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Maternal Isolation During the First Two Postnatal Weeks Affects Novelty-Induced Responses and Sensitivity to Ethanol-Induced Locomotor Activity During Infancy

ABSTRACT: *Animals exposed to chronic maternal separation (MS) exhibit enhanced ethanol self-administration and greater hormonal and behavioral responsiveness to stress in adulthood. Whether the effects of MS are immediately evident in infancy or whether they appear only later on development is still an unanswered question This study tested sensitivity to ethanol's behavioral stimulating effects in infant rats that experienced MS from postnatal Day 1–14. MS infants exhibited significantly greater reactivity to the motor stimulating effects of 1.25 g/kg ethanol than control animals, yet greater motor suppression after 2.5 g/kg ethanol. Baseline level of response to novelty was altered in MS infants, in a norbinaltorphimine insensitive manner, that is, despite modified activity of the kappa-opioid system. These results indicate that the consequences of chronic maternal isolation emerge early in ontogeny, affecting ethanol sensitivity in infancy.* © *2013 Wiley Periodicals, Inc.* Dev Psychobiol 9999: 1–13, 2013.

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A negative association between number of early adverse experiences and age of alcohol initiation has been found (Enoch, 2012; Rothman, Edwards, Heeren, & Hingson, 2008). Subjects who had experienced early life stress were also more likely to report stress coping as a motive for drinking during the first year of drinking (Rothman et al., 2008). Early onset of drinking, in turn, increases the risk for stress-related drinking (Dawson, Grant, & Li, 2007) and predicts alcohol abuse and dependence (DeWit, Adlaf, Offord, & Ogborne, 2000).

Effects of early-life stress on reactivity to ethanol can be assessed through the maternal separation (MS) paradigm (Francis & Kuhar, 2008; Kosten & Kehoe, 2010; Kosten, Zhang, & Kehoe, 2003; Plotsky & Meaney, 1993; Spivey, Barrett, & Padilla, 2008; Zimmerberg, Rosenthal, & Stark, 2003). In this preparation, rats experience 180 or 360 min of MS, every day from postnatal Day (PD) 1 to PD14 or until weaning on PD21 (Champagne, Weaver, Diorio, Sharma, & Meaney, 2003; Kawakami, Quadros, Takahashi, & Suchecki, 2007). Early work indicated that maternally separated animals exhibited, when tested at adulthood, enhanced alcohol self-administration and greater hormonal and behavioral responsiveness to stress (Huot, Thrivikraman, Meaney, & Plotsky, 2001) than animals under normal animal facility rearing conditions (AFR). Later work confirmed that early MS results in exacerbated operant self-administration of ethanol at adulthood (Cruz, Quadros, Planeta, & Miczek, 2008).

The facilitative effects of protracted MS on later ethanol intake may be due to alterations in the motivational effects of ethanol. Early MS could increase

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sensitivity to the rewarding or anti-anxiety effects of ethanol or lessen the ability of the drug to induce aversive effects such as sedation or motor incoordination. The appetitive, aversive and negative reinforcing effects of ethanol significantly modulate ethanol seeking and intake and occur early in life, during developmental periods in which initiation and experimentation with ethanol is pervasive (Pautassi, Nizhnikov, & Spear, 2009; Pilatti, Godoy, Brussino, & Pautassi, 2013).

Few animal studies assessed effects of MS soon after termination of this treatment, during infancy or adolescence. It is still an open question whether the effects of MS are immediately evident or whether they appear only later in development. The few studies that did not impose a delay between MS treatment and testing on adulthood revealed alterations in open field activity and play behavior in infant and adolescent rats that had been exposed to chronic MS (Arnold & Siviy, 2002). More recently, we observed that repeated MS altered ethanol-induced motivational learning (Pautassi, Nizhnikov, Fabio, & Spear, 2012).

The present study assessed effects of chronic MS on sensitivity to ethanol's effects in infant rats. Daily length of MS was shorter than that used in most of the previous studies, and the resulting changes in behavior toward ethanol were assessed soon after termination of early stress, when rats were preweanlings. In Experiment 1 preweanlings were tested for dose-response, ethanol-induced motor activation and motor depression, 60 min after withdrawal from their home cage. Experiment 2 removed the acute, pre-test isolation, and tested novelty and ethanol-induced locomotor activity (LMA) in MS and AFR pups right after removal from their homecage. We expected MS animals to be more sensitive to ethanol-induced activation or less sensitive to ethanol-induced motor depression.

The kappa opioid receptor (KOR) system mediates acute and chronic stress (Land et al., 2009) and blockade of KOR through administration of norbinaltorphimine (nor-BNI) inhibited potentiation by stress of ethanol reward (Sperling, Gomes, Sypek, Carey, & McLaughlin, 2010). It has been also suggested that opioidergic transmitter systems are altered by MS (Vazquez et al., 2005). Experiment 3, tested the hypothesis that proximal treatment with nor-BNI (24 hr before testing) would counteract the effects of repeated maternal isolation.

GENERAL MATERIALS AND METHODS

Subjects

Four hundred forty-five Wistar rat pups born and reared in the vivarium at INIMEC-CONICET (Córdoba, Argentina) were used. Number of animals and litter representation in each experiment was as follows: Experiment 1, 248 animals (30 litters, 15 experienced conventional AFR, 15 experienced daily episodes of MS); Experiment 2, 59 animals (17 litters, 9 AFR, 8 MS); and Experiment 3, 138 animals (20 litters, 8 AFR, 10 MS). Births were checked daily and the day of birth was considered as PD0. Subjects used in a given Experiment were never re-tested in subsequent experiments. This is, subjects were naïve to experimental procedures in each experiment. Litters were housed in standard maternity cages and given ad-libitum access to water and lab chow. Room temperature was kept at 22–24°C and lighting followed a12 h/12 h light/dark cycle (lights on at 8:00 AM). The experimental protocol was approved by the National University of Cordoba and complied with the regulations of the Guide for Care and Use of Laboratory Animals (National Research Council, 1996). Litter effects across experiments were controlled by including no more than one animal per litter in any group condition.

Across experiments, body weight was measured through the use of a precision scale (portable Ohaus L2000; Ohaus, Pine Brook, NJ), featuring a sensitivity of 1/100 g. During the weighing procedure animals were gently strapped in a smooth cloth, thus allowing brief (2–3 s) immobility and therefore the desired amount of precision.

Ethanol and Nor-BNI Preparation and Administration Procedures

Ethanol and nor-BNI administrations were conducted intragastrically (i.g.) and intraperitoneally (i.p.), respectively, as described in Pautassi, Nizhnikov, Molina, and Spear (2007). Nor-BNI was given on PD14, at noon. Ethanol was administered on PD15 (Experiments 1–3), prior to motor activity measurements.

