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Genetic Factors Involved in the Effects of Developmental Low-Level Alcohol Induced Behavioral Alterations in Rats

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Behavioral and neurochemical effects of perinatal alcohol exposure (3% v/v solution from Day 15 of gestation to Day 7 after parturition) have been investigated in Sardinian alcohol-preferring (sP) and alcohol-nonpreferring (sNP) rat lines, selectively bred for opposite alcohol preference and consumption. In an elevated zero-maze model of anxiety, sucrose-exposed sP rats (sP-S): (i) spent significantly less time on the open arms (TO); (ii) exhibited a significantly lower number of head dips (HDIPS); and (iii) showed a higher number of stretched attend-postures (SAP) than sucrose-exposed sNP rats (sNP-S) at 90 and 180 days of age. The two rat lines displayed different emotional reactivity in response to alcohol exposure. Subtle differences in sexual behavior and ultrasonic emission (latency to the first intromission and to the first 50 kHz call) were observed between sP-S and sNP-S rats. sP-alcohol exposed (sP-A) offspring exhibited a higher latency to the first intromission than sNP-alcohol (sNP-A) treated rats. Moreover, a lower number of sP-A rats exhibited both intromission and ejaculation with respect to sNP-A animals. sP-S rats were significantly slower in recover of the righting reflex than sNP-S animals after a challenge dose of alcohol (3 g/kg, i.p.). Perinatal alcohol did not affect either onset or duration of sleep time in either line. Neurochemical experiments have shown that perinatal alcohol did not influence basal dopamine levels or amphetamine-induced dopamine increase in the prefrontal cortex of either sP or sNP offspring. These results, showing an endpoint-specific differential sensitivity of sP and sNP lines to perinatal low alcohol exposure, indicate that genetic factors could be responsible for selective susceptibility to behavioral alterations induced by developmental treatment with this drug of abuse.

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Previous data suggest that genetic factors could contribute to differences in susceptibility to alcohol-related neurodevelopmental disorders (ARNDs) (Gilliam et al. 1987; Gilliam and Irtenkauf 1990; Govoni et al. 1994; Hannigan 1996; Riley and Lochry 1982; Riley et al. 1993). A recent study in the present series has shown that offspring of Sardinian alcohol-preferring (sP) and Sardinian alcohol-nonpreferring (sNP) rats, selectively bred (from Wistar rats) for opposite alcohol preference and consumption, exhibited a different behavioral profile in response to perinatal exposure to this drug of

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abuse, at doses below those associated with gross malformations and/or overt neurotoxic effects (Tattoli et al. 2001). Specifically, alcohol exposure significantly reduced the rate of ultrasonic emission in sP male pups, whereas it did not influence this indicator of emotional reactivity in sNP neonates. Therefore, it appeared of interest to further investigate the effects of developmental alcohol exposure in the sP and sNP lines, using both some of the offspring from litters of the study just mentioned (Tattoli et al. 2001) and the offspring of additional litters. As in the previous study, the aim was to investigate the consequences of a low level alcohol exposure clearly under the threshold for gross toxic and/or teratogenic effects, but still capable to provide information on the pathogenesis of ARNDs falling short of the fully fledged Fetal Alcohol Syndrome (FAS).

A first experiment was to determine if perinatal alcohol exposure might affect homing behavior, which is a simple form of learning based on ultrasonic and olfactory cues (Altman and Sudarshan 1975; Fechter and Annau 1980). In this regard, it could be hypothesized that the decreased rate of ultrasonic emission observed in sP-A pups exposed to alcohol during development (Tattoli et al. 2001) might influence their performance in this test.

Secondly, since it has previously been shown that sP rats exhibit a greater innate degree of anxiety than sNP ones (Colombo et al. 1995), it was also of interest to explore further the effects of developmental alcohol treatment on emotional reactivity, using a test appropriate for adults (elevated zero-maze test). On the basis of the aforementioned findings (Tattoli et al. 2001) concerning the reduction by developmental alcohol exposure of emotional reactivity in sP, but not in sNP neonates, the intent was to check whether or not a similar differential effects profile would persist over time (i.e., at 3 and 6 months).

