

EFFECTS OF PRENATAL AND POSTNATAL MATERNAL ETHANOL ON OFFSPRING RESPONSE TO ALCOHOL AND PSYCHOSTIMULANTS IN LONG EVANS RATS

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Abstract—An important factor that may influence addiction liability is exposure during the early life period. Exposure to ethanol, early in life, can have long-lasting implications on brain function and drugs of abuse response later in life. In the present study we investigated the behavioral responses to ethanol and to psychostimulants in Long Evans rats that have been exposed to pre- and postnatal ethanol. Since a relationship between heightened drug intake and susceptibility to drug-induced locomotor activity/sensitization has been demonstrated, we tested these behavioral responses, in control and early life ethanol-exposed animals. The young adult male and female progeny were tested for locomotor response to alcohol, cocaine and *d*-amphetamine. Sedative, rewarding effects of alcohol and alcohol consumption were measured. Our results show that early life ethanol exposure behaviorally sensitized animals to subsequent ethanol and psychostimulants exposure. Ethanol-exposed animals were also more sensitive to the hyperlocomotor effects of all drugs of abuse tested and to those of the dopamine receptor agonist apomorphine. Locomotor sensitization to repeated injections of cocaine was facilitated in ethanol-exposed animals. Ethanol-induced conditioned place preference was also facilitated in ethanol-exposed animals. Ethanol consumption and preference were increased after early life ethanol exposure and this was associated with decreased sensitivity to the sedative effects of ethanol. The altered behavioral responses to drugs of abuse were associated with decreased striatal dopamine transporter and hippocampal NMDAR binding. Our results outline an increased vulnerability to rewarding and stimulant effects of ethanol and psychostimulants and support the epidemiological and clinical data that suggested that early chronic exposure to ethanol may increase the propensity for later self-administration of ethanol or other substances. © 2009 IBRO. Published by Elsevier Ltd. All rights reserved.

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Abbreviations: ANOVA, analysis of variance; BEL, blood ethanol level; CPA, conditioned place aversion; CPP, conditioned place preference; DAT, dopamine transporter; LORR, loss of righting reflex; NMDA, *N*-methyl-D-aspartate; NMDAR, *N*-methyl-D-aspartate receptor; RM, repeated measures.

Ethanol use during pregnancy is one of the most common known causes of preventable birth defects, and can result in long-term deficits in physical and cognitive growth and development. The combined incidence of fetal alcohol syndrome and its partial forms, fetal alcohol effects and alcohol-related neurodevelopmental disorder, has been estimated to 9.1 per 1000 (Sampson et al., 1997). Brain imaging studies have identified structural changes in various brain regions of children exposed to prenatal ethanol including basal ganglia, corpus callosum, cerebellum and hippocampus that may account for the cognitive deficits (Mattson et al., 2001). Clinical studies reported that fetal ethanol exposure may increase risk of later ethanol and other drug dependence (Famy et al., 1998; Yates et al., 1998; Baer et al., 1998, 2003; Alati et al., 2006).

This epidemiological evidence has also been suggested in preclinical studies. Numerous neurobehavioral effects have been detected in animals following pre- and/or postnatal ethanol exposure and among them, early ethanol exposure can yield later enhancement of ethanol intake in rodents (Spear and Molina, 2005; Chotro et al., 2007). Earlier studies demonstrated altered response to the hypothermic effects of different drugs of abuse such as ethanol (Abel et al., 1981; Taylor et al., 1981) and morphine (Nelson et al., 1986), after prenatal ethanol exposure.

The long-term effects of early life ethanol exposure on drugs of abuse vulnerability and the neurobiological substrates involved in this vulnerability remain to be elucidated, even though numerous studies identified some mechanisms involved in CNS dysfunctions observed after *in utero* ethanol exposure (see for review Guerri, 2002). In addition to ethanol, we also analyzed the effects of both amphetamine and cocaine in order to test if early life ethanol exposure affects only the future response to ethanol or also alters the response to other drugs of abuse or dopaminergic agents. In this regard, previous studies (Xu and Shen, 2001; Choong and Shen, 2004) have shown that psychostimulants can restore the alterations of the neuronal activity of the ventral tegmental area (a critical brain area involved in addiction) by prenatal ethanol exposure, thus suggesting that animals exposed to prenatal ethanol may respond differently to the effects of psychostimulants. It is noteworthy, that a previous report has shown an increase in ethanol responding after prenatal exposure to cocaine in mice (Kelley and Middaugh, 1996), suggesting a potential heterosensitization.

In order to understand the mechanisms underlying the increased vulnerability to drugs of abuse disorders induced by early life ethanol exposure, we used a pre- and post-

natal ethanol exposure paradigm in rat (Naassila and Daoust, 2002; Othman et al., 2002; Dubois et al., 2006, 2008; Kervern et al., 2009) to investigate the long-term alterations on both behavioral responses to drugs of abuse and neurotransmission systems.

EXPERIMENTAL PROCEDURES

Drugs

All chemicals were obtained from Sigma Chemicals (Paris, France). [³H]MK-801, [³H]raclopride, [³H]mazindol, [³H]muscimol and [³H]SCH23390 were obtained from PerkinElmer (Courtaboeuf, France). Cocaine hydrochloride, *d*-amphetamine, quinpirole, sulpiride and SCH23390 were obtained from Sigma Chemicals (Paris, France). Ethanol (95% v/v) was obtained from Carlo Erba réactifs (Val de Reuil, France). Ethanol was diluted to 20% v/v in physiological saline prior to i.p. injection. Cocaine and *d*-amphetamine injections were made in volumes of 1 ml/100 g and ethanol injections were made in volumes of 1.25 ml/100 g. Saline injections were made in volumes equal to that of the corresponding drug for each animal.

Animals and prenatal ethanol exposure

Adult male and virgin female Long Evans rats (200–300 g) were obtained from Elevage Janvier (Le Genest-Saint-Isle, France), maintained on a 12-h light/dark cycle (light on between 7 a.m. and 7 p.m.) and were used at least after 1 week of habituation in our facilities. The procedures described comply with ethical principles and guidelines for care and use of laboratory animals adopted by the European Community, Law 86/609/EEC and were approved by the Animal Care and Use Committee responsible for our institution. One hundred forty females were randomly assigned to two groups. The ethanol-treated group received as sole drinking fluid a 10% v/v ethanol solution, prepared from 95% ethanol, for 4

weeks before mating and had unlimited access to standard rat chow (UAR, France; standard diet). To avoid any dehydration, the forced ethanol consumption group had limited access to water every day for 2 h in the morning (during the light phase). Importantly, there was no difference in the daily total fluid consumption between the two groups (see Table 1). The *ad libitum* control group had unlimited access to standard rat chow and water. After successful mating, the ethanol-treated group was maintained on 10% ethanol solution until after delivery and also throughout gestation and lactation periods (Othman et al., 2002; Naassila and Daoust, 2002; Dubois et al., 2006, 2008; Kervern et al., 2009). This ethanol exposure is associated with an ethanol intake of 7–9 g/kg body weight/day in the dams before and during gestation and with an ethanol intake of 16–20 g/kg body weight/day during the two last weeks of lactation and peak BELs reached an average of 100 mg/dl (see Table 1) (Naassila and Daoust, 2002).