The ethanol doses of 1.25, 2.5, and 3.0 g/kg were derived from ethanol solutions at 10.5, 21.0, and 25.2% v/v, respectively (Porta Hnos, Cordoba, Argentina); vehicle: tap water). Volume of administration was 0.015 ml of solution per gram of body weight. Ethanol doses $\geq$1.25 g/kg were selected based on previous studies that assessed ethanol-induced locomotor activity in preweanling rats (Pautassi et al., 2012). The nor-BNI doses of 1.0, 5.0, and 10.0 mg/kg (Tocris, Bristol, UK) were derived from a 1 mg/10 ml, 5 mg/10 ml, and 1 mg/ml solution (vehicle, 0.9% saline), respectively. Injection volume was 0.01 ml/g for all doses and for the animals treated with vehicle. The range of nor-BNI doses was selected based on previous work. Doses of 2.5 and 10 mg/kg of nor-BNI have been observed to facilitate expression of ethanol-induced conditioned taste preference (Nizhnikov, Pautassi, Varlinskaya, Rahmani, & Spear, 2012) and to block stress potentiation of ethanol-induced CPP (Sperling et al., 2010), in preweanling and adult rats, respectively.

Rearing Conditions Across PDs 1–14 (Experiments 1, 2, and 3)

Births were checked daily and litters were culled on PD0 to 10 animals (5 males and 5 females, whenever possible). On PD1 litters were randomly assigned to be housed under normal animal facility conditions or to experience 240 (Experiments 1, 2, and 3) min of daily MS, once daily during PDs 1–13. In other words, length of daily MS treatment was

4 hr in Experiments 1, 2, and 3 (see Fig. 1. Control animals experienced similar, conventional AFR conditions across Experiments.

Maternal separation began at 8:00 AM and followed procedures described in Pautassi et al. (2012). Briefly, pups were removed from the dam, transported to an adjacent room and placed, as a litter, in a standard, clean maternity cage kept warm (35˚C) through the use of heating pad. All litters experienced changes in maternal cages and beddings twice a week. This procedure was conducted by the same experimenters that conducted the maternal isolation and the behavioral testing.

In Experiment 3 pups were injected on PD14, about 18 hr before the assessment of spontaneous and ethanol-induced motor activity, with one of four nor-BNI treatments (0.0, 1.0, 5.0, or 10 mg/kg, i.p.; see Fig. 1). The rationale for the delay between injection and testing is that immediately after its injection, nor-BNI acts as a general opioid antagonist and only after 3 or 4 hr the selectivity for kappa antagonism emerges (Metcalf & Coop, 2005). Studies indicate that once achieved nor-BNI induced KOR blockade is long lasting, in the order of several weeks (Broadbear, Negus, Butelman, de Costa, & Woods, 1994).

Assessment of Spontaneous or Ethanol-Induced Locomotor Activity (LMA) (Experiments 1, 2, and 3)

Across experiments, measurements of LMA were conducted via an automatic activity monitoring system (ITCOMM,

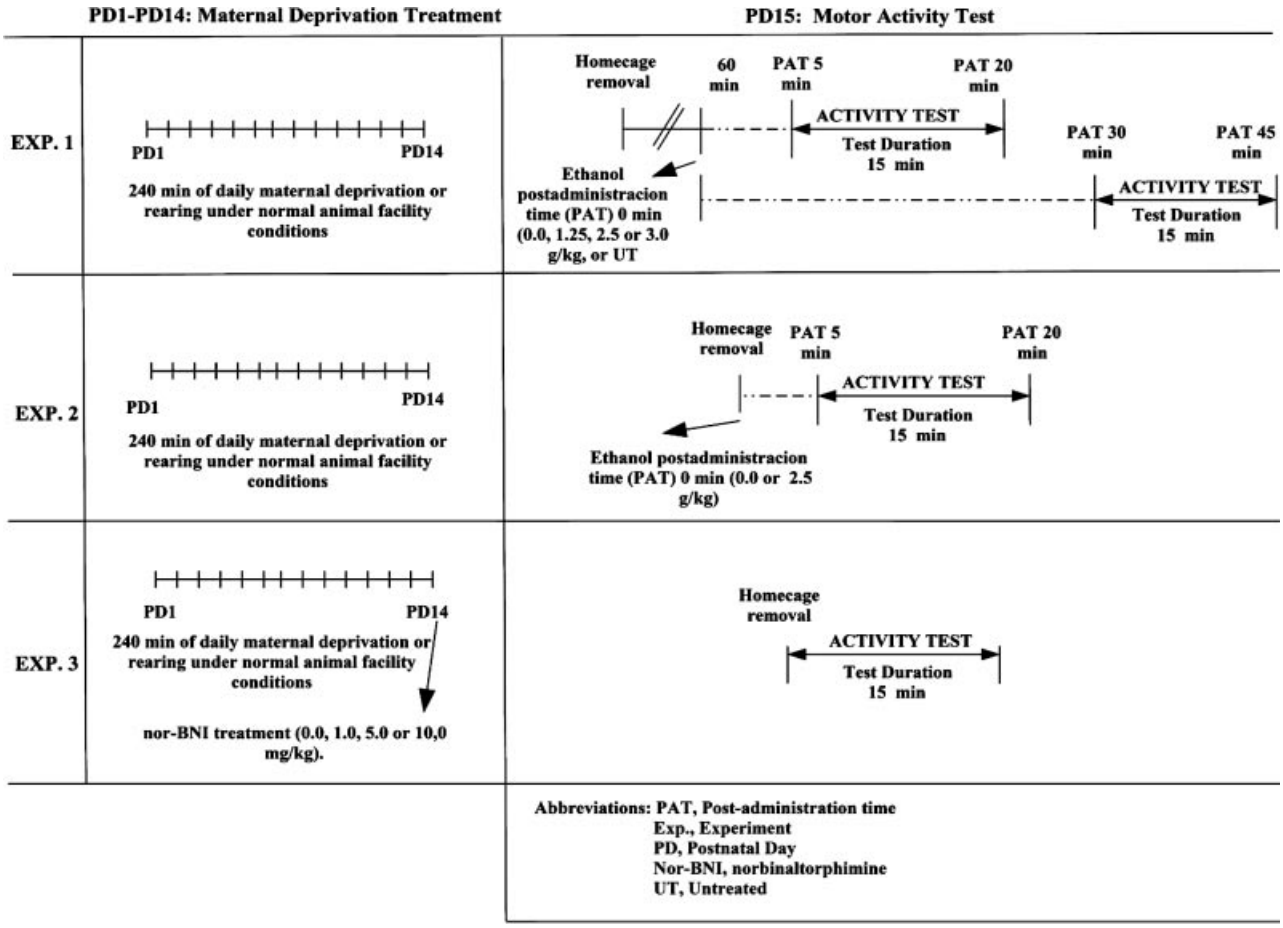

FIGURE 1 Methods for the analysis of the effects of early maternal deprivation on spontaneous and ethanol-induced motor activity in infant rats, in Experiments 1, 2, and 3. Across experiments, animals were given 240 min of maternal separation, each day from postnatal Day 1–14. In Experiment 3, after termination of the last episode of maternal separation on postnatal Day 14, animals were given varying doses of a long lasting kappa opioid receptor antagonist (norbinaltorphimine: 0.0, 1.0, 5.0, or 10.0 mg/kg). A motor activity test (duration: 15 min) was conducted on postnatal Day 15. In Experiment 1 the test was conducted 60 min after homecage removal, whereas in Experiments 2 and 3, the test was conducted immediately after homecage removal. In Experiments 1 and 2 animals were administered varying doses of ethanol [Experiment 1: 0.0, 1.25, 2.5, or 3.0 g/kg and a group of untreated (UT) animals; Experiment 2: 0.0 or 2.5 g/kg], whereas in Experiment 3 animals were tested for spontaneous, novelty-induced motor activity. Testing in Experiments 1 and 2 began either 5 or 30 min after ethanol administration.

