factors, at any of the CpG sites measured. The ANOVA for DNA methylation scores at VTA (CpG site 1) yielded significant main effects of prenatal treatment and age ($F_{1,24} = 8.20$, $p < .001$, $\eta^2p = 0.25$ and $F_{1,24} = 15.50$, $p < 0.001$, $\eta^2p = 0.39$, respectively). The interaction between these factors also achieved significance ($F_{1,24} = 14.07$, $p < .001$, $\eta^2p = 0.37$). The *post-hoc* tests indicated that the level of methylation at CpG site 1 was significantly lower in PEE than in PV infants. On the other hand, methylation levels at CpG site 1 were similar between PEE and PV adolescents. The ANOVA conducted on DNA methylation of the KOR gene promoter at CpG 4 yielded no significant main effect or significant interaction, whereas those conducted on scores obtained at CpGs 2, 3 or 5 revealed a significant main effect of age ($F_{1,24} = 14.92$, $p<0.001$, $\eta^2p = 0.39$; $F_{1,18} = 10.69$, $p < 0.005$, $\eta^2p = 0.37$; and $F_{1,24} = 8.35$, $p < 0.01$, $\eta^2p = 0.26$; respectively), with infants showing significantly higher methylation than adolescents (Table 4).

3.4. Baseline and ethanol-induced motor activity

As shown in Fig. 7, PEE animals exhibited significantly less distance travelled (significant main effect of prenatal treatment, $F_{1,53} = 6.99$, $p < 0.05$, $\eta^2p = 0.12$), and ethanol induced a brief, yet significant, behavioral stimulation (significant main effect of minute of evaluation and significant interaction between ethanol treatment and minute of evaluation, $F_{14,742} = 91.74$, $p < .001$, $\eta^2p = 0.63$ and $F_{14,742} = 6.38$, $p < .001$, $\eta^2p = 0.11$, respectively). The planned comparisons indicated significantly greater distance travelled in minutes 1, 2 and 3, in animals given ethanol before the OF than in peers administered vehicle. This activating effect of ethanol was statistically similar in PEE and PV rats.