This procedure of forced oral ethanol (10%–18% in drinking water) consumption by dams has been used in numerous other studies (Jänicke and Coper, 1993; Othman et al., 2002; Naassila and Daoust, 2002; Carneiro et al., 2005; Dubois et al., 2006, 2008; Nowak et al., 2006; Servais et al., 2007; Kervern et al., 2009). The exposure to ethanol during the early postnatal period (i.e. the three weeks of lactation) was maintained since part of this period corresponds to the third trimester in human neural development (Andersen, 2003). Naive rats were used in each experiment and a particular animal was used only in a single behavioral test. One thousand eighty-four offspring born from 140 dams were used in the present study. A maximum of two siblings per litter (one male and one female) was randomly assigned to each of the experimental groups. All behavioral experiments have been conducted with two-month-old offspring and only the experiments on behavioral sensitization to cocaine have been conducted in both two and three-month-old offspring. Females have been tested for their basal locomotor activity, the hypnotic effects of ethanol and the locomotor effects of drugs of abuse. Hippocampal and striatal brain regions were dissected from two-month-old male pups. The animals used in

Table 1. Ethanol consumption, total fluid consumption, dam weight gain, litter size, pup weight at birth and at postnatal day 60 and BELs measured in offspring and dams during the 2nd week of gestation and the 3rd week of lactation

Measure	Ethanol-exposed group	Control group
Daily ethanol consumption (g ethanol/kg body weight) (<i>n</i> =20)		
Before gestation (4 weeks)	6.88±0.34	—
Gestation	8.62±0.42	—
Lactation week 1	13.19±0.93	—
Lactation week 2	16.12±0.56	—
Lactation week 3	20.35±1.17	—
Daily total fluid consumption (ml/dam)	(<i>n</i> =20)	(<i>n</i> =20)
Before gestation (4 weeks)	40.1±4.05	37.9±2.10
Gestation	46.2±6.52	47.3±3.32
Lactation week 1	62.3±7.94	65.8±6.30
Lactation week 2	78.6±5.56	83.6±8.09
Lactation week 3	75.10±6.64	78.8±7.40
Mean weight gain (g)	62±6.9 (<i>n</i> =20)	55.82±16.25 (<i>n</i> =20)
Litter size	9.46±0.69 (<i>n</i> =213)	9.02±0.43 (<i>n</i> =201)
Mean pup birth weight	6.5±0.5 (<i>n</i> =213)	7.7±0.7 (<i>n</i> =201)
Body weight at PND 60	201.7±3.5 (<i>n</i> =213)	206.1±4.2 (<i>n</i> =201)
Mean BELs in suckling offspring (mg/dl)	19.1±5.5 (<i>n</i> =10)	—
Maximum BELs in pregnant dams (mg/dl)	124.8 (<i>n</i> =10)	—
Minimum BELs in pregnant dams (mg/dl)	8.7 (<i>n</i> =10)	—
Maximum BELs in lactating dams (mg/dl)	161.6 (<i>n</i> =10)	—
Minimum BELs in lactating dams (mg/dl)	10.0 (<i>n</i> =10)	—

Mean BELs were determined from the blood samples collected at 08:00 h. BELs in dams are reported as minimum and maximum values obtained at that time of blood sampling. Values represent the means±SEM. —, Not applicable.

the brain assays were not used in behavioral tests. All behavioral experiments were conducted between 8 AM and 3 PM.

Measurement of blood ethanol concentration

We took about 20 μ l of tail blood samples obtained in a separate set of 10 dams (during the 2nd week of gestation and the 3rd week of lactation) and in 10 suckling offspring. In order to study the ethanol clearance in 60-day-old offspring, BELs were measured at the indicated time-points after an i.p. injection of ethanol 4.0 g/kg body weight (20% (w/v) prepared in saline) in males ($n=4$) and females ($n=4$). Samples were microcentrifuged for 10 min (14,000 rpm) at 4 °C and analyzed immediately. BELs were measured using an Analox AM1 analyzer available from IMLAB (Lille, France). The reaction is based on the oxidation of ethanol by ethanol oxidase in the presence of molecular oxygen ($\text{ethanol} + \text{O}_2 \rightarrow \text{acetaldehyde} + \text{H}_2\text{O}_2$). Under the conditions of the assay, the rate of oxygen consumption is directly proportional to the ethanol concentration. Single point calibration is done for each set of samples with reagents, provided by Analox Instruments that read 100 mg/dl (21.7 mmol/l). The sensitivity of the assay is 1 mg/dl, the precision is 1%–2% and the curve is linear up to 400 mg/dl.

Ethanol consumption in offspring

Fluid intake and body weight were assessed every two days. Young adult (two-month-old) control and ethanol-exposed rats ($n=12$ /group) were individually housed in plastic cages with access *ad libitum* to standard rodent chow and had a 10% v/v ethanol bottle as the only source of fluid for three weeks. Free choice ethanol consumption was also measured in ethanol naive rats with a 10% v/v ethanol solution for three weeks.

Sensitivity to ethanol-induced LORR

LORR was measured after i.p. injection of ethanol 4.0 g/kg, 20% (w/v) mixed in isotonic saline ($n=6$ –8/group). At the onset of ethanol-induced sedation, each rat was placed on its back in a plastic U-shaped trough. The time (in min) that elapsed between the ethanol injection and righting of the rat on all four paws, measured three times within a 30-s interval, was used as the index of time to regain the righting reflex.

CPP apparatus and procedures

The CPP apparatus (BIOSEB, Chaville, France) consisted of two compartments (30 \times 20 \times 20 cm) with distinct visual and tactile cues. One of the compartments had grey colored walls and a light-colored stainless steel floor and the opposite compartment had black and white striped walls and a dark smooth floor. The two compartments were separated by a guillotine door. Distance and time spent in each compartment were measured by computer-interfaced infrared photobeams (16 \times 16 cm). Both compartments were illuminated by dim light with 40 lx brightness. The experiment consisted of three distinct phases: preconditioning phase (day 1), conditioning phase (days 2–5), and post-conditioning test (day 6) as previously described (Houchi et al., 2005). To control possible innate preferences for one of the two conditioning compartments, rats underwent a single preconditioning session. Initial place preference was determined for each rat by the side in which they spent more than 600 s out a 20 min trial. Place preference conditioning was conducted using an unbiased procedure (Cunningham et al., 2003).

During the conditioning phase, animals were randomly assigned to undergo either drug conditioning in the morning and saline conditioning in the afternoon, or vice versa. Animals received a total of two injections per day. Immediately after ethanol (2.0 and 3.0 g/kg) or saline injection, each subject ($n=7$ –10/group) was confined to the appropriate compartment for 20 min (guillotine door closed). The drug- and saline-paired conditioning compart-

ments and the time of the day of the drug or saline conditioning session (morning or afternoon) were randomly assigned and counterbalanced across all groups. Conditioning sessions were conducted twice daily for 4 days, with a duration of 7 h between conditioning sessions in order to achieve a total clearance of ethanol before the 2nd session. Post-conditioning test was conducted by placing animals between the two compartments (guillotine door removed) and allowing free access to both conditioning compartments for 20 min. CPP was determined by comparing time spent (in s) in drug-paired compartment before (preconditioning) and after (test) conditioning session.

Locomotor activity

The analysis of spontaneous and drug-induced horizontal locomotion and rearing was conducted in the LE 88811 IR motor activity monitor (BIOSEB, Chaville, France). Animals were confined to 45 cm² clear acrylic plastic chamber, in which locomotion was measured from photocell beam interruptions. Photocell beams transected the chamber 2 cm above the floor at 16 sites along each side. Test chambers were shielded from external noise and light, but each test field was illuminated with a white fluorescent light and was fully ventilated. Rats ($n=6$ –11/group) were injected i.p. with saline or cocaine (5.0, 10, 15 and 25 mg/kg) or amphetamine (0.6, 1.2 and 2.4 mg/kg) and were immediately placed in activity monitors for a 20-min test duration. Each rat received only a single injection of either saline or drugs. Locomotor response was also measured after repeated injections of cocaine (25 mg/kg on day 1 and 12.5 mg/kg the two following days). Three days after the last injection (on day 6) rats were challenged with an acute injection of cocaine (12.5 mg/kg) before their introduction into the activity boxes, and their locomotor activity was measured for 20 min. This evaluation of the locomotor response to repeated administration of cocaine was performed in 2-month-old rats. Because the locomotor response to cocaine on day 1 was different between the control and ethanol-exposed groups, this evaluation was also performed in 3-month-old rats, a time point at which both groups displayed similar response to cocaine on day 1.

In another set of experiments, spontaneous activity was recorded for 30 min (habituation to the experimental procedure) then rats ($n=6$ –11/group) were injected i.p. with apomorphine (2 mg/kg) or quinpirole (0.25 mg/kg) or SKF38393 (8 mg/kg) or with increasing doses of MK-801 (0.1, 0.3 and 0.5 mg/kg) and locomotor responses were recorded for an additional 30–60 min period. The apomorphine, quinpirole and SKF38393 doses were chosen based on previous studies (Breese et al., 1987; Archer et al., 2003) and on our pilot experiments to produce optimal locomotor stimulation. For example, increasing doses of quinpirole (0.1, 0.25 and 0.5 mg/kg) produced a biphasic effect with a little stimulation, a significant stimulation and no effect at the higher dose (hypolocomotor effects), respectively.