Córdoba, Argentina). Animals were placed in square chambers (25 cm × 25 cm × 25 cm) made of clear Plexiglas and equipped with photocell beams. These beams allowed measurement of distance traveled (cm) and Vertical behavior. Vertical behavior was measured by registering breaks of a photocell beam located about 6 cm from the floor. Vertical behavior was, therefore, was a combination of wall-climbing and rearing behaviors.

Data Analysis

Across experiments, body weight at PD15 was analyzed through a factorial ANOVA (independent factors: rearing condition and sex).

The main dependent variables in Experiments 1, 2, and 3 were distance traveled (cm) in the activity chamber and frequency of vertical behaviors. In Experiments 1 and 2 data obtained for both variables were divided into three 5 min bins (i.e., post-administration intervals of 5–9 or 30–34 min, 10–14 or 35–39 min, and 15–19 or 40–44 min) and processed through repeated measures Analysis of Variance (ANOVAs). Sex, early rearing condition, ethanol treatment, and post-administration time of assessment were the between factors, whereas bin of assessment (bins 1–3) was the within factor.

In Experiment 1, a sex × group (0.0 g/kg or untreated) × testing bin mixed ANOVA was employed to analyze, in each rearing condition, differences between untreated animals and animals given vehicle intubations.

In Experiment 3, distance traveled during the 15 min testing phase was also divided into three 5 min bins. In Experiment 3, distance traveled was analyzed through analysis of variance (ANOVA). Sex, nor-BNI treatment (0.0, 1.0, 5.0, or 10.5 mg/kg), and rearing condition were considered the between-factors whereas bin of assessment (bins 1–3) served as within-measure factor. A similar ANOVA analyzed frequency of vertical behaviors.

The loci of significant main effects or significant interactions were analyzed using follow-up ANOVAs and post-hoc tests. Planned comparisons were also employed. Specifically, Fisher's LSD tests were employed for analysis of simple main effects or interaction comprising "between" factors. The significant main effect of within-subject measures or any interaction comprising repeated measures was analyzed through orthogonal planned comparisons. The rationale for the use of planned comparisons was that there is no unambiguous choice of pertinent error terms for post-hoc comparisons involving between-by-within factors (Winer, Brown, & Michels, 1991). Alpha level for rejection of null hypothesis was ≤ 0.05.

EXPERIMENT 1

This experiment analyzed the effects of early neonatal stress (240 min of daily MS, from PD1 to 14) on sensitivity to the activating or depressing effects of ethanol, in 15-day old male and female preweanling pups. Ethanol-induced LMA was assessed for 15 min, beginning 5 or 30 min following ethanol administration. According to previous studies (e.g., Arias, Molina, Mlewski, Pautassi, & Spear, 2008), at these time points, the activating and depressing effects of 2.5 g/kg ethanol are respectively maximal. Ethanol administration was given 60 min after removal from homecage, following procedures from previously published studies (Arias et al., 2008; Pautassi, Nizhnikov, Fabio, & Spear, 2011). The hypothesis was greater ethanol-induced LMA in MS animals.

Untreated groups were employed to control for potential locomotor-activating effects resulting from the intubation. Animals in these untreated (UT) control conditions were tested for activity in the open field 60 min after being removed from the dam but did not receive ethanol or vehicle intubations.

Experimental Design

Experiment 1 was defined by a 2 (sex: male or female) × 2 (early rearing condition: AFR or MS) × 2 (post-administration time of assessment: 5–20 min or 30–45 min) × 4 (ethanol treatment: 0.0 [water vehicle], 1.25, 2.5, or 3.0 g/kg) factorial design. Four untreated control conditions (one for each sex and for each early rearing condition) were also tested. Number of animals in each group can be found in Table 1.

Methods

A brief and graphical depiction of the experimental procedures can be found in Figure 1. More in detail, animals were separated from the dam 60 min prior to LMA testing. This is the standard MS interval in studies assessing ethanol-induced behavioral stimulation (Arias et al., 2008; Pautassi et al., 2011). The pups were roomed with a same-sex peer in a holding cage lined with clean pine shavings and warmed with a heating pad. After the 60-min period the pups were weighed, administered one of four ethanol doses (0.0, 1.25, 2.5, or 3.0 g/kg) and returned to the warmed holding cage. They were then tested for locomotor activity in the automatic activity monitoring system at the assigned post-administration time: 5–20 or 30–45 min. These time points represent two distinct periods of the blood ethanol curve. During the 5–20 min phase blood alcohol levels are rising and ethanol's appetitive and activating effects are presumably maximal, whereas at 30–45 min ethanol has reached maximal blood levels (see Nizhnikov, Pautassi, Truxell, & Spear, 2009).

Half of the vehicle-treated control animals (i.e., the .0 g/kg group) was tested 5 min after the intubation, whereas the other half was tested 30 min after the intubation with vehicle (post-administration time: 5–20 and 30–45 min, respectively). Two untreated groups

Table 1. Number of Subjects in Each of the Experimental Conditions of Experiments 1, 2, and 3

		Experiment 1	Experiment 2		Experiment 3	
Early Rearing Condition	Ethanol Treatment	PAT 5–20 min	PAT 30–45 min		Nor-BNI Dose	
AFR (normal animal facility rearing)	Untreated group	13 ♂, 12 ♀	—	—		—
	0.0 g/kg ethanol group	8 ♂, 7 ♀	7 ♂, 7 ♀	9 ♂, 7 ♀	0.0 mg/kg	8 ♂, 8 ♀
	1.25 g/kg ethanol group	8 ♂, 7 ♀	7 ♂, 7 ♀		1.0 mg/kg	8 ♂, 8 ♀
	2.5 g/kg ethanol group	8 ♂, 5 ♀	7 ♂, 7 ♀	9 ♂, 6 ♀	5.0 mg/kg	8 ♂, 8 ♀
	3.0 g/kg ethanol group	8 ♂, 7 ♀	5 ♂, 8 ♀		10.0 mg/kg	7 ♂, 9 ♀
MS (maternal separation)	Untreated group	8 ♂, 10 ♀	—	—		—
	0.0 g/kg ethanol	7 ♂, 7 ♀	7 ♂, 7 ♀	6 ♂, 8 ♀	0.0 mg/kg	10 ♂, 10 ♀
	1.25 g/kg ethanol group	8 ♂, 6 ♀	6 ♂, 6 ♀		1.0 mg/kg	9 ♂, 11 ♀
	2.5 g/kg ethanol group	6 ♂, 6 ♀	6 ♂, 7 ♀	7 ♂, 7 ♀	5.0 mg/kg	9 ♂, 8 ♀
	3.0 g/kg group	7 ♂, 7 ♀	6 ♂, 7 ♀		10.0 mg/kg	9 ♂, 8 ♀