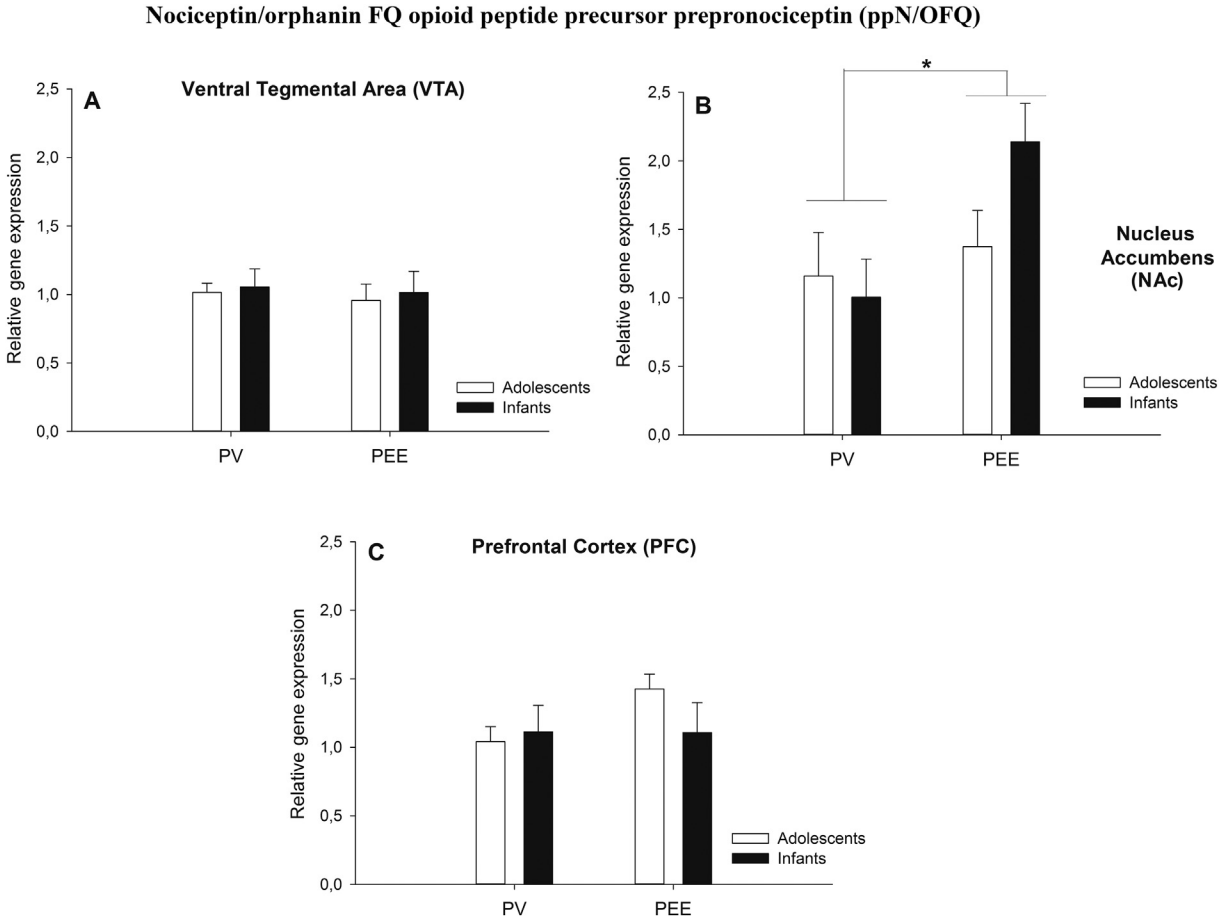

Fig. 5. Effects of prenatal ethanol exposure on mRNA levels of the nociceptin/orphanin FQ opioid peptide precursor prepronociceptin (ppN/OFQ) in the ventral tegmental area (VTA, Panel A), nucleus accumbens (NAc, Panel B) and prefrontal cortex (PFC, Panel C), in male and female infant and adolescent rats as a function of prenatal treatment (daily administration of 0.0 g/kg or 2.0 g/kg ethanol, gestational days 17–20; PV and PEE groups, respectively). For visualization purposes, the data were collapsed across males and females. An ANOVA indicated that sex did not significantly interact with prenatal treatment. All the mRNA measures were analyzed via factorial ANOVAs (prenatal treatment × age), and the locus of significant main effects and significant interactions in the ANOVAs was analyzed using Tukey's post hoc test. The asterisk (*) sign in panel B indicates a significantly heightened expression of ppN/OFQ, at NAc, in PEE than in PV rats ($p < 0.05$), regardless of age. The data are expressed as relative expression ($2^{-\Delta\Delta Ct}$) of the gene of interest as a function of the calibrator (i.e., normalised to β-Actin and GADPH), and described as mean ± SEM.

4. Discussion

The main new finding of the present study was that PEE increased the expression of genes that are related to the PDYN/KOR system in key brain areas, relevant for ethanol's rewarding effects (McBride et al. 1999). Moreover, these effects on the PDYN/KOR system occurred in conjunction with changes in the genes related to ppN/OFQ and NOR. We identified epigenetic changes that likely underlie some of the changes in the gene expression of the PDYN/KOR system, and PEE-induced alterations in the regulation of genes transcription were associated with an anxiety-prone phenotype. The rats exhibited heightened avoidance of the dangerous areas of the light-dark box and concentric square field, heightened shelter-seeking in the concentric square field test, and a reduction of exploration in all of the behavioral tasks.