Thirdly, it is known that fetal alcohol-exposed male rats can show demasculinization and/or feminization of both reproductive and nonreproductive sexually dimorphic behaviors and of brain structures (see Blanchard and Hannigan 1994; Kelly et al. 2000 for references); therefore, the effects of developmental alcohol exposure on sexual behavior of male sP and sNP rats were evaluated.

Since the mesolimbic dopaminergic system plays an important role in the regulation of rat sexual behavior (Gessa and Tagliamonte 1975) and differences in DA levels between sP and sNP rats (not affected by developmental alcohol exposure) have been found in the nucleus accumbens (Tattoli et al. 2001), the intent was to verify whether adult sexual behavior would be similarly or differentially affected by early alcohol exposure in the two lines. This included the analysis of ultrasonic vocalization during copulatory activity of male offspring, which is considered a sensitive detector of subtle changes in sexual motivation (Cagiano et al. 1989a,b).

Fourthly, based on data showing that sP rats are more sensitive to the sedative/hypnotic effects of alcohol that sNP rats (Colombo et al. 2000), an experiment assessed whether perinatal alcohol treatment could affect time to lose and to recover the righting reflex after the acute administration of a challenge dose of this agent.

Finally, it has been shown (Tattoli et al. 2001) that perinatally alcohol-exposed sP rats, but not sNP rats, apparently lost the capacity to discriminate between the novel and the familiar object when subjected to the novel exploration object test, which provides a valid and relatively pure measure of working memory (Ennaceur and Delacour 1988; Ennaceur et al. 1997). Dopaminergic mechanisms in the prefrontal cortex play an important role in this cognitive end-point (Mizoguchi et al. 2000). Thus, neurochemical experiments (*in vivo* microdialysis) were performed in order to measure the extracellular levels of dopamine (DA) as well as the biochemical responsiveness to a challenge dose of amphetamine in the prefrontal cortex of adult offspring perinatally exposed to alcohol. The intent was to verify whether prefrontal DA in the two lines would respond to early alcohol exposure differently from, or similarly to, accumbens DA (no alcohol-related differences, see above), which would account or viceversa not account for the differential changes in discriminative capacity.

MATERIALS AND METHODS

Animals and Treatment Schedule

The experiments have been conducted in accordance with guidelines released by Italian Ministry of Health (D.L. 116/92), the Declaration of Helsinki, and the "Guide for the Care and Use of Laboratory Animals" as adopted and promulgated by the National Institutes of Health.

Primiparous alcohol-naive sP and sNP female rats from the 42nd generation of both lines (Dept. of Neuroscience "Bernard B. Brodie", University of Cagliari, Italy) weighing 280–350 g were used. A randomly bred control line was not established in this selection experiment. It was deemed that the use of animals from the original colony (Wistar from Charles River, Italy) after such a long time (about 20 years) from the start of the selection could not provide appropriate control data.

The animals were individually housed in standard plastic cages and allowed free access to food and water, with constant room temperature (20–22°C) under a reversed 12/12 h light-dark cycle (light on: 21.00 h to 09.00 h). Each female was placed with a male rat in the early morning and vaginal smears were taken daily at 19.00 h. The day on which sperm were present was des-