Males and females are indicated by the signs ♂ and ♀, respectively. PAT indicates ethanol post-administration time. Nor-BNI is the abbreviation of the kappa opioid receptor norbinaltorphimine (nor-BNI). Across experiments animals were reared under normal animal facility conditions or experienced daily, 240 min episodes of maternal deprivation during postnatal Days 1–14.

(one for each rearing condition) were also included. Untreated animals (one male and one female per litter) were separated from the dam and 60 min later tested for LMA, but they did not experience intubations or manipulations before testing. All untreated animals were tested at the same time, 60 min after homecage removal.

Results

A factorial ANOVA (independent factors: rearing condition and sex) indicated a significant main effect of rearing condition. Body weight at PD15 was significantly lower in MS pups than in AFR counterparts, ($F_{1,244} = 7.97$, $p < .01$; Means and SEM were $35.49 \pm .38$ and $33.60 \pm .55$, for AFR and MS pups, respectively]. Weight differences alter the sensitivity of the automated behavior tracking system and therefore introduce unwanted random variability. Data from Experiment 1 was thus analyzed via separate analyses of variance (ANOVAs) for MS and AFR pups. Specifically, each variable was analyzed through a four-way mixed ANOVA [between-group factors were sex (male or female), ethanol treatment (0.0, 1.25, 2.5, or 3.0 g/kg) and post-administration time of assessment (5–20 or 30-min)]. Due to apparatus malfunction, wall-climbing data for nine animals was lost.

Distance Traveled Scores

Animal Facility Reared Pups (AFR). The ANOVA for distance traveled in the control rearing condition (AFR pups) revealed significant main effects of time at test and bin of assessment ($F_{1,93} = 4.21$, $p < .05$; $F_{2,186} = 137.79$, $p < .0001$). The two-way interaction between ethanol treatment and time at test, and between time and bin of assessment, achieved significance ($F_{3,93} = 6.16$, $p < .001$; $F_{2,186} = 3.40$, $p < .05$, respectively). The three way interaction between ethanol treatment, time at test and bin of assessment also reached significance ($F_{6,186} = 5.27$, $p < .001$).

Planned comparisons conducted for the scores during 5–20 min postadministration (see Fig. 2, upper panel) revealed that AFR pups exhibited dose-response, ethanol-induced LMA. Specifically, during the first bin of assessment, AFR pups given 3.0 g/kg ethanol exhibited significantly higher LMA than the remaining pups. Animals treated with 2.5 g/kg, but not those given the lowest ethanol dose (i.e., 1.25 g/kg), exhibited significantly greater LMA than control, vehicle treated animals. The planned comparisons also indicated that, during the first bin of assessment of the late phase of the intoxication (i.e., pups tested during post-administration time 30–45 min) AFR animals treated with 1.25 and 3.0 g/kg exhibited significantly greater and less distance traveled, respectively, than vehicle treated controls. These behaviors, indicative of activating and sedative effects of ethanol, respectively, were no longer observed on bins 2 and 3. These results are depicted in the upper panel of Figure 2.

The ANOVA that compared distance traveled in untreated and vehicle-treated animals revealed only a significant main effect of testing bin ($F_{2,100} = 149.52$, $p < 0.001$). Distance traveled in AFR untreated pups at testing bins 1–3 was $1{,}362 \pm 54.35$, 908.88 ± 43.35 and 787.88 ± 42.70, respectively.

Maternally Isolated Pups (MS). Distance traveled in pups that had been exposed to daily episodes of maternal isolation indicated significant main effects of ethanol treatment and bin of assessment, $F_{3,90} = 4.73$; $F_{2,180} = 139.14$, $ps < .005$. The interactions between time at test and bin; and the three-way interaction