Membrane preparation and binding studies

Membrane preparation and binding experiments were performed as previously described (Naassila et al., 1998). Briefly, hippocampi from individual pups (10 different litters per group) were rapidly removed on ice and homogenized in five volumes of 0.32 M sucrose using an Elvehjem type potter. After the first centrifugation (3000 \times g, 4 °C, 15 min), the supernatant was further centrifuged (48,000 \times g, 4 °C, 15 min). The pellet was carefully rinsed at least 10 more times using five volumes of Tris–HCl buffer (pH 7.4, 20 °C). The final pellet was resuspended in a final volume of 1.5 ml and was frozen (–18 °C) until use and a 10 μ l aliquot was used for protein measurement by the method of Lowry et al. (1951). The assay conditions for each of the ligands were as follows: (i) D1R binding: [³H]SCH23390 (1.5 nM, s.a. 85 Ci/mmol) was incubated for 1 h at 30 °C in assay buffer (50 mM Tris–HCl, 4 mM MgCl₂, pH 7.4). (ii) D2R binding: [³H]raclopride (1.5 nM, s.a.

80 Ci/mmol) was incubated for 1 h 30 at 25 °C in assay buffer (50 mM Tris–HCl, 1 mM CaCl₂, 5 mM MgCl₂, 5 mM KCl, 120 mM NaCl, 0.1% ascorbate, pH 7.4). (iii) DAT binding: [³H]mazindol (4 nM, s.a. 19.5 Ci/mmol) was incubated for 1 h 30 at 4 °C in (50 mM Tris–HCl, 120 mM NaCl, 5 mM KCl, pH 7.4, 20 °C). (iv) NMDAR binding: [³H]MK-801 (2.5 nM, s.a. 17.1 Ci/mmol) was incubated for 1 h 30 at 30 °C in assay buffer (50 mM Tris–HCl, pH 7.4 containing 100 μM glutamate and 30 μM glycine). (v) GABA_A binding: [³H]muscimol (2 nM, s.a. 28.5 Ci/mmol) was incubated for 30 min at 4 °C in assay buffer (50 mM Tris–citrate, pH 7.1). One micromolar SCH23390, 10 μM sulpiride, 10 μM mazindol, 100 μM MK-801 and 1 mM GABA were used to define non-specific binding for D1R, D2R, DAT, NMDAR and GABA_AR, respectively. The radioligand concentrations were near the K_d value. After incubation in a final volume of 500 μl assay buffer with 100–150 μg proteins, samples were filtered through Whatman GF/B (45 μm pore size) glass fiber filters pre-soaked in 0.5% polyethylenimine (PEI) and washed with an additional 2×5 ml ice cold assay buffer. Radioactivity was determined using 5 ml of ACS scintillation fluid and counted in a Wallac 1414 Winspectral liquid scintillation counter (PerkinElmer, 60% efficiency for [³H]).

Statistical analysis

All values are presented as means ± SEM. A two-way ANOVA and Tukey's post hoc test were used to analyze locomotor responses and LORR results. A two-way RM ANOVA and Tukey's post hoc test were used to analyze locomotor response to repeated cocaine administration, blood ethanol metabolism and ethanol consumption results. A three-way (ethanol dose × pre-treatment [control or early life ethanol] × session) RM-ANOVA and Tukey's post hoc test were used to analyze CPP experiments. A three-way (dose × pre-treatment [control or early life ethanol] × gender) RM-ANOVA and Tukey's post hoc test were used to analyze the locomotor response to drugs of abuse. The gender factor was analyzed only in the LORR and locomotion experiments.

RESULTS

Ethanol consumption by dams, body weight and litter size

The average daily ethanol consumption expressed as pure ethanol intake (g/kg body weight, g/kg bwt) was stable before mating and during the gestation period reaching 6.88 ± 0.34 g/kg bwt and increased during the lactation period reaching 20.35 ± 1.17 g/kg bwt during the last week of lactation (see Table 1). This level of ethanol consumption is consistent with previous studies and this procedure produced pharmacologically significant BELs in dams and suckling offspring (Naassila and Daoust, 2002; Othman et al., 2002; Dubois et al., 2006; Servais et al., 2007; see Table 1). The BELs range (maximum and minimum values) obtained in dams reflects the variability in ethanol intake during 24 h. The litter size, mean pup birth weight and maternal weight gain of the ethanol group were not different compared to control group.

Ethanol consumption in male offspring

When 2-month-old rats were submitted to a forced 10% ethanol solution exposure during three weeks, early life ethanol-exposed rats displayed an increase in ethanol consumption (main effects of pre-treatment $F_{(1,379)}=5.32$; $P<0.05$; and time $F_{(2,379)}=11.04$; $P<0.001$; and significant pre-treatment × time interaction $F_{(2,379)}=1.54$; $P=0.05$). Tukey's post hoc analysis revealed that early life ethanol

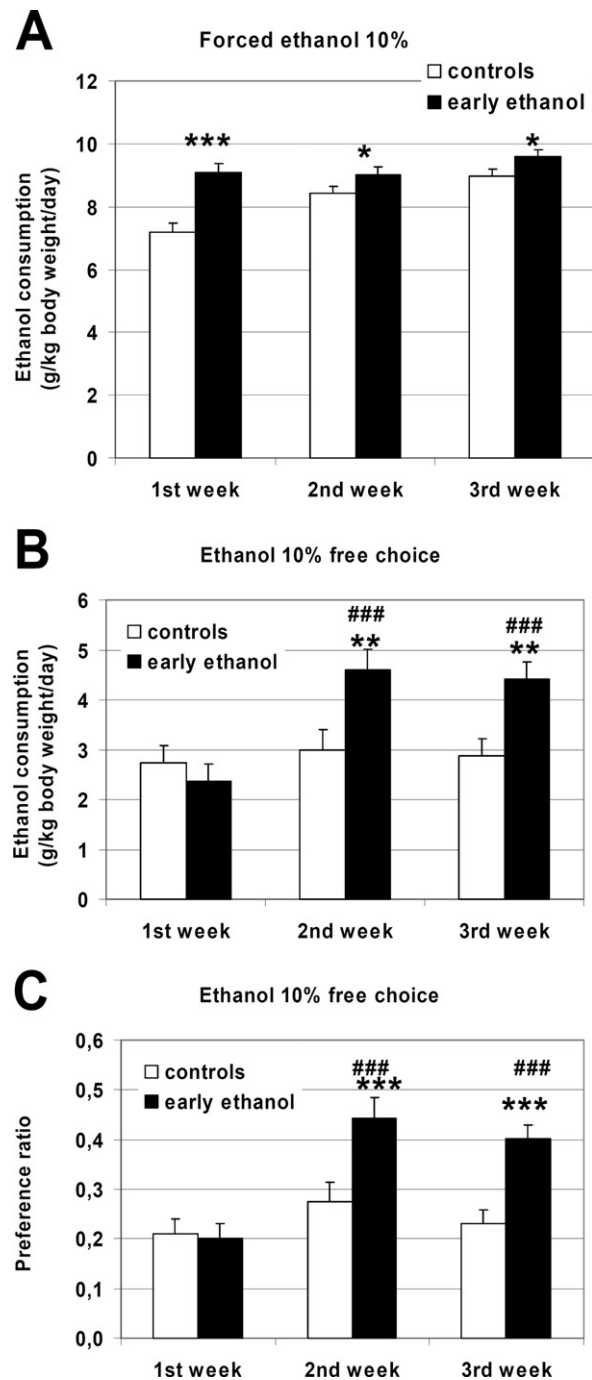


Fig. 1. Ethanol consumption in control and early life ethanol-exposed male rats. Forced ethanol (10%) solution consumption (g pure ethanol/kg body weight/day) was measured for 3 weeks (A). Free choice ethanol (10%) solution consumption (g pure ethanol/kg body weight/day, B) and preference (C) were measured for 3 weeks. * $P<0.05$, ** $P<0.01$, *** $P<0.001$ compared to respective control group and #### $P<0.001$ compared to respective value of the first week (Student's *t*-test). $n=12$ Per group.

exposure induced an increase in ethanol consumption (+27% the first week, $P<0.001$ and +7% during the two following weeks, $P<0.05$; Fig. 1A).