ignated Day 0 of gestation. In order to avoid the systemic administration of alcohol, the treatment schedule had to be established on the basis of preliminary experiments (Tattoli et al. 2001) aimed at assessing the intake of alcohol solution by pregnant sNP females and its temporal distribution. Specifically, the least biased procedure appeared to be that of controlling the alcohol solution intake by sP females as well as the control solution intake by both sP and sNP female controls (see below) on the basis of the "ad libitum" alcohol solution intake by sNP females. As previously reported (Tattoli et al. 2001): (i) sNP female rats consuming "ad libitum" a 3% (v/v) aqueous solution of alcohol from Day 15 of gestation to Day 7 after parturition (as the only available beverage), exhibited a significant increase (one-way ANOVA for repeated measures: $F = 19.52$; $df = 12/60$, $p < .01$) in alcohol intake during the lactation period (Figure 1); and (ii) the total daily volume of alcohol solution consumed by pregnant sNP rats did not significantly differ from that of sNP animals receiving tap water "ad libitum." This indicated that controlling fluid intake in three of the four experimental groups (see the treatment schedule described below) on the basis of the "ad libitum" intake of alcohol solution by sNP females was unlikely to produce harmful effects due to fluid restriction.

Experimental Procedure

The exposure schedule used in the present study was as follows:

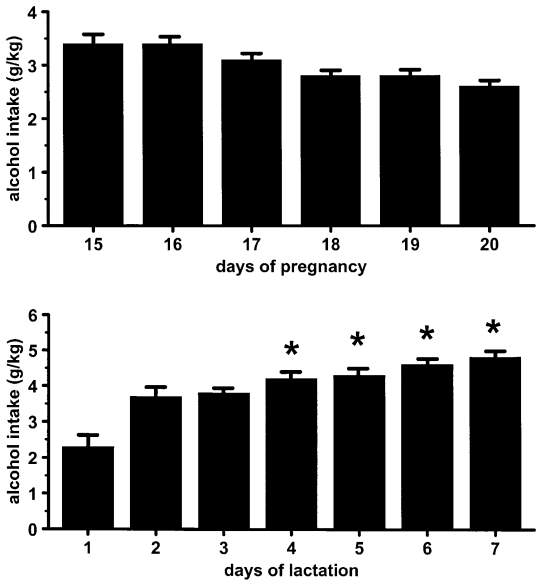

Figure 1. Daily 3% (v/v) alcohol solution intake in sNP dams during pregnancy (from Day 15 to Day 20) and lactation (from Day 1 to Day 7 after delivery). Each colum represents the mean ± S.E.M. *$p < .05$ vs. Day 15 of gestation (two tail Dunnett's test).

— sNP females were given "ad libitum" a 3% (v/v) aqueous solution of alcohol, from Day 15 of gestation to Day 7 after parturition, as the only available fluid intake (sNP-A);
— sP females were given daily, from Day 15 of gestation to Day 7 after parturition, the same volume of aqueous solution of alcohol (3% v/v) consumed by the sNP group (sP-A). In order to mimic the daily drinking pattern shown by sNP females, sP dams were offered half of the volume at the beginning of the dark period and the remaining volume 6 h later;
— control sNP and sP dams received, as the only available fluid (from Day 15 of gestation up to Day 7 after parturition), the same volume of a solution in which alcohol was substituted by an equicaloric amount of sucrose (sNP-S and sP-S, respectively). Half of the volume of the solution was given at the beginning of the dark period and the remaining volume was administered 6 h later.

The exposure schedule resulted in a daily consumption of alcohol by sP dams equivalent to that exhibited by sNP dams. Moreover, daily total fluid intake in sucrose-exposed sNP and sP dams was equivalent to that observed in alcohol-exposed sNP and sP females. There were no differences in food consumption either between lines or between alcohol and sucrose solution groups.

All litters were reduced to a standard size of six male pups per litter (when possible) within 24 h after birth. All pups were weaned at 21 days of age. One male pup per litter from different litters per treatment group was used in all experiments.

As observed previously (Tattoli et al. 2001), perinatal alcohol exposure does not affect dam weight gain, number of dams giving birth, pregnancy length, litter size at birth, pup weight gain, and postnatal mortality (data not shown).