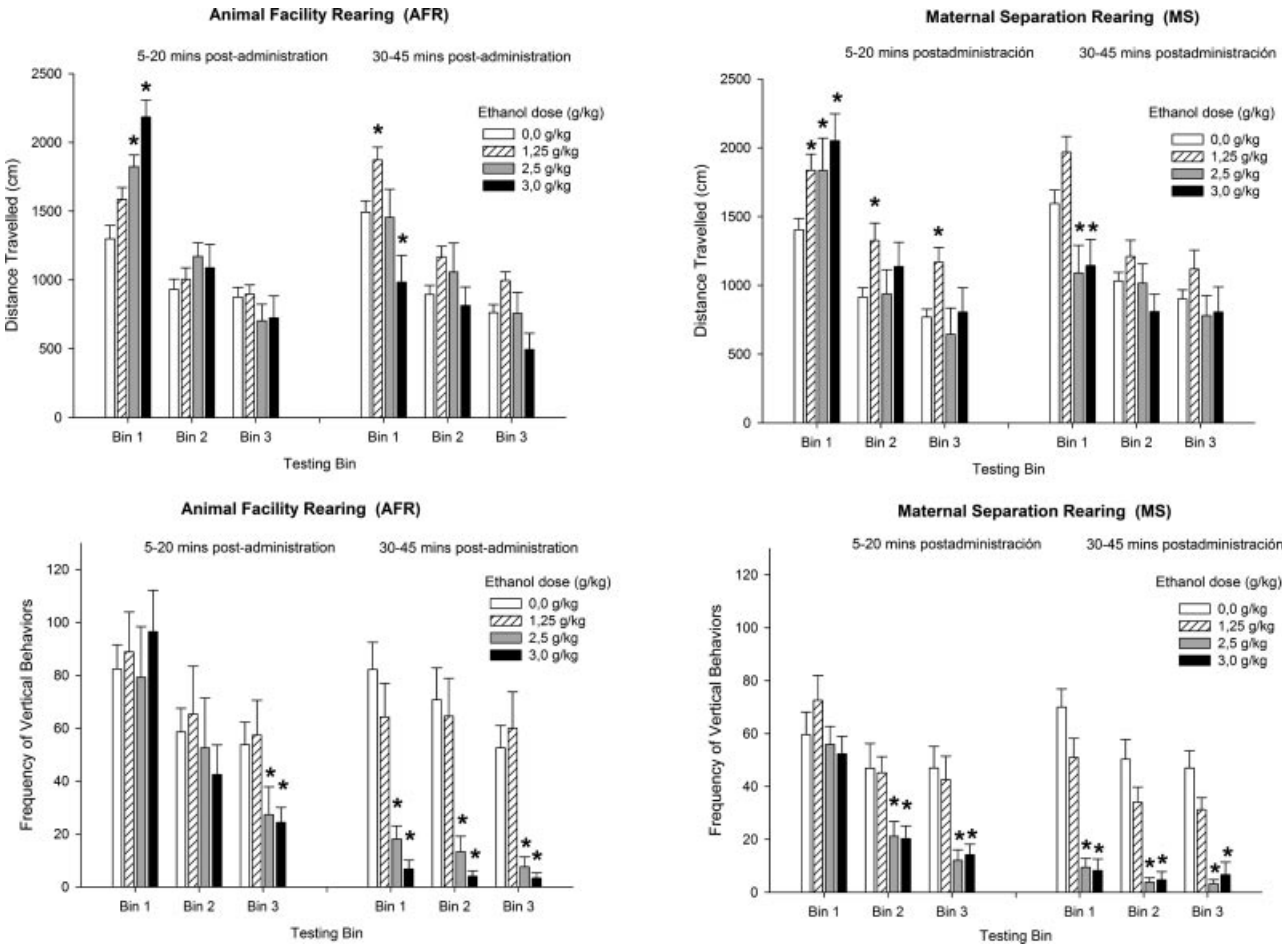

FIGURE 2 Ethanol-induced locomotor activity (distance traveled, cm; upper panel) and frequency of vertical behavior (lower panel) 5–9, 10–14, and 15–19 min or 30–34, 35–39, and 40–44 min after ethanol administration (bins 1, 2, and 3, respectively) in infant male and female rats that experienced either normal animal facility rearing (AFR, left panels) or daily episodes of maternal separation (MS, right panels) during postnatal Days (PD) 1–14. ANOVAs for distance traveled indicated that 3.0 g/kg and 2.5 g/kg, but not 1.25 g/kg, ethanol induced significant motor activation in AFR animals, during bin 1 of the 5–20 post-administration time. In MS animals, all ethanol doses exerted similar motor activation during testing bin 1 of the 5–20 post-administration time. MS animals treated with 1.25 g/kg ethanol, but not AFR counterparts, exhibited greater motor activity than vehicle-treated controls during testing bins 2 and 3. The ANOVAs for vertical behaviors revealed that MS and AFR animals treated with the highest ethanol doses (2.5 or 3.0 g/kg) exhibited significantly lower wall-climbing scores than vehicle-treated counterparts. The asterisk indicates a significant difference ($p < .05$) between an ethanol-treated group and its corresponding vehicle-treated control. Vertical bars indicate the SEM.

ethanol treatment × time at test × bin also reached significance, $F_{2,180} = 9.36$, $p < .001$; $F_{6,180} = 6.30$, $p < .001$.

Planned comparisons revealed that, during the first bin of assessment of the early time of assessment, all ethanol doses yielded similar locomotor activation. Specifically, MS pups given 3.0, 2.5, or 1.25 g/kg had greater distance traveled than control pups during minutes 5–9. During the subsequent second and third testing bins, MS pups given 1.25 g/kg ethanol, but not those given 2.5 or 3.0 g/kg, locomoted significantly more than vehicle-treated controls. During the late phase of the intoxication (postadministration time 30–45 min) significant differences as a function of ethanol dose were observed only during the first testing bin. During postadministration time 30–34 min animals given 2.5 or 3.0 g/kg exhibited less locomotion than controls. This difference was no longer found in

subsequent bins. Distance traveled in MS animals as a function of ethanol treatment, bin of assessment and time at test is depicted in Figure 2, upper right panel.

Untreated and vehicle-treated MS animals exhibited similar distance traveled across test. The ANOVA only yielded a significant main effects of testing bin ($F_{2,82} = 117.48$, $p < .0001$). Mean and SEM distance traveled in MS untreated pups was 1470.72 ± 75.87, 1006.44 ± 82.66, 875.83 ± 59.97; for testing bins 1–3, respectively.

Vertical Behavior Scores

Animal Facility Reared Pups (AFR). The ANOVA revealed significant main effects of treatment, time at test and bin of assessment, $F_{3,82} = 6.17$; $F_{1,82} = 9.80$; $F_{2,164} = 90.56$; $ps < .005$. The interactions between time at test and bin; bin and treatment and the three-way interaction ethanol treatment x time at test × bin also reached significance, $F_{2,164} = 35.46$, $p < .001$; $F_{6,164} = 2.42$; $F_{6,164} = 5.93$,$p < .001$, respectively. Post-hoc tests indicated that during bin 3 of the early testing time and throughout the late testing phase animals given 2.5 or 3.0 g/kg ethanol exhibited reduced vertical behavior than animals given 0.0 or 1.25 g/kg ethanol (see Fig. 2, lower panels).

Maternally Isolated Pups (MS). Significant main effects of treatment, time at test and bin were found ($F_{3,89} = 20.49$; $F_{1,89} = 11.31$; $F_{2,178} = 98.00$, $ps < .005$), alongside significant interactions between bin and time ($F_{2,178} = 16.80$, $p < .001$), and between ethanol treatment, time at test and bin ($F_{6,178} = 6.45$, $p < .001$). The post-hoc test revealed a pattern similar to that observed in AFR pups. Animals treated with the highest ethanol doses (2.5 or 3.0 g/kg) exhibited significantly lower wall-climbing scores during bin 2 and 3 of the early testing time and across the late testing phase.

Discussion

Taken together, these data suggest that MS induces greater sensitivity to ethanol-induced locomotor activity. MS, but not AFR, pups exhibited greater distance traveled than control counterparts following administration of a moderate (i.e., 1.25 g/kg) ethanol dose. Moreover, AFR animals exhibited dose-response ethanol-induced LMA during the first testing bin. This graded response was absent in MS pups, which exhibited similar ethanol-induced locomotor activity across the ethanol doses under analysis. Vertical behaviors revealed sedative effects of ethanol, which were fairly similar across rearing conditions.

EXPERIMENT 2

Experiment 1 suggested early stress-related differences in sensitivity to ethanol-induced motor activation, at a moderate (i.e., 1.25 g/kg) but not at high (i.e., 2.5 or 3.0 g/kg) ethanol dose. In Experiment 1, however, motor activity was assessed 60 min after home cage removal on PD15. This delay may have introduced further isolation-induced stress and perhaps differentially so in AFR and MS pups. Experiment 2 tested pups immediately after removal from homecage and during post-administration time 5–20 min (ethanol dose: 2.5 g/kg). The aim was to assess if MS and AFR groups differed in ethanol-induced sensitivity immediately after removal from the dam, at a dose that in Experiment 1 had yielded similar behavioral activation in AFR and MS. Our expectation was to find a difference in reactivity to 2.5 g/kg ethanol, between AFR and MS animals, after removing the additional stress of pre-test isolation.