An innovative aspect of the present study was that we used a relatively brief pattern of PEE and a relatively moderate dose of ethanol. The majority of studies that assessed the effects of PEE on gene expression used either sustained (e.g., throughout gestation; (Gangisetty et al. 2017) or heavy (e.g., 5.8 g/kg on gestational day 9; (Downing et al. 2012) ethanol exposure. Such models of drinking patterns may likely result in fetal alcohol syndrome and are not comparable to the sporadic pattern of drinking that is reported by most women who drink during gestation (Lopez et al. 2017). An important limitation, however, is that we used a single ethanol dose during the prenatal exposure and a single ethanol dose during the OF testing, which limits the scope and generalization of the findings. Another important limitation is the lack of epigenetic and gene expression measurements at amygdala (Ranjbar et al. 2017; Sagarkar et al. 2017), a key structure in the regulation of anxiety responses.

4.1. Changes in PDYN/KOR system gene expression

It is important to contextualize the present findings within the relevant literature on ontogenetic differences in opioid gene expression after PEE. Using prenatal ethanol exposure similar to that of our study, Bordner and Deak (2015) reported heightened expression of the PDYN mRNA transcript in VTA, in PEE vs. control rats. This effect was observed at PD4, but not at PDs 8 or 12. Moreover, PEE enhanced PDYN and KOR expression in NAc. Consistent with this, we found, in infants exposed to prenatal ethanol, a selective upregulation of PDYN mRNA in the VTA, and of KOR mRNA in the PFC. The rats exposed to ethanol in-utero also exhibited increased levels of KORmRNA at VTA, an effect that was particularly noticeable at adolescence. KOR activation hyperpolarizes dopamine neurons in the VTA (Ford et al. 2007) and inhibits dopamine release in regions receiving VTA input (Carlezon et al., 2006), resulting in dysphoria, anhedonia and depressive-like effects. The present results are consistent with a previous study by our group (D'Addario et al. 2013), in which we observed greater activity of