Blood Alcohol Levels (BALs)

Subgroups of alcohol-exposed sNP and sP dams (eight females for each group) were implanted under anaesthesia (Equithesin 3 ml/Kg, i.p.) with catheters in the abdominal aorta on Day 20 of gestation, on Day 7 after parturition, and 24 h after alcohol withdrawal. Maternal BALs were measured by a spectrophotometric method (UV-method, Boehringer Mannheim Kit, detection limit 0.22 mmol/l). Drawing time was randomly chosen for each rat during the last six hours of the dark phase of the daily dark/light cycle. Blood alcohol determinations were also made in fetuses on Day 20 of gestation. All fetuses obtained via hysterotomy were immediately decapitated and blood collected from the neck vessels. Blood samples from fetuses from the same litter were pooled. On postnatal Day 7 and 24 h after mater-

nal alcohol withdrawal pups were decapitated and blood of each pup was collected from the neck vessels.

Statistical Analysis. Maternal BALs were analysed by an overall two-way ANOVA, followed by a post-hoc test (Tukey's test). Fetal and pup BALs were analysed by Student's t test.

Behavioral studies

Homing Behavior. The apparatus and procedures have been described previously (Altman and Sudarshan 1975). Briefly, the apparatus consisted of a central circular area, 11 cm in diameter and surrounded by a wire fence. The orientating platform was positioned between two enclosed alleys, 7.5 cm long, that opened into the home cage (containing mother and siblings) on one side and into an empty cage on the opposite side. The size of the fenced-in area limited locomotion and, to facilitate scoring, the circular area was demarcated by octagonally oriented lines.

The animal was placed randomly facing right or left at a right angle to the entrances; these were the null points. Turning toward the home cage or empty cage was scored as $+1$ or -1, and turning half-way to the two entrances was scored as $+1/2$ or $-1/2$. Each animal was individually removed from the home cage and placed into the fenced-in circle for 180 sec. and its orientation was recorded every 10 sec.

For each animal scores were summed and the mean of all animals was calculated (maximum individual score was $+18$ if the animal was oriented at all observation periods fully towards the opening of the home cage). Because homing performance could be influenced by the level of locomotor activity, this parameter was also evaluated.

To score locomotor activity, the fenced-in area was divided in nine identical surfaces (eight circular sectors surrounding a central circular area) and the number of lines crossed by each rat was measured during the test session. The test was carried out at 10 days of age and one pup per litter from eight different litters in each experimental group was used.

STATISTICAL ANALYSIS. The statistical analysis of orientation scores and of number of lines crossed was based on an overall two-way ANOVA. Post-hoc tests (Tukey's test) were used for individual between group comparisons.

Elevated Zero-Maze Test

Apparatus. According to the technique previously described by Shepherd and coworkers (1994) and modified by Bickerdike and coworkers (1994), the maze comprised a black Perspex annular platform (105 cm diameter, 10 cm width) elevated to 65 cm above ground level, divided equally into four quadrants. Two opposite quadrants were enclosed by black Perspex walls (27 cm high) on both the inner and outer edges of the platform, while the remaining two opposite quadrants were surrounded only by a Perspex "lip" (1 cm high) which served as a tactile guide to animals on these open areas.

The apparatus was illuminated by dim red light arranged in such a manner as to provide similar lux levels in open and closed quadrants (40–60 lux). A video camera, connected to video equipment in a separate observation room, was mounted overhead in order to record behavior on the maze for subsequent analysis.

Procedure. Subjects were placed on a closed quadrant and a 5-min test period was recorded on videotape for subsequent analysis. The maze was cleaned with 5% ethanol/water solution and dried thoroughly between test sessions. Behavioural measures were as follows:

A. Percent time spent on the "open" quadrants (% TO) expressed as the percentage of the total time of the test. Time on the "open" quadrants was timed from the moment all four paws of the rat were placed on an open section and ended when all four paws re-entered a closed quadrant.
B. Number of exploratory head dips (HDIPS) made over the edge of the platform, either from the exit of the "closed" quadrant, or whilst on the "open" quadrant;
C. Number of stretched-attend postures (SAP) made from the exit of a "closed" quadrant towards an "open" quadrant. This exploratory posture can be described as a forward elongation of the body, with static hind-quarters, followed by a retraction to the original position.