Experimental Design

The design was defined by the following factors: sex (male or female), early rearing condition (AFR or MS, 240 min per day) and ethanol dose (0.0 or 2.5 g/kg). Number of animals in each group can be found in Table 1.

Methods

Experiment 2 measured ethanol-induced locomotor activity (.0 g/kg or 2.5 g/kg) in pups derived from AFR or MS litters. In this Experiment, animals were immediately transferred from the maternal homecage to the testing room, and administered ethanol (see Fig. 1, intermediate panel). The rationale for this procedure was to avoid the 60-min pre-testing isolation period, which by itself may alter reactivity to ethanol's motor effects. The ethanol dose was chosen because in Experiment 1 it had yielded similar motor activating response in MS and AFR pups. All animals were tested for ethanol-induced LMA during post-administration time 5–20 min. The automatic activity monitoring system, described in Experiment 1, was employed during tests.

Results

A factorial ANOVA (early rearing × sex) on body weights indicated the lack of significant main effects or significant interactions. In other words, no weight differences were found between MS and AFR animals. Mean weight and SEM (grams) was 35.66 ± 0.60 and 37.11 ± 0.62; for AFR and MS pups, respectively. Distance traveled and frequency of vertical behaviors

were separately analyzed through a four-way mixed ANOVA (between factors: early rearing condition, sex, and ethanol treatment; within factor: bin of assessment).

Distance Traveled. Figure 3 suggests that motor activity was greater in MS than in AFR pups regardless ethanol treatment. The ANOVA for distance traveled confirmed this impression, yielding a significant main effect of early rearing condition, $F_{1,51} = 23.21$, $p < .001$. It seems that, when tested immediately after removal of homecage, MS pups are significantly more sensitive to the mild stress induced by the unfamiliar environment. Given this baseline difference in novelty-induced motor activity, and following a strategy similar to that used in previous studies (e.g., Varlinskaya & Spear, 2012), ethanol-induced distance traveled was analyzed separately for MS and AFR pups.

The ANOVA for AFR pups revealed a significant main effect of bin of testing and a significant bin × ethanol treatment interaction, $F_{2,54} = 46.31$, $p < .001$; $F_{2,54} = 14.28$, $p < .001$. As observed in Figure 3, upper left section, and confirmed by planned comparisons, animals reared under normal facility conditions exhibited significant ethanol-induced motor activation during the initial testing bin. This ethanol-induced behavioral activation was, however, transient and turned to motor depression during testing bin 3. Specifically, during post-administration time 15–19 min, ethanol-treated AFR pups exhibited reduced distance traveled when compared to vehicle-treated counterparts.

The ANOVA for MS pups revealed a significant main effect of bin of testing, $F_{2,48} = 58.11$, $p < .0001$; and a significant bin × ethanol treatment interaction, $F_{2,48} = 14.03$, $p < 0.0001$. Planned comparisons revealed that MS pups exhibited ethanol-induced motor activation during the initial testing bin, yet they appeared to be resistant to ethanol-induced motor depression. Specifically, MS pups given 2.5 g/kg ethanol exhibited greater distance traveled than control pups during post-administration time 5–9 min; whereas distance traveled scores were similar across groups during the second and third testing bins (i.e., 10–14 and 15–19 min, respectively). These results have been depicted in Figure 3, upper right panel.

Vertical Behavior Scores. The ANOVA indicated significant main effects of ethanol treatment and testing bin, $F_{1,51} = 6.62$, $F_{2,102} = 66.71$, $ps < .05$. The interaction between testing bin and ethanol treatment achieved significance, $F_{2,102} = 6.95$, $p < .005$. The ANOVA also indicated a significant three-way interaction between ethanol treatment, bin of testing and early rearing condition, $F_{2,102} = 7.28$, $p < .005$. Planned comparisons indicated that ethanol-treated AFR controls displayed

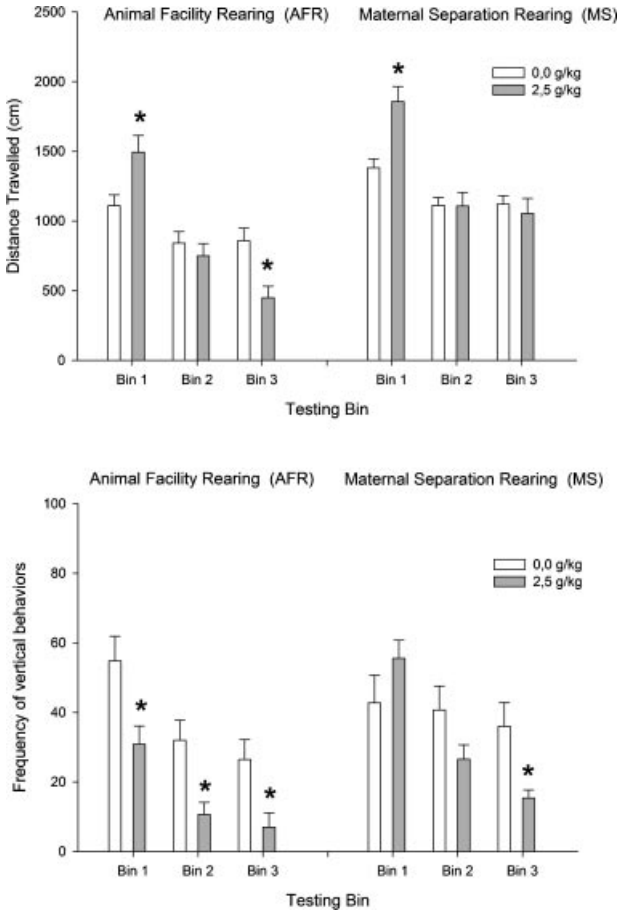

FIGURE 3 Ethanol-induced locomotor activity (distance traveled, cm; ethanol dose: 2.5 g/kg; see upper panels) and frequency of vertical behavior (lower panels) 5–9, 10–14, and 15–19 min after ethanol administration (bins 1, 2, and 3, respectively) in infant male and female rats that experienced either normal animal facility rearing (AFR, left panel) or daily episodes of maternal separation (MS, right panel) during postnatal days (PD) 1–14. The ANOVAs for distance traveled indicated significant overall greater activity scores in MS than in AFR pups. The analysis also revealed that ethanol induced significant motor activation during testing bin 1 and that this effect was similar across AFR and MS conditions. During testing bin 3 AFR animals treated with ethanol exhibited significantly less motor activity than vehicle-treated counterparts. This effect, indicative of ethanol-induced motor depression, was not observed in MS animals. ANOVAs also indicated an ethanol-induced suppression in vertical behaviors, which was more pronounced in AFR than in MS pups. The asterisk indicates a significant difference ($p < .05$) between an ethanol-treated group and its corresponding vehicle-treated control. Vertical bars indicate the SEM.

significantly less vertical behavior than vehicle-treated counterparts, throughout testing. The sedative effect of ethanol was not as clear in MS animals. Planned comparisons revealed a similar amount of vertical

behaviors in MS animals treated with ethanol or vehicle during testing bins 1 and 2. During bin 3 ethanol-treated animals had significantly fewer vertical behaviors than control counterparts (see Fig. 3, lower panel).