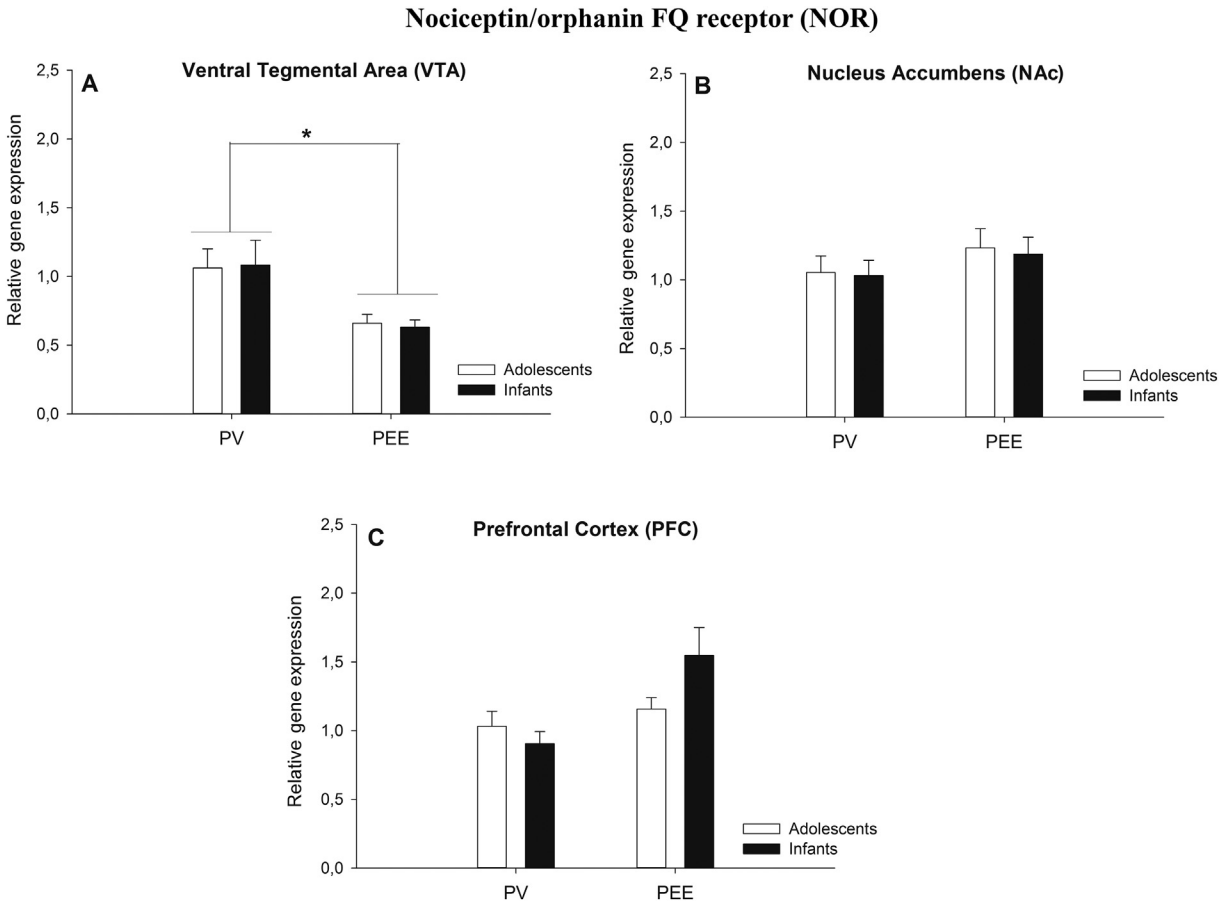

Fig. 6. Effects of prenatal ethanol exposure on mRNA levels of the nociceptin/orphanin FQ receptors (NOR) in the ventral tegmental area (VTA, Panel A), nucleus accumbens (NAc, Panel B) and prefrontal cortex (PFC, Panel C), in male and female infant and adolescent rats as a function of prenatal treatment (daily administration of 0.0 g/kg or 2.0 g/kg ethanol, gestational days 17–20; PV and PEE groups, respectively). For visualization purposes, the data were collapsed across males and females. An ANOVA indicated that sex did not significantly interact with prenatal treatment. All the mRNA measures were analyzed via factorial ANOVAs (prenatal treatment × age), and the locus of significant main effects and significant interactions in the ANOVAs was analyzed using Tukey's post hoc test. The asterisk (*) sign in panel A indicates a significantly reduced expression of NOR, at VTA, in PEE than in PV rats ($p < 0.01$), regardless of age. The data are expressed as relative expression ($2^{-\Delta\Delta Ct}$) of the gene of interest as a function of the calibrator (i.e., normalised to β-Actin and GADPH), and described as mean ± SEM.

dynorphinergic systems after repeated postnatal administration of ethanol in infant rats. The results also support the hypothesis that repeated exposure to ethanol recruits anti-reward systems (Walker and Koob 2008) that render the individual more sensitive to stress and anxiety-related stimuli and thus more sensitive to the negative reinforcing effects of ethanol.

In the present study, the heightened PDYN gene expression after PEE was generally greater in infants than in adolescents, and this gene upregulation was associated with a significant reduction in DNA methylation at the PDYN promoter in the VTA. Similarly, PEE-induced upregulation of KOR at VTA was associated with reduced DNA methylation at the KOR promoter. There was, however, a relative dissociation between the epigenetic mark and gene expression at VTA. In the latter structure, the PEE-induced upregulation of KOR mRNA transcript levels was particularly noticeable in adolescents, whereas the PEE-induced reduction in DNA methylation at the KOR promoter was

Table 4
DNA methylation levels at the KOR gene promoter.