A separate set of offspring was used to measure ethanol (10%) consumption in a free choice situation (Fig. 1).

The results showed that early life ethanol-exposed rats displayed an increase in both ethanol consumption (main effects of pre-treatment $F_{(1,379)}=7.94$; $P<0.005$; time $F_{(2,379)}=5.90$; $P<0.005$; and significant pre-treatment \times time interaction $F_{(2,379)}=4.06$; $P<0.05$) (Fig. 1B) and preference (main effects of pre-treatment $F_{(1,379)}=13.13$; $P<0.001$; time $F_{(2,379)}=8.18$; $P<0.001$; and significant pre-treatment \times time interaction $F_{(2,379)}=4.10$; $P<0.05$, Fig. 1C). Tukey's post hoc analysis revealed that early life ethanol-exposed animals displayed an increase in both ethanol consumption (+35%, $P<0.001$) and preference (+40%, $P<0.001$) but only during the second and third weeks.

Ethanol (4.0 g/kg)-induced LORR and ethanol metabolism

Male and female rats exposed to ethanol during early life were less sensitive to the sedative/hypnotic effects of ethanol

(main effects of early life treatment: $F_{1,32}=11.92$, $P<0.001$, gender $F_{1,32}=15.58$, $P<0.005$ and significant early life treatment \times gender interaction $F_{1,32}=3.58$, $P<0.05$; Fig. 2A). Tukey's post hoc analysis indicated that both male and female rats exposed to early life ethanol were more resistant to the hypnotic effects of ethanol ($P<0.05$). Early life ethanol exposure did not affect rate of metabolism of alcohol (Fig. 2B).

Ethanol-induced place preference

In order to study differences in the rewarding properties of ethanol in rats exposed to ethanol, we analyzed drug reward in the place preference paradigm where rats learned to associate the characteristics of the experimental environment with the effects of an injection of either saline or drug. The difference in time spent in either the saline- or

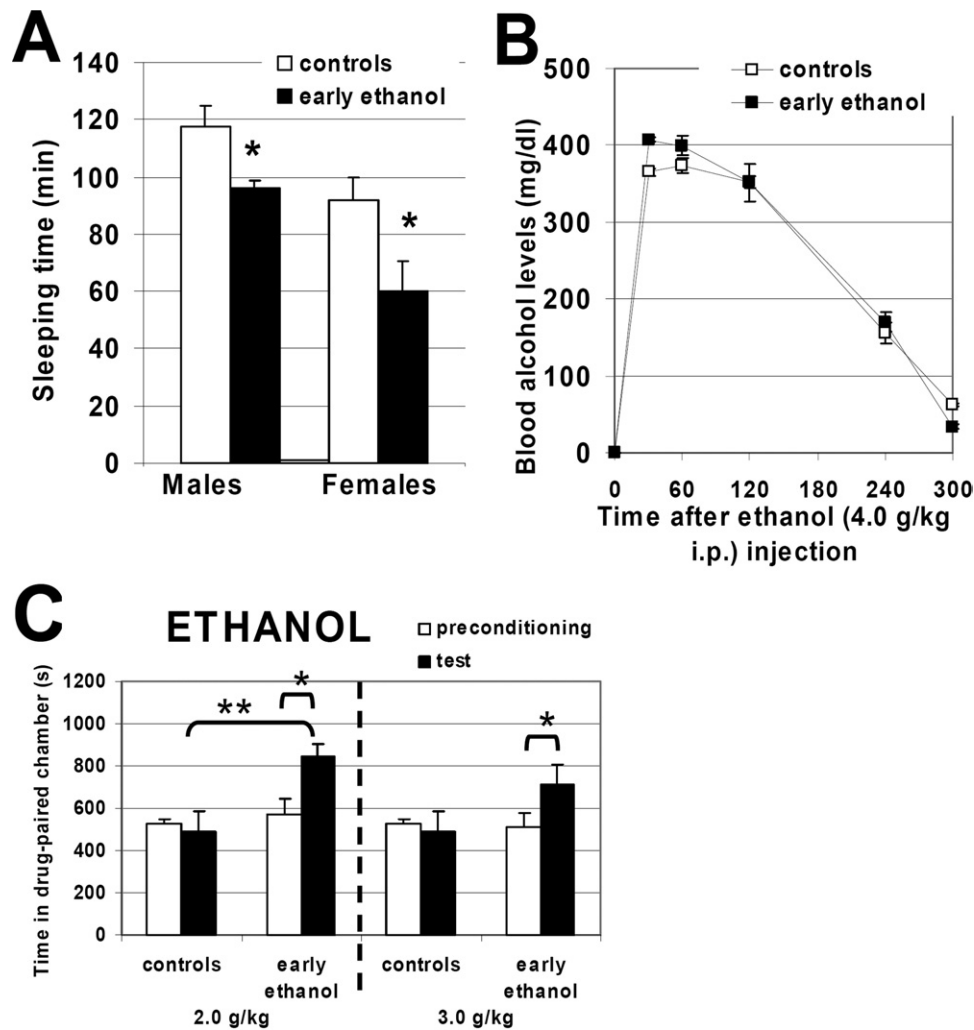


Fig. 2. Ethanol-induced LORR (A), ethanol metabolism (B) and ethanol-induced CPP (C) in control and early life ethanol-exposed rats. Ethanol (4.0 g/kg)-induced sedation in male and female rats exposed pre- and postnatally to ethanol and controls (A). Control and ethanol-exposed groups ($n=6-8$ /group) were injected i.p. with 2.0 or 3.0 g/kg ethanol. * $P<0.05$, ** $P<0.01$ and *** $P<0.005$ compared to respective control group. (B) Blood alcohol levels measured after ethanol (4.0 g/kg) injection ($n=4$ /group). (C) Ethanol-induced CPP ($n=7-10$ /group). Data are expressed as mean time spent \pm SEM in the drug-paired chambers during the 20-min preconditioning (□) and 20-min test phases (■). * $P<0.05$ compared to respective preconditioning session and ** $P<0.01$ compared to preconditioning or controls as indicated.

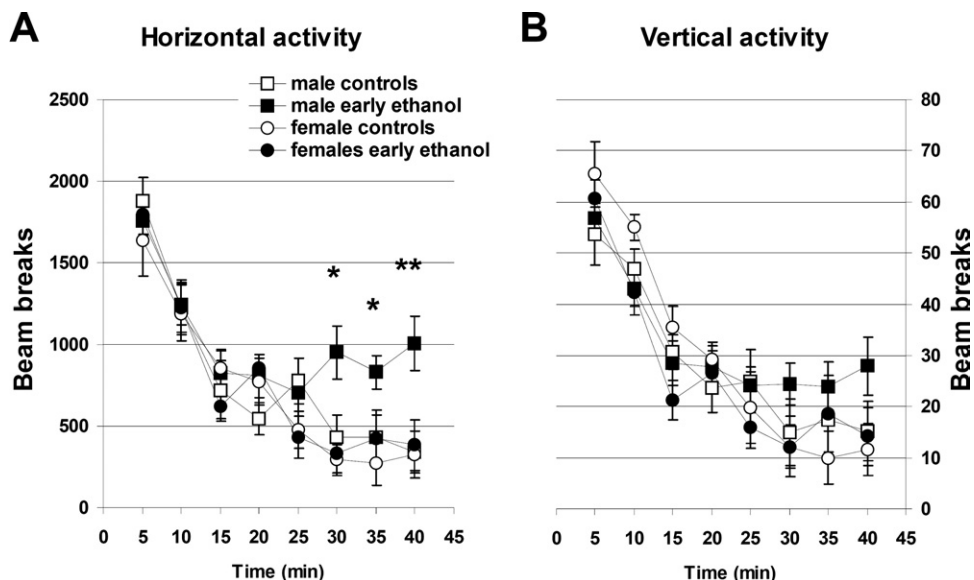


Fig. 3. Spontaneous locomotor activity. Basal locomotor activity was measured in animals that did not receive saline i.p. injections. (A) Horizontal activity expressed as numbers of beam breaks/40 min. (B) Vertical activity expressed as number of rearings. Activity habituated during the 40 min in all groups. The effects of early life ethanol exposure mainly oppose the decrease in locomotion (habituation) which normally occurs in control animals. * $P < 0.05$, ** $P < 0.01$ compared to respective control group.

the drug-paired compartment between the pre-conditioning and post-conditioning sessions at each dose of drug was used as a measure to reflect CPP.