Discussion

The hypothesis was that removal of acute pre-test isolation would result in differences in sensitivity to the motor activating effects of 2.5 g/kg ethanol, between AFR and MS animals. Animals from both rearing conditions exhibited similar behavioral stimulation during the first testing bin. As testing progressed control AFR, but not MS, pups displayed significant ethanol-induced motor depression. This suggests lessened sensitivity to the sedative effects of ethanol in MS animals. Congruent with this suggestion, ethanol rapidly induced a significant and substantial reduction in frequency of vertical behaviors in AFR animals, whereas MS pups only exhibited a similar decrease in the final testing bin. An important result was that overall motor activity was greater in MS than in AFR animals.

EXPERIMENT 3

Experiment 2 revealed alterations in baseline level of exploration of the activity chamber in pups exposed to early neonatal stress. This may be the consequence of an exaggerated response to the stress of novelty in MS pups. The KOR system is thought to mediate some of the effects of environmental stress (Land et al., 2009). The aim of Experiment 3 was to reverse MS-induced alterations in spontaneous exploration through antagonism of the endogenous kappa opioid system.

Experimental Design

Experiment 3 assessed effects of kappa receptor blockade on novelty-induced motor activity. A 2 (sex) $\times$ 2 early rearing condition (AFR or MS) $\times$ 4 (nor-BNI treatment on PD14: 0.0, 1.0, 5.0, or 10.0 mg/kg) factorial design was employed. Number of animals that composed each group can be found in Table 1.

Methods

Throughout the first 2 weeks of life pups experienced daily MS or normal rearing conditions, following the conditions described in the previous experiments. On PD14, after the last episode of maternal deprivation, animals from both early rearing conditions were administered with the long-lasting kappa antagonist nor-BNI (0.0, 1.0, 5.0, or 10.0 mg/kg). Eighteen hours later they were removed from the homecage, weighed, and assessed for spontaneous motor activity during 15 min. These procedures have been depicted in Figure 1. Distance traveled and vertical behavior were measured through the automatic activity monitoring system described in the previous experiments.

Results

Mean weight and SEM (g) was 35.36 ± 0.60 and 35.52 ± 0.54; for AFR and MS pups, respectively. A factorial ANOVA (early rearing $\times$ sex) confirmed that MS and AFR animals exhibited similar body weight. Specifically, the ANOVA indicate the lack of significant main effects or significant interactions. Distance traveled and vertical behaviors were separately analyzed through a three-way mixed ANOVA (between factors: early rearing condition and sex; within factor: bin of assessment).

The ANOVA for distance traveled indicated a nearly significant main effect of nor-BNI, $F_{3,122} = 2.67$, $p = .051$, and a significant main effect of bin of testing, $F_{2,244} = 18.74$, $p < .05$. The interaction between early rearing condition and bin of testing also achieved significance, $F_{2,244} = 7.52$, $p < .01$. Post-hoc tests indicated that animals given the highest nor-BNI dose exhibited significantly less overall distance traveled than those given vehicle or 1.0 mg/kg nor-BNI 24-h before test. Total distance traveled (cm) in pups given 0.0, 1.0, 5.0, and 10.0 mg/kg nor-BNI was $2{,}933 \pm 124.46$, $2{,}988 \pm 108.01$, $2{,}850 \pm 119.52$, and $2{,}543.97 \pm 115.35$, respectively.

The loci of the two-way significant interaction was explored through planned comparisons and follow-up repeated measures ANOVA for each early rearing treatment. The planned comparisons revealed that MS animals exhibited, when compared to AFR controls, significantly less distance traveled during the first testing bin, yet they exhibited significantly more locomotion during the last testing bin. Moreover, ANOVAs revealed that AFR, but nor MS pups, exhibited significantly greater locomotion during the first testing bin than in bins 2 and 3 (significant effect of bin of testing, $F_{3,126} = 30.35$, $p < .001$). As depicted in Figure 4 and confirmed by planned comparisons, locomotion in control pups dropped sharply from testing bin 1 to bins 2 and 3. That was not the case for MS animals, in which locomotion remained stable across bins.

The ANOVA for vertical behavior scores revealed a significant interaction between early rearing condition and bin of testing, $F_{2,234} = 5.47$, $ps < .005$. Subsequent follow-up repeated measures ANOVA for each early rearing treatment revealed that vertical behavior decreased from bin 1 to bins 2 and 3 in AFR pups

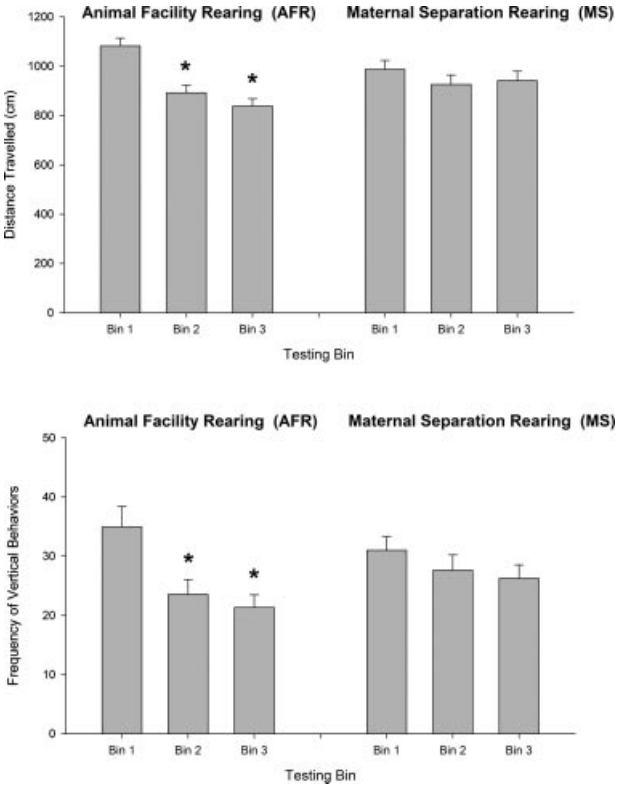

FIGURE 4 Spontaneous, novelty-induced locomotor activity (distance traveled, cm; upper panels) and frequency of vertical behavior (lower panel) at 1–4, 5–9, and 10–14 min of testing (bins 1, 2, and 3, respectively) in male and female infant rats that experienced either normal animal facility rearing (AFR, left panel) or daily episodes of maternal separation (MS, right panel) during postnatal Days (PD) 1–14. Twenty-four hours before testing animals were given 0.0, 1.0, 5.0, or 10.0 mg/kg nor-BNI. ANOVAs indicated that distance traveled and vertical behaviors in AFR animals were greater in testing bin 1 than in testing bins 2 and 3. These effects were not observed in MS animals, which displayed similar scores across testing bins. Data is depicted collapsed across sex and nor-BNI treatment. These factors did no exert significant main effects nor were involved in significant interactions. The asterisk indicates a significant difference ($p < .05$) between motor scores in testing bin 2 or 3 and motor scores in testing bin 1.Vertical bars indicate the SEM.