	Infants				Adolescents			
	PV		PEE		PV		PEE	
	VTA	PFC	VTA	PFC	VTA	PFC	VTA	PFC
S1	5.25 ± 0.78	3.17 ± 0.60	2.49 ± 0.07*	3.73 ± 0.58	2.04 ± 0.28	2.94 ± 0.16	2.41 ± 0.32	3.01 ± 0.16
S2	4.70 ± 0.88	2.48 ± 0.71	4.33 ± 0.64	3.05 ± 0.62	2.76 ± 0.38	3.25 ± 0.52	2.22 ± 0.15	2.26 ± 0.25
S3	5.67 ± 1.32	2.72 ± 0.41	5.27 ± 0.87	4.05 ± 0.79	3.20 ± 0.39	3.89 ± 1.03	2.95 ± 0.16	2.85 ± 0.28
S4	2.03 ± 0.31	1.64 ± 0.62	2.61 ± 0.46	2.12 ± 0.48	1.40 ± 0.35	1.67 ± 0.37	2.47 ± 0.82	1.79 ± 0.26
S5	4.50 ± 0.79	3.09 ± 1.23	3.81 ± 0.45	2.47 ± 0.48	2.42 ± 0.33	2.60 ± 0.51	2.99 ± 0.43	2.43 ± 0.07

DNA methylation levels (mean ± SEM) at five cytosine-guanine dinucleotide (CpG) sites, within the KOR gene promoter, in the ventral tegmental area (VTA) and prefrontal cortex (PFC) of infant and adolescent subjects, as a function of prenatal treatment (daily administration of 0.0 g/kg or 2.0 g/kg ethanol, gestational days 17–20; PV and PEE groups, respectively). ANOVAs and subsequent *post-hoc* tests indicated that the level of methylation at CpG site 1 of VTA was significantly lower in PEE than in PV infants. This effect is indicated by the asterisk ($p \leq 0.001$). Refer to the text for a full account of statistical analysis.