Three-way RM-ANOVA revealed that there was a difference between control and early life ethanol-exposed animals (main effects of pre-treatment: $F_{1,32} = 18.52$, $P < 0.0001$; session: $F_{1,32} = 3.83$, $P < 0.05$ and a significant pre-treatment \times session interaction $F_{1,32} = 15.79$, $P < 0.0005$; Fig. 2C). There was no main effect of the ethanol dose ($F_{1,32} = 1.37$, $P > 0.05$). Tukey's post hoc analysis indicated that both doses of ethanol induced a significant CPP in early life ethanol-exposed animals ($P < 0.05$).

Locomotor activity

The effects of early life ethanol exposure mainly oppose the decrease in locomotion (habituation) which normally occurs in control animals. Basal locomotor activity was different between the two groups (pre-treatment: controls vs. early life ethanol-exposed animals) (Fig. 3A). A two-way ANOVA showed a main effect of pre-treatment ($F_{1,138} = 7.92$, $P < 0.01$) and time ($F_{7,138} = 15.47$, $P < 0.001$) and a significant pre-treatment \times time interaction ($F_{7,138} = 3.55$, $P < 0.05$). Tukey's post hoc analysis indicated that ethanol-exposed rats exhibited higher horizontal activity than controls at 30 and 35 ($P < 0.05$) min and at 40 min ($P < 0.01$). No differences were observed between female groups. Vertical activity expressed as the number of rears was not different between the groups (Fig. 3B).

Amphetamine produced greater locomotor-activating effects in early life ethanol-exposed animals than control rats (Fig. 4A). A three-way RM-ANOVA found significant main effects of pre-treatment (control vs. ethanol-exposed group, $F_{1,106} = 33.43$, $P < 0.001$) and dose ($F_{3,53} = 36.12$,

$P < 0.001$), no main effect of gender and a significant pre-treatment \times dose interaction ($F_{3,53} = 4.8$, $P < 0.005$).

Similarly, cocaine produced greater locomotor-activating effects in early life ethanol-exposed animals than control rats (Fig. 4B). A three-way RM-ANOVA found significant main effects of pre-treatment (control vs. ethanol-exposed group, $F_{1,165} = 38.79$, $P < 0.001$), dose ($F_{4,165} = 122.36$, $P < 0.001$) and gender ($F_{1,165} = 43.64$, $P < 0.001$). There were also significant pre-treatment \times dose ($F_{4,165} = 7.39$, $P < 0.001$) and gender \times dose ($F_{4,165} = 11.75$, $P < 0.001$) interactions.

Increasing doses of ethanol induced a biphasic locomotor response in males (main effect of dose: $F_{4,80} = 5.45$, $P < 0.001$) and females (main effect of dose: $F_{6,94} = 7.93$, $P < 0.001$) (Fig. 4C). Early life ethanol exposure did not alter locomotor response to ethanol in males (main effect of early life treatment: $F_{1,80} = 0.005$, $P > 0.05$) while it increased locomotor response to ethanol in females (main effect of early life treatment: $F_{1,94} = 18.2$, $P < 0.001$ and significant interaction dose \times early life treatment $F_{6,94} = 4.75$, $P < 0.01$) and Tukey's post hoc analysis revealed that their locomotor response was increased at higher doses of ethanol ($P < 0.05$). A three-way RM-ANOVA also found significant main effect of gender ($F_{1,146} = 76.63$, $P < 0.001$) and significant pre-treatment \times gender ($F_{1,102} = 7.39$, $P < 0.001$) and gender \times dose ($F_{3,102} = 11.75$, $P < 0.001$) interactions. These results indicated that the locomotor response to ethanol was elevated in female rats compared to male rats and that early life ethanol exposure increased the sensitivity to the locomotor effects of ethanol only in females.

Locomotor response to increasing doses of MK-801 was not changed in early life ethanol-exposed animals (Fig. 5A–C). At the 0.1 mg/kg dose, a two-way RM-ANOVA revealed significant main effect of time ($F_{17,287} = 11.49$, $P < 0.001$), no significant effect of pre-treatment ($F_{1,287} = 0.003$, $P > 0.05$)

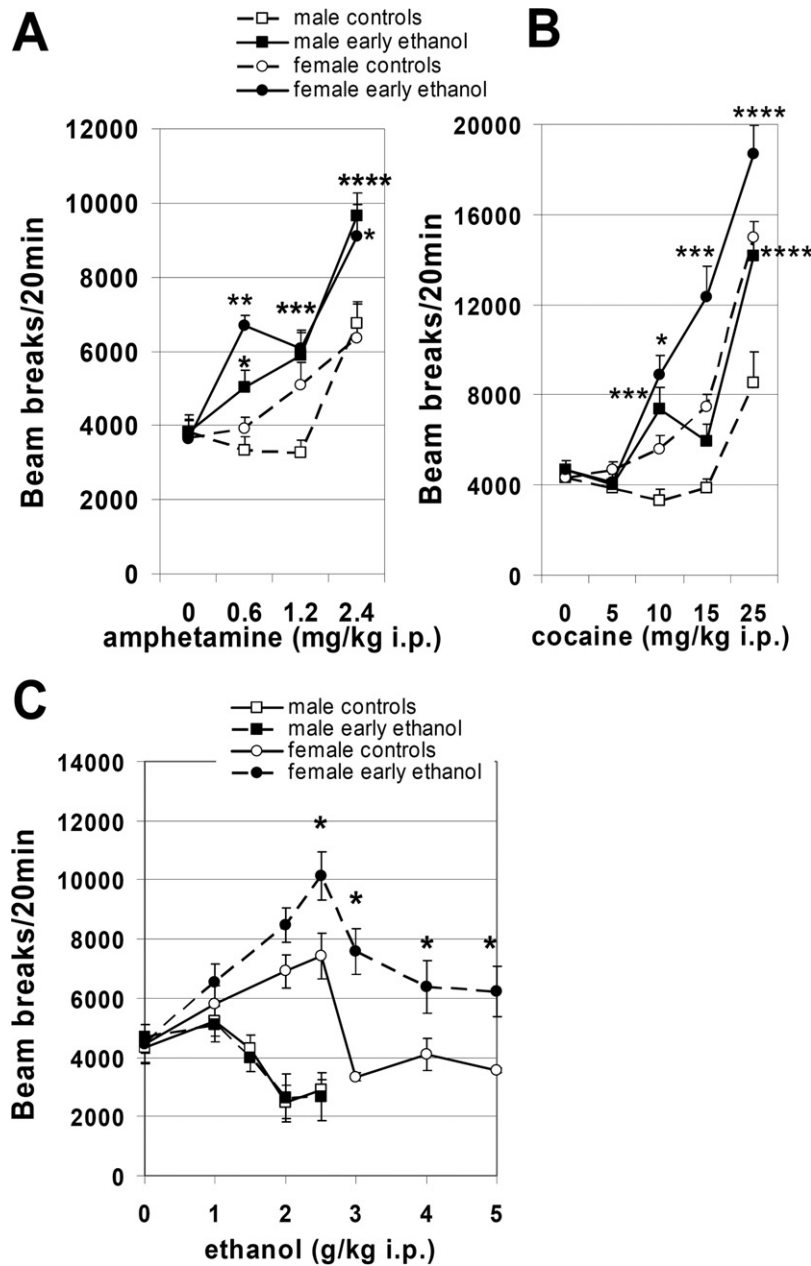


Fig. 4. Locomotor response to psychostimulants and ethanol in both males and females after early life ethanol exposure. (A) Horizontal activity expressed as numbers of beam breaks/20 min, immediately after injection of saline or amphetamine 0.6, 1.2 and 2.4 mg/kg. (B) Horizontal activity expressed as numbers of beam breaks/20 min, immediately after injection of saline or cocaine 5, 10 and 15 and 25 mg/kg. (C) Horizontal activity expressed as numbers of beam breaks/20 min, immediately after injection of saline or ethanol 1, 1.5, 2.0, 2.5, 3.0, 4.0 and 5.0 g/kg. Early life ethanol exposure increased locomotor response to psychostimulants in males and females and to ethanol in females, compared to control animals. $N=6-11/\text{group}$, * $P<0.05$, ** $P<0.01$, *** $P<0.001$ and **** $P<0.0001$ compared to respective control group.

and no significant interaction ($F_{11,287}=0.52$, $P<0.05$). At the 0.3 mg/kg dose, a two-way RM-ANOVA revealed significant main effects of time ($F_{17,358}=8.45$, $P<0.001$), and of pre-treatment ($F_{1,358}=13.51$, $P<0.001$) and no significant interaction ($F_{17,358}=0.30$, $P>0.05$). At the 0.5 mg/kg dose, a two-way RM-ANOVA revealed significant main effect of time ($F_{17,287}=4.56$, $P<0.001$), no significant effect of pre-treatment ($F_{1,287}=0.08$, $P>0.05$) and no significant interaction ($F_{11,287}=0.77$, $P>0.05$).