($F_{2,126} = 21.37$, $ps < .001$), but remained stable across testing bins in MS animals, $F_{2,136} = 1.26$, $ps > .20$. These results can be found in the lower panel of Figure 4.

Discussion

These data confirmed that MS pups exhibit alterations in baseline level of exploration of the activity chamber. Our hypothesis of nor-BNI blocking this effect of early neonatal stress, however, was not corroborated. Treatment with the KOR antagonist induced a similar effect in MS and in AFR animals.

GENERAL DISCUSSION

The main finding of the present study is that the effects of early MS, occurring during the first and second weeks of life, emerged quickly during development. MS altered responsivity to novelty and ethanol-induced motor stimulation as well as depression during infancy.

During post-administration time 5–20 min MS and AFR infants exhibited similar motor stimulation after 2.5 or 3.0 g/kg ethanol. A novel and important finding was the greater sensitivity of MS animals to the motor stimulating consequences of a moderate ethanol dose. MS, but not AFR, pups displayed significant behavioral activation after 1.25 g/kg ethanol, throughout the rising phase of the blood ethanol curve. Enhanced sensitivity to ethanol-induced activation may reflect greater vulnerability to ethanol reinforcement. Ethanol's appetitive effects are presumably maximal during the rising limb of the blood ethanol curve (Risinger & Cunningham, 1992).

Persistence of ethanol-induced locomotion in pups exposed to MS may be due to their greater resistance to ethanol-induced sedation. This hypothesis received some support in Experiment 2: a relatively high (2.5 g/kg) ethanol dose induced a transient, yet similar, enhancement of distance traveled in AFR and MS pups. Early-stressed animals, however, were insensitive to the ethanol-induced depression of distance traveled exhibited by control animals in testing minutes 14–19. MS pups also exhibited increased resistance to the suppressive effects of 2.5 g/kg ethanol on vertical behaviors. The sedative consequences of 2.5 g/kg ethanol on vertical behaviors emerged rapidly in AFR pups, but were only expressed at the last testing bin in MS counterparts.

It has been suggested that ethanol-induced sedation can acutely reduce ethanol intake (Schramm-Sapyta et al., 2009). Lessened sensitivity to ethanol-induced motor depression, therefore, may put MS subjects at risk for escalating in alcohol intake. There is, however, a caveat to this hypothesis of reduced sensitivity to ethanol-induced motor depression. When testing occurred during postadministration time 30–45 min, MS animals given 2.5 g/kg ethanol had less motor activity than vehicle-treated MS animals, yet only a higher dose (3.0 g/kg) induced motor depression in animals reared under normal animal facility conditions.

Altogether, the results suggest that chronic MS alters the balance between ethanol's activating, and presumably appetitive, effects and the sedative effects of the drug, which are commonly assumed to be aversive.

This is congruent with a previous report of MS altering ethanol-induced conditioned place preference in infant rats (Pautassi et al., 2012). Another important result is that AFR pups exhibited a direct relationship between ethanol dose and motor stimulation during the 5–20 min postadministration time (Experiment 1). This graded, dose-response pattern was absent in MS pups, which exhibited similar stimulation by ethanol, regardless of dose.

It could be argued that ethanol-related differences between AFR and MS animals can be attributed to alterations in the metabolism of ethanol. Yet, in our previous study (Pautassi et al., 2012) no differences in blood ethanol levels were found across rearing conditions.

Overall locomotion scores were higher in MS than in AFR animals in Experiment 2. In Experiment 3 animals with early deprivation of maternal care exhibited a transient decrease in spontaneous exploration of the open field, yet by the end of testing they continued locomoting significantly more than control, unstressed counterparts, and also failed to exhibit habituation to locomotion. This pattern is in agreement with Meaney, Brake, and Gratton's (2002) suggestion that MS animals display higher initial levels of freezing in an open field, thus resulting in low overall levels of locomotion. As testing progresses, however, MS animals usually exhibit motor hyperactivity.

The KOR system mediates acute and chronic stress (Land et al., 2009); and it has been suggested that MS induce alterations of opioidergic transmitter systems (Vazquez et al., 2005). We hypothesized that blockade of KOR would ameliorate MS-induced alterations in exploratory behavior. This expectation, however, was not corroborated. Overall locomotion scores in Experiment 3 were still higher in MS than in AFR pups, after the administration of nor-BNI (a specific, long lasting KOR antagonist). The highest dose of nor-BNI similarly decreased levels of activity in MS and AFR.

In Experiment 1, AFR subjects seem to display greater vertical behavior than MS counterparts, regardless ethanol doses. Wall climbing is often a reliable index of unconditioned and conditioned aversive responding (Arias, Pautassi, Molina, & Spear, 2010). Therefore, it is possible that lower level of vertical behaviors in MS than in AFR animals reflected reduced level of aversive response to the novelty of the activity chamber. This difference, however, was not observed in Experiments 2 and 3. It should also be taken into account that distance traveled and vertical behavior are topographically incompatible behaviors.

In general, the present results are consistent with previous work. In one study open field activity at 15 days of age was greater in MS than in control animals and MS adolescent animals exhibited subtle, yet significant alterations, in play behavior (Arnold & Siviy, 2002). Spivey et al. (2008) exposed Holtzman rats, known for their greater predisposition to stress-induced depression, to 360 min of daily separation, throughout most of the first 2 weeks of life. These animals exhibited significantly reduced exploration in a novel open field and reduced time spent in the bright, exposed side of a light-dark apparatus at adolescence.

A caveat of the present study is that distance traveled and rearing behavior are within-subject, repeated measures, but they were separately analyzed. The substantial difference in scale across these variables precluded conducting an overall analysis. Another caveat is that the significant differences in body weight between MS and AFR pups, observed in Experiment 1, was not found in Experiments 2 or 3.

Overall, the present study suggests that early environmental stress alters spontaneous motor activity during infancy, enhances responsivity to ethanol-mediated motor stimulation and lessens responsivity to ethanol-induced sedation. To our knowledge, this is the first time that significant alterations in ethanol-induced psychomotor effects as a function of early neonatal stress are reported. The stimulant effects of ethanol, particularly those occurring during the rising phase of the blood ethanol curve, have been considered an index of ethanol-induced reinforcement (Pautassi et al., 2009; Quoilin et al., 2012); and heightened sensitivity to these effects have been found in humans at-risk for developing alcohol-related problems (Conrod et al., 1997; King et al., 2002). The idiosyncratic pattern of response to ethanol observed in early-stressed subjects may put them at risk for subsequent engagement in problematic alcohol intake.

NOTES

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