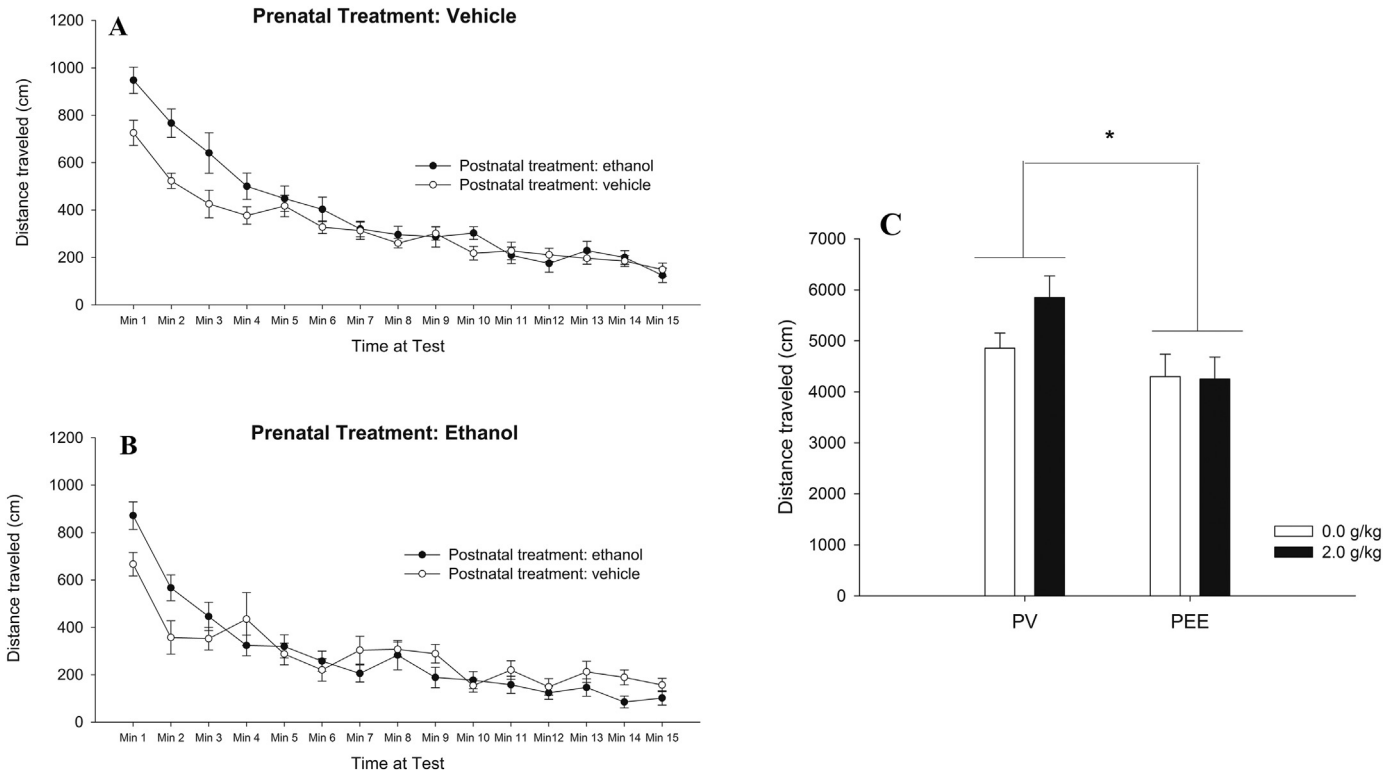

Fig. 7. Prenatal ethanol exposure affects baseline level of locomotion in an open field (OF) test. Distance travelled (cm), in male and female adolescent rats as a function of prenatal treatment [daily administration of 0.0 g/kg or 2.0 g/kg ethanol, gestational days 17–20; PV (panel A) and PEE (panel B) groups, respectively] and postnatal ethanol treatment given 5 min before the OF test (0.0 [vehicle] or 2.0 g/kg of ethanol). Panel C depicts cumulative distance travelled by the rats during the 15 min test. For visualization purposes, the data were collapsed across males and females. An ANOVA indicated that sex did not significantly interact with prenatal treatment. Distance travelled was analyzed via a repeated measure ANOVA [comparative factors between groups: prenatal treatment and ethanol treatment before OF; within-factor: minute of evaluation (1–15)], and the locus of significant main effects in the ANOVA was analyzed using Tukey's post hoc test. The statistical analysis revealed that PEE animals exhibited significantly less distance travelled, regardless of ethanol or vehicle postnatal treatment, than PV counterparts ($p < 0.05$; this effect is indicated by the asterisk in panel C); and that rats treated postnatally with ethanol exhibited significantly greater locomotion during the first three minutes of the test, when compared to peers administered vehicle ($p < 0.001$). The data are described as mean ± SEM.

only observed in infant rats. Overall, these results point out to the relevance of evaluating epigenetic marks at early developmental stages, in accordance with a recently proposed model of plasticity to stress mainly restricted to periods of rapid epigenetic remodeling during early ontogeny (Zannas and Chrousos 2017).

4.2. Changes in ppN/OFQ/NOR system gene expression

The ppN/OFQ/NOR receptor system has structural similarities to the PDYN/KOR system, but they appear to exert opposite effects. For instance, it has been suggested that CRF-mediated KOR activation that induces stress effects is antagonized by nociceptin (Land et al. 2008). In the present study we found a reduction of NOR gene expression in the VTA in both infants and adolescents exposed to PEE, thus opposite to the heightened mRNA levels of KOR. Previous studies reported that subchronic intracerebroventricular injection of nociceptin/orphanin FQ decreased home-cage ethanol drinking (Ciccocioppo et al. 1999) and operant self-administration of ethanol (Ciccocioppo et al. 2004; Martin-Fardon et al. 2000) in genetically selected Marchigian Sardinian alcohol-preferring (msP) rats. Based on this literature, NOR activation should result in the inhibition of ethanol drinking, thus it can be hypothesized that the reduction of NOR gene expression reflects increased vulnerability to ethanol consumption in PEE animals. According to our previous studies, the prenatal ethanol exposure here employed (i.e., 2.0 g/kg, GDs 17–20) has promoting effects upon ethanol intake (Fabio et al. 2015a) and ethanol-induced conditioned preference (Pautassi et al. 2012b).