Early life ethanol-exposed animals were more sensitive to the hyperlocomotor effects of apomorphine (Fig. 5D). A two-way RM-ANOVA revealed significant main effects of time ($F_{11,286}=25.16$, $P<0.001$), pre-treatment ($F_{1,286}=24.96$, $P<0.001$) and a significant time \times pre-treatment interaction ($F_{11,286}=2.07$, $P<0.01$). Early life ethanol exposure did not modify the locomotor response either to SKF38393 (Fig. 5F) or to quinpirole (Fig. 5E). For the locomotor response to quinpirole, there was a significant effect of time

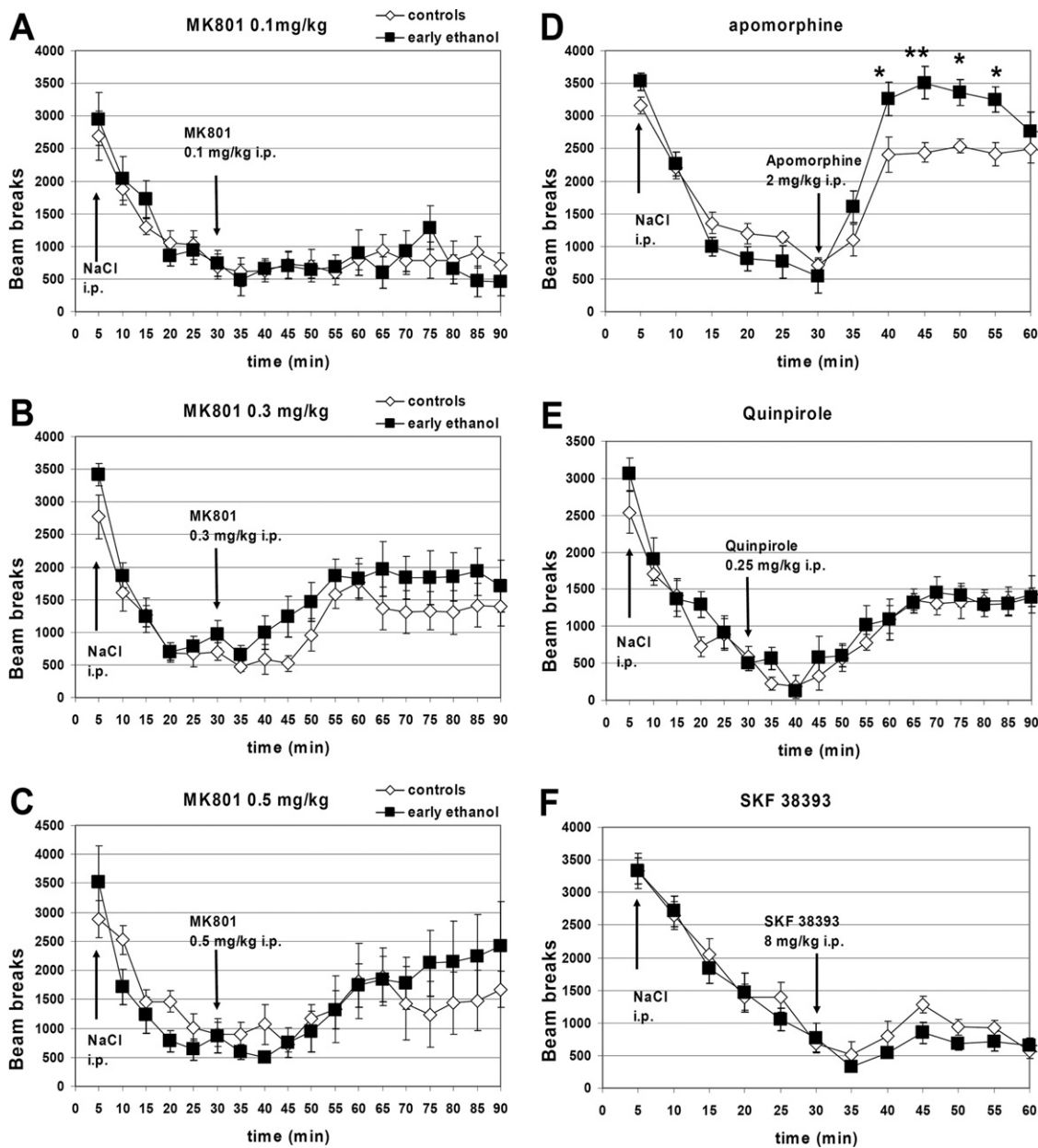


Fig. 5. Locomotor effects of MK-801 (0.1, 0.3 and 0.5 mg/kg) (A–C), apomorphine 2 mg/kg (D), quinpirole 0.25 mg/kg (E) and SKF38393 8 mg/kg (F) in control and early life ethanol-exposed male rats. Data are expressed as mean horizontal activity counts \pm SEM of control (\square) and early life ethanol-exposed (\blacksquare) rats ($n=6-11$ /group). Rats were injected with saline and immediately introduced in the chamber activity for 30 min (habituation phase). After the habituation period, rats were injected with the NMDA or dopamine receptor pharmacological agent and locomotor activity was measured for an additional 30 or 60 min period. * $P<0.05$, ** $P<0.01$ compared to respective control group.

($F_{17,283}=21.32$, $P<0.001$), no significant effect of pre-treatment ($F_{1,283}=2.15$, $P>0.05$) and no significant interaction ($F_{17,283}=0.51$, $P>0.05$). For the response to the locomotor effects of SKF38393, there was a significant effect of time ($F_{10,227}=46.67$, $P<0.001$), no significant effect of pre-treatment ($F_{1,227}=2.96$, $P>0.05$) and no significant interaction ($F_{10,227}=0.5$, $P>0.05$).

Early life ethanol exposure increased locomotor response to repeated cocaine injections (early life treatment \times day of cocaine injection interaction, $F_{4,103}=7.3$, $P<0.001$) in 2-month-old rats (Fig. 6A). Locomotor re-

sponse to cocaine was decreased in control rats after the second injection on day 2 while a high level of response was maintained in ethanol-exposed animals with a lower dose of cocaine on day 2 and also on day 6 after 3-day-withdrawal period. In three-month-old rats, motor stimulant effects of cocaine were also increased in early life ethanol-exposed animals (early life treatment \times day of cocaine injection interaction, $F_{4,122}=2.99$, $P<0.05$; Fig. 6B). Higher response was observed on day 2 and on day 6 (Tukey's post hoc, $P<0.001$). No difference was observed on day 1 while a