Testing was carried out in 90 and 180 days old offspring between 13.00 and 17.00 hrs. Each group consisted of 10 animals.

STATISTICAL ANALYSIS. According to previous studies (Shepherd et al. 1994; Bickerdike et al. 1994), % TO produced normal data, whereas both SAP and HDIPS produced skewed non-parametric data (Poisson distribution). Therefore, square root transformations of SAP and HDIPS were carried out, thereby producing normal distribution of data and allowing parametric analyses. % TO and transformed SAP and HDIPS data were analysed by two-way ANOVAs followed by Duncan's Multiple Comparison test or Tukey's test where appropriate. For brevity, in the Results section square root head dips and square root stretched-attend postures are referred to simply as head dips and stretched-attend postures.

Sexual Behaviour and Ultrasonic Emission

The technique has been previously described by Cagiano et al. (1988, 1998a,b). sP and sNP heterosexually na-

ive male rats were tested for sexual behavior at 80 days of age (10-min session); thereafter, animals were subjected to further two 10-min sessions (the inter-session interval was 15 days). Rats were tested again at 180 days of age (30-min session) for sexual activity.

As stimulus females bilaterally ovariectomized rats were used in which oestrus had been induced by subcutaneous injection of estradiol benzoated (8 μg/rat) and progesterone (200 μg/rat) dissolved in 0.2 ml of sesame oil, 52 and 4 h before the test session, respectively.

Each male rat was tested for sexual behavior with a stimulus female. Sexual behavior was recorded by a JVC video-tape recorder. Ultrasonic calls, detected by a QMC ultrasonic microphone connected to a receiver (QMC Bat Detector S200) which transformed, in real time, the ultrasonic calls into audible sounds, were sent to the video tape-recorder through the microphone connection terminal of the video camera. Microphone signals were relayed, via the high frequency output socket, to the Bruel & Kjaer (model 2033) High Resolution Signal Analyzer in order to visualize and count the ultrasonic calls.

The experiments were carried out in a sound-attenuating chamber (amplifon G-type chamber). Each male rat was observed alone for 5 min; an oestrous female was then introduced into the centre of the arena and the behavior of the male was recorded. Video tape-recordings were later replayed and analyzed (in slow motion when necessary) and the following parameters were measured: (M/L) mount latency (time between the introduction of the female into the mating cage and the first mount); (I/L) intromission latency (time between the introduction of the female into the mating cage and the first intromission in the ejaculatory series); (M/IF) mount-intromission frequency (number of mounts or intromissions in each ejaculatory series); (EjL) ejaculation latency (time between the first intromission and ejaculation in each ejaculatory series); (EjF) ejaculation frequency (number of ejaculations in each test session); (PejI) post-ejaculatory interval (interval between each ejaculation and the next intromission in each ejaculatory series); (TM/IF) total mount/intromission frequency (total number of mounts or intromissions during the test session); (ICI) inter-copulatory interval (ejaculation latency/intromission frequency in each ejaculatory series).

The following ultrasonic parameters were evaluated: (L50) latency from the introduction of the stimulus female into the mating cage and the onset of the first 50 kHz call; (L22) time from the ejaculation and the beginning of the 22 kHz vocalization in each ejaculatory series; (D22) duration of the 22 kHz post-ejaculatory vocalization in each ejaculatory series. Each group consisted of 10 animals.

Statistical Analysis. Both sexual and ultrasonic parameters were evaluated by using non-parametric statistics due to a highly significant heteroschedasticity in all instances. Statistical analysis was based on Kruskal-Wallis ANOVAs followed by post-hoc tests (Dunn's Multiple Comparison test). Fisher's exact-test was used where appropriate.