We also observed a significant increase in ppN/OFQ mRNA transcript levels in PEE vs. PV rats. The latter effect, which was found at NAc, was statistically similar at both ages but noticeably greater in infants than in adolescents. Moreover, this significant increase in ppN/OFQ mRNA was concomitant with a slight, yet significant, PEE-induced decrease in PDYN mRNA at NAc. It is interesting to note that the positive motivational effects of drugs of abuse seem to involve hedonic hot spots in the NAc (Berridge and Kringelbach 2008), and ppN/OFQ activation in this area suppresses dopamine release (Narayanan et al. 2004).

Based on these results, one possibility is that the changes in gene expression that, in the present study, were observed after PEE resulted from an interplay between the nociceptin/orphanin FQ system and PDYN/KOR systems. Under this hypothesis, the upregulation of the expression of genes that are related to the ppN/OFQ or NOR receptor system in the NAc and PFC may reflect an attempt to counteract PEE-induced changes in the genes that are related to the PDYN/KOR system. This hypothesis requires further testing.

4.3. Behavioral correlates of PEE-induced changes in gene expression

PEE rats exhibited an anxiety-prone phenotype, which is consistent with the upregulation of the genes related to KOR signaling, a system that is largely responsible for the induction of aversive, stress-like effects and dysphoria. As reviewed by Van't Veer and Carlezon (2013), the administration of KOR agonists induces anxiety-like behavior in rodents. In our study PEE rats exhibited greater avoidance of the white and brightly lit areas of the light-dark box and concentric square field, heightened preference for the sheltered area of the concentric square

field, and an overall reduced pattern of locomotor activity that was evident across tests. The latter outcome, however, can be viewed as a limitation, since altered spontaneous locomotion can affect the interpretation of animal behavior in the tests that measure anxiety in rodents.

In previous studies, we have found greater ethanol-induced conditioned place preference in PEE than in PV rats (Pautassi et al. 2012b). Hence, we expected that PEE rats would exhibit greater sensitivity to ethanol-induced motor activity in Experiment 2. Ethanol-induced forward locomotion is considered a proxy of ethanol's appetitive effects (Camarini and Pautassi 2016). This expectation, however, was not corroborated. Ethanol-induced stimulation was brief and similarly exhibited by PEE and PV adolescent rats.

5. Conclusions

Establishing causal relationships between changes in mRNA levels and changes in complex behaviors is difficult, especially when considering the variety of effects of ethanol upon biological systems. The present results suggest that PEE alters gene expression of the PDYN/KOR system, which is correlated with an anxiety-prone phenotype at the behavioral level. These effects should render the organism more sensitive to the negative reinforcing effects of ethanol or could enhance the sensitivity to the appetitive motivational effects of ethanol and hence could underlie the greater propensity for ethanol intake in individuals that are exposed to ethanol early in life.

Ethical Statement

All of the procedures were certified by the Institutional Animal Care and Use Committee at INIMEC-CONICET-UNC, complied with the Declaration of Helsinki, the ARRIVE guidelines, and the Guide for the Care and Use of Laboratory Animals (National-Research-Council, 1996) as promulgated by the NIH and the EU.

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Author contribution

RMP designed the study, with significant input by AWB and CD; and CD, SMM, MP, FB, AWB and RMP wrote the protocol. AWB, SMM and RMP run the behavioral assays, whereas AWB, FB and MP run the gene expression and DNA methylation assays. AWB, SMM, MP, FB and RMP run the statistical analysis. AWB, RMP and CD wrote the first draft of the manuscript. All authors contributed and approved the final manuscript.

Competing interests statement

We declare having no competing interest nor conflict of interest related to our MS or its results.

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