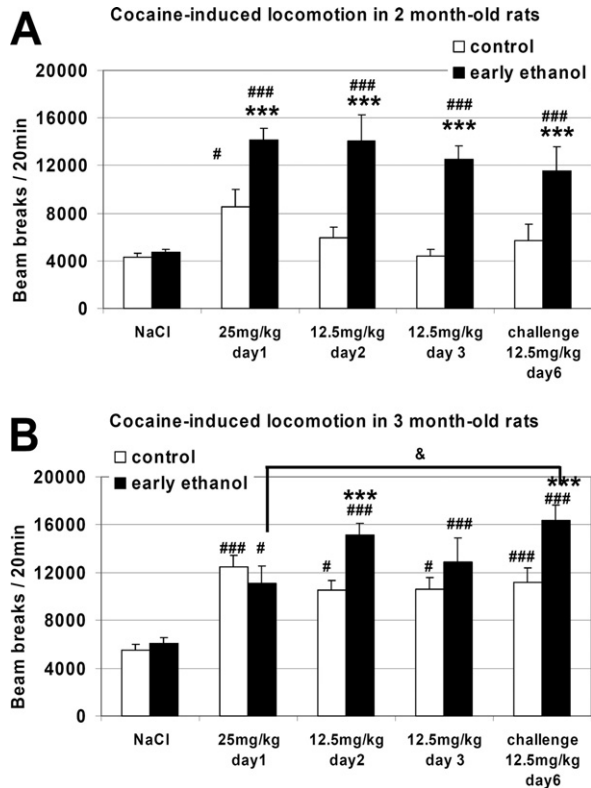


Fig. 6. Locomotor effects of repeated cocaine injections in 2-month-old (A) and 3-month-old (B) control and early life ethanol-exposed male rats. Data are expressed as mean horizontal activity counts/20 min \pm SEM of control (\square) and early life ethanol-exposed (\blacksquare) rats ($n=6-11$ /group). Rats were injected with saline the first day of experiments, with cocaine (25 mg/kg) the following day (day 2) and with cocaine (12.5 mg/kg) for two more consecutive days. Rats were tested on day 6 with a challenge dose (12.5 mg/kg) of cocaine after 2 days of withdrawal. *** $P<0.001$ compared to respective control group; # $P<0.05$, ### $P<0.001$ compared to respective NaCl group and & $P<0.05$ vs. respective day 1 as indicated.

significant difference was observed for the first injection in 2-month-old rats. An increased response was also observed when comparing day 1 (25 mg/kg cocaine) and day 6 (12.5 mg/kg cocaine) in ethanol-exposed animals, revealing the development of behavioral sensitization (Tukey's post hoc, $P<0.05$).

Radioligand binding

Fig. 7 shows the results of the radioligand binding studies. In the striatum, the 2-month-old early life ethanol-exposed rats had a significant decrease in DAT binding (27%, $P<0.05$) and no significant alteration of D1 and D2 receptor binding. In the hippocampus, early life ethanol-exposed animals had a significant decrease in NMDAR binding (51%, $P<0.05$) and no significant alteration in GABA_A receptor binding.

DISCUSSION

The present study demonstrated that early life ethanol exposure increased later propensity to drink ethanol

solutions and facilitated the induction of CPP to ethanol. Ethanol-exposed animals were also less sensitive to the sedative effects of ethanol and their locomotor responses to psychostimulants and to ethanol were enhanced. Striatal DAT and hippocampal NMDAR binding was found to be decreased in early life ethanol-exposed animals.

The results of the experiments reported here show that early life ethanol exposure increased the propensity to drink ethanol solution in adulthood when ethanol is freely available or in a situation of forced exposure. These results confirm earlier observations (Bond and DiGiusto, 1976; Phillips and Stainbrook, 1976; Holloway and Tapp, 1978; Randall et al., 1983; Molina et al., 1987; Spear and Molina, 2005) suggesting that prenatal or perinatal ethanol exposure may influence vulnerability to ethanol consumption. Ethanol consumption and preference increased over time in the early life ethanol-exposed group suggesting the development of tolerance and/or sensitization to the reinforcing effects of ethanol. Early life ethanol-exposed rats acquired voluntary alcohol drinking behavior within two weeks. Indeed, mean daily alcohol intake was 2.5 g/kg on week 1 and rose to 4.5 g/kg on week 2 and this was associated with a twofold increase in ethanol preference.

Since early life stress experiences and stress reactivity at adulthood have been shown to increase the propensity of an individual to develop drug self-administration (Piazza and Le Moal, 1998; Koehl et al., 2002), we measured the reactivity of the HPA-stress axis in animals exposed to early life ethanol. We did not find any difference either in the basal levels of ACTH or of corticosterone (data not shown). The increase in the levels of these hormones induced by environmental stress (electric foot shock or confinement in the open arm of a plus maze) was not altered by early life ethanol exposure (data not shown). Thus these results indicate that a potential stress induced by early life ethanol exposure can be ruled out. It is noteworthy that ethanol consumption during pregnancy has

Fig. 7. Binding studies. Dopamine D1 ($[^3\text{H}]$ SCH23390), D2 ($[^3\text{H}]$ raclopride) receptors and DAT ($[^3\text{H}]$ mazindol) measurements in the striatum from 2-month-old and NMDAR ($[^3\text{H}]$ MK-801) and GABA_A receptor (GABA_AR) ($[^3\text{H}]$ muscimol) measurements in the hippocampus from 2-month-old rats ($n=10$ /group). * $P<0.05$ compared to respective control group (Student's *t*-test).

been shown to increase corticosterone in dams during the late period of gestation (Sinha et al., 1997) and it is therefore possible that ethanol-induced alteration of fetal and/or maternal pituitary-adrenal function could be involved in long-term modification of drug responsiveness observed in the offspring.

The increased ethanol consumption is associated with a decreased sensitivity to ethanol-induced sedation while ethanol metabolism is not altered. This profile is consistent with an abundance of animal data showing an inverse correlation between ethanol sensitivity and motivation to consume ethanol, as well as human findings of an inverse relation between alcoholism risk and ethanol sensitivity (Naassila et al., 2002, 2004; Schuckit, 1987).

Interestingly, our results show that the increased propensity to drink ethanol solutions is also associated with a facilitation to induce a CPP with ethanol, thus indicating that CPP is more easily induced when animals voluntarily consume more ethanol, in line with the idea that both of these behaviors are related to the reinforcing effects of ethanol (Green and Graham, 2008). In the majority of studies that used non-selected rat lines ethanol induced no effect or CPA (see for review Tzschentke, 2007). In our experimental conditions, controls did not show significant ethanol-induced CPP at both doses, thus these conditions may have allowed the effect of early life ethanol exposure to be detected. Numerous previous data indicated that ethanol did not induce CPP in rat and that it preferentially induced a CPA. In this regard results in rat have been variable in alcohol place conditioning in that some studies find a CPP (Ciccocioppo et al., 1999; Reid et al., 1985; Bozarth, 1990; Colombo et al., 1990; Cole et al., 2003); and others find a CPA (Bormann and Cunningham, 1997; Schechter, 1992) or no effect of alcohol (Philpot et al., 2003). Earlier studies also demonstrated development of sensitization to the rewarding effects of ethanol after prolonged exposure to ethanol (Reid et al., 1985; Bińkowski et al., 1995). Our observations also support the idea that chronic early life ethanol exposure sensitized the animals to the rewarding properties of ethanol assessed in the CPP paradigm and facilitated the induction of place preference by ethanol and/or decreased its aversive properties.

In the current study there was evidence of a sex-dependent increase in exploratory behavior due to early life ethanol exposure. Hyperactivity is the most frequently reported behavioral problem in children with fetal alcohol effects (Driscoll et al., 1990). However, studies on adult rats brought controversial results (Dursun et al., 2006) and our results show that basal horizontal locomotor activity (no saline injection) was altered in male rats. The effects of early life ethanol exposure mainly oppose to the decrease in locomotion (habituation) which normally occurs in control animals.

Early life ethanol exposure sensitized animals to subsequent ethanol and psychostimulants exposure. There was an increase in the sensitivity to the locomotor effects of acute cocaine or amphetamine. This appears to be in accordance with previous studies showing that prenatal ethanol exposure increased the locomotor activation in-

duced by amphetamine (Hannigan and Pilati, 1991) but is contradictory to previous studies on Long Evans rats showing no modification of the locomotor response to amphetamine and methylphenidate (Blanchard et al., 1987; Randall and Hannigan, 1999). Interestingly, modafinil has been shown to mainly oppose to the habituation phenomenon which normally occurs in control animals (Simon et al., 1996). Modafinil may act by stimulating α -adrenoreceptors, suppressing GABA release, weakly inhibiting the DAT, or elevating extracellular levels of glutamate in numerous brain regions (Ballon and Feifel, 2006).