Sleep Time

One hundred and eighty days old animals were injected with an acute dose of alcohol (3 g/kg, i.p.). According to the technique used by Colombo et al. (2000), after alcohol injection, each rat was placed on its back once every 30 sec until it was unable to right itself within 30 sec. The time between ethanol injection and the start of the 30-sec interval when the rat was unable to right itself was measured as onset of the righting reflex loss. Then each rat was left undisturbed on its back until it spontaneously regained its righting reflex (determined as having at least three paws under its body). Complete recovery of the righting reflex was defined as the rat being able to turn itself upright at least twice within 1 min. If this criterion was not fulfilled, the rat was left undisturbed until it again spontaneously regained its righting reflex. The time between loss and recovery of righting reflex for each rat was taken as its sleep time. Each group consisted of the following animals: sP-S: $n = 8$; sP-A: $n = 6$; sNP-S: $n = 8$; and sNP-A: $n = 9$.

Statistical Analysis. The data were analysed by an overall two-way ANOVA followed by a post-hoc test (Tukey's test).

Neurochemical Studies

Ninety-days old rats were anaesthetized with Equithesin (3 ml/kg, i.p.) and placed on a stereotaxic apparatus (David Kopf Instruments, Tujunga, CA, USA). Microdialysis was performed in the prefrontal cortex. Stereotaxic coordinates were as follows: AP = 11.6, H = 8.0 from the interaural line with the incisor bar set at −2.4 mm according to the Paxinos and Watson atlas (1986). A short piece of dialysis fiber made of copolymer of acrylonitrile sodium methallyl sulfonate (AN69 Hospal S.p.A; 20.000 Daltons cut-off) was covered with epoxy glue to confine dialysis to the area of interest (12 mm glue-free zone). The rat's skull was exposed and two holes were made on the lateral surface at the level of the head of the prefrontal cortex. A dialysis fiber, held straight by a tungsten wire inside, was inserted transversely into the brain so that the glue-free zone was located exactly in the target area. The tungsten wire was withdrawn and stainless-steel cannulae (22-gauge diameter, 15 mm long) were glued to the ends of the fibers. These ends were bent up and fixed vertically to the skull using dental cement and modified Eppendorf tips. Finally, the skin was sutured and the rats

were allowed to recover from anaesthesia for at least 15 h before neurotransmitter release study.

Experiments were performed on freely moving rats. On the day of the experiment, fibers were perfused with Ringer solution containing (mM): NaCl 145, KCl 3, $CaCl_2$ 2.2, $MgCl_2$ 1 in distilled water. The solution was buffered at pH 7.4 with a 2 mM sodium phosphate buffer, filtered (0.22 μm) and degassed. Fibers were perfused at a constant flow rate of 2 μl/min with a CMA/100 microinjection pump (Carnegie Medicine, Stockholm, Sweden). After a 60 min wash-out period, consecutive 20-min samples of perfusate were collected and injected into a high performance liquid chromatography (HPLC) system equipped with an electrochemical detector (ESA Coulochem II, Bedford, MA, USA) in order to quantitate DA levels (Pozzi et al. 1995). The first electrode was set at +300 mV (oxidation) and the second at −225 mV (reduction). Separation was obtained by using a reverse phase column (LC-18 DB, 15 cm, 5 μm particle size, Supelco). The mobile phase, consisting of 13.6 g/l of sodium acetate, 37 mg/l of disodium EDTA dihydrate, 80 mg/l of octyl sodium sulfate, 60 ml/l of methanol, pH 4.1 with acetic acid, was pumped at a constant flow rate of 1.0 ml/min (Shimadzu LC-10AD).

According to our previous studies (Cagiano et al. 1998b; Pozzi et al. 1995; Tattoli et al. 2001) once a stable basal DA output was obtained (no more than 10% difference between two consecutive samples) rats were given a challenge dose of amphetamine (0.5 mg/kg). d-Amphetamine sulfate (Sigma Chemical Company, St. Louis, MO, USA) was dissolved in saline and administered (as the salt) s.c. in a volume of 2 ml/kg.

The exact position of the microdialysis probe was verified by histological procedures at the end of each experiments, and only rats in which probe tracks were