For ethanol, the effects were gender-specific and the increased sensitivity to its locomotor effects was observed only in female rats. In ethanol-exposed offspring, the locomotor stimulating effect following an ethanol challenge has been reported to be either augmented (Rockman et al., 1989) or blunted (Becker et al., 1993). Our results indicate that early life ethanol-exposed female rats are more sensitive to the locomotor stimulant effects of ethanol, thus confirming the existence of long-term consequences onto ethanol response after ethanol exposure during early life. It has been suggested that locomotor-activation effect caused by low doses of ethanol (Wise and Bozarth, 1987) and insensitivity to ethanol's depressant effects (Schuckit and Smith, 2001) may be a marker for abuse potential. In this context, the increased sensitivity to the locomotor stimulant effects of ethanol observed after early life ethanol exposure may explain, together with the increased sensitivity to the rewarding effects of ethanol and the decreased sensitivity to its sedative effects, the increased risk for ethanol abuse.

Several lines of evidence have indicated that the positive reinforcing and locomotor-stimulating effects of ethanol result from activation of common biological mechanisms involving dopamine pathways. Low to moderate doses of ethanol have been extensively reported to increase the firing rate of ventral tegmental dopaminergic neurons (Gessa et al., 1985) and, in turn, dopamine release in the nucleus accumbens that has been implicated in stimulating spontaneous locomotor activity in rodents (Imperato and Di Chiara, 1986). Since the dopamine system seems involved in the observed alterations of the behavioral effect of ethanol we measured the sensitivity of rats to the locomotor effects of dopaminergic agents. Our data, in agreement with a previous study (Hannigan et al., 1990), indicated that the hyperlocomotor effect of the D1/D2 agonist apomorphine is enhanced in ethanol-exposed animals while there was no difference in the sensitivity to the D1 or D2/D3-like receptor agonists, SKF38393 and quinpirole, respectively. These results suggest that early life ethanol exposure may induce functional alteration of both receptors and that the enhanced sensitivity to apomorphine may require both D1 and D2 receptor stimulation for the expression of the non-selective dopamine agonist hyperresponsiveness (balance between functional D1 and D2 receptor response). This hypothesis has been suggested with injection of dopamine agonists into the nucleus accumbens (Dreher and Jackson, 1989). Alternatively, given that early life ethanol-exposed rats show

greater apomorphine-induced locomotion while no difference was observed in dopamine receptor binding, the difference with apomorphine challenge may be related to indirect rather than direct mechanisms of dopamine receptor stimulation.

We have also demonstrated that there is no modification in the striatal D1 and D2 binding site density that is consistent with previous studies. Previous reports have shown that prenatal ethanol exposure reduces (Druse et al., 1990; Carneiro et al., 2005) moderately (5%–10%) increases (Gillespie et al., 1997) or does not alter (Randall and Hannigan, 1999) the number of striatal dopamine D1 in the rodent brain. Striatal D2 receptors have been shown to be decreased (Nio et al., 1991; Randall and Hannigan, 1999) or unchanged depending on the dose of ethanol (Druse et al., 1990; Randall and Hannigan, 1999). This discrepancy in the results may be due to variations in ethanol exposures, ethanol dosing, route of administration and/or the radioligands used. Variation in the basal number and/or affinity of DAT binding sites could explain differences in drug-induced locomotor activity. Our results also demonstrate that early life ethanol exposure decreased striatal DAT binding (–27%) and this is consistent with previous study that has shown a decrease in the number of dopamine uptake sites (approximately 25% decrease in V_{max}) at 35 days (Druse et al., 1990). Previous results also demonstrated that prenatal ethanol exposure reduced DAT mRNA in the ventral tegmental area (VTA) (–68%) and in the substantia nigra pars compacta (–81%) of adult male offspring (Szot et al., 1999). Our results show that the decrease in striatal DAT binding is not associated with an alteration of dopamine receptors. To our knowledge this is the first study on early life ethanol exposure that examined both dopamine receptors and DAT. Developmental studies indicated that D1R and D2R expression precedes DAT expression, which implies that receptor expression is an intrinsic property of neurons and does not require the presence of endogenous dopamine which suggests that the expression of these receptors is independent of dopaminergic innervation (Schambra et al., 1994). In addition, a previous report has shown in DAT knock-down mice that there was no change in postsynaptic D1 or D2 receptor levels and there was a 50% reduction in D2 autoreceptor levels leading to the hypothesis that a shift in the balance between dopamine auto- and heteroreceptor function is observed after a decrease in DAT expression (Zhuang et al., 2001).

The results of the present study show that the decreased DAT binding is associated with alteration of the locomotor stimulating effects of psychostimulants and ethanol. Inhibition of striatal DAT is clearly critical for psychostimulant-induced behaviors. Since an inverse correlation between the number of striatal DATs and locomotor activity induced by psychostimulants is reported here it is possible to argue that the decrease in the number of DAT may be related to an alteration of its functional properties (i.e. increase sensitivity to inhibitory effects of psychostimulants).

As we have previously demonstrated on the same model that developmental expression of the NMDAR sub-

units was altered (Naassila and Daoust, 2002) and since there is considerable evidence indicating the critical role of NMDA and GABA_A receptors in the development of ethanol dependence, we tested the locomotor effects of MK-801 and measured the number of hippocampal [³H]MK-801 and [³H]muscimol binding sites. Our results show a 51% decrease in the number of binding sites while no difference was observed in the hyperlocomotor effects of MK-801. A study has also demonstrated a decrease in NMDA-sensitive [³H]-glutamate binding site density in 45-day-old rats (Savage et al., 1991).

As initial sensitivity to the locomotor activating effects of psychostimulants in outbred rats has also proven to be predictive for drug-induced locomotor sensitization and because the behavioral sensitization produced by repeated drug treatment may represent the neural adaptations underlying some of the features of addiction in humans, we also investigated the development of behavioral sensitization induced by repeated injection of cocaine in both 2-month-old and 3-month-old rats. Our results show that when 2-month-old rats were treated with 25 mg/kg cocaine on day 1 and with 12.5 mg/kg for the next 2 days and the challenge day (day 6), control rats did not respond to the 12.5 mg/kg dose whereas the level of locomotor response was still increased in ethanol-exposed animals. The decreased response observed in control rats on day 2 was expected since the dose is reduced and insufficient to induce sensitization in control rats using our paradigm (Todtenkopf et al., 2006). In 3-month-old rats the enhanced response to the first injection of cocaine is not observed in ethanol-exposed animals compared to controls, and only ethanol-exposed animals developed cocaine sensitization with an enhanced response to cocaine challenge on day 6 compared to the first day of cocaine injection. In line with these results, a previous study has shown that male but not female rats exposed to prenatal alcohol showed an apparent sensitization to 10 mg/kg amphetamine not seen in control rats between PN28 and PN42 (Hannigan and Pilati, 1991). These results indicate that early life ethanol exposure induced alteration in the sensitivity to the locomotor effects of cocaine but also in the development of behavioral sensitization. The mechanism by which ethanol did alter the development of behavioral sensitization is currently unknown but it is possible to argue that the observed decreased number of DAT binding sites could be involved. In this regard, it has been recently shown that behavioral sensitization to cocaine is associated with alteration of DAT mRNA expression (Belin et al., 2007). We also report a decrease in the number of striatal [³H]mazindol binding sites after early life ethanol exposure. Only very few studies investigated the GABA_A receptor after early life ethanol exposure, a previous study demonstrated that prenatal ethanol exposure enhanced sensitivity of GABA-stimulated $^{36}\text{Cl}^-$ flux to positive modulators (Allan et al., 1998) and our results show that there is no modification of hippocampal [³H]muscimol binding sites.

CONCLUSION

In summary our results indicate that the long-term vulnerability to addiction after early-life ethanol exposure reported by clinical studies is also observed in our animal model of early life ethanol exposure. In addition, our results confirm that there is a long-term alteration not only in the ethanol response but also in response to other drugs of abuse.

As addiction is increasingly formulated as a developmental disorder, identifying how early developmental exposures influence later responses to drugs of abuse is important to our understanding of substance abuse neurobiology.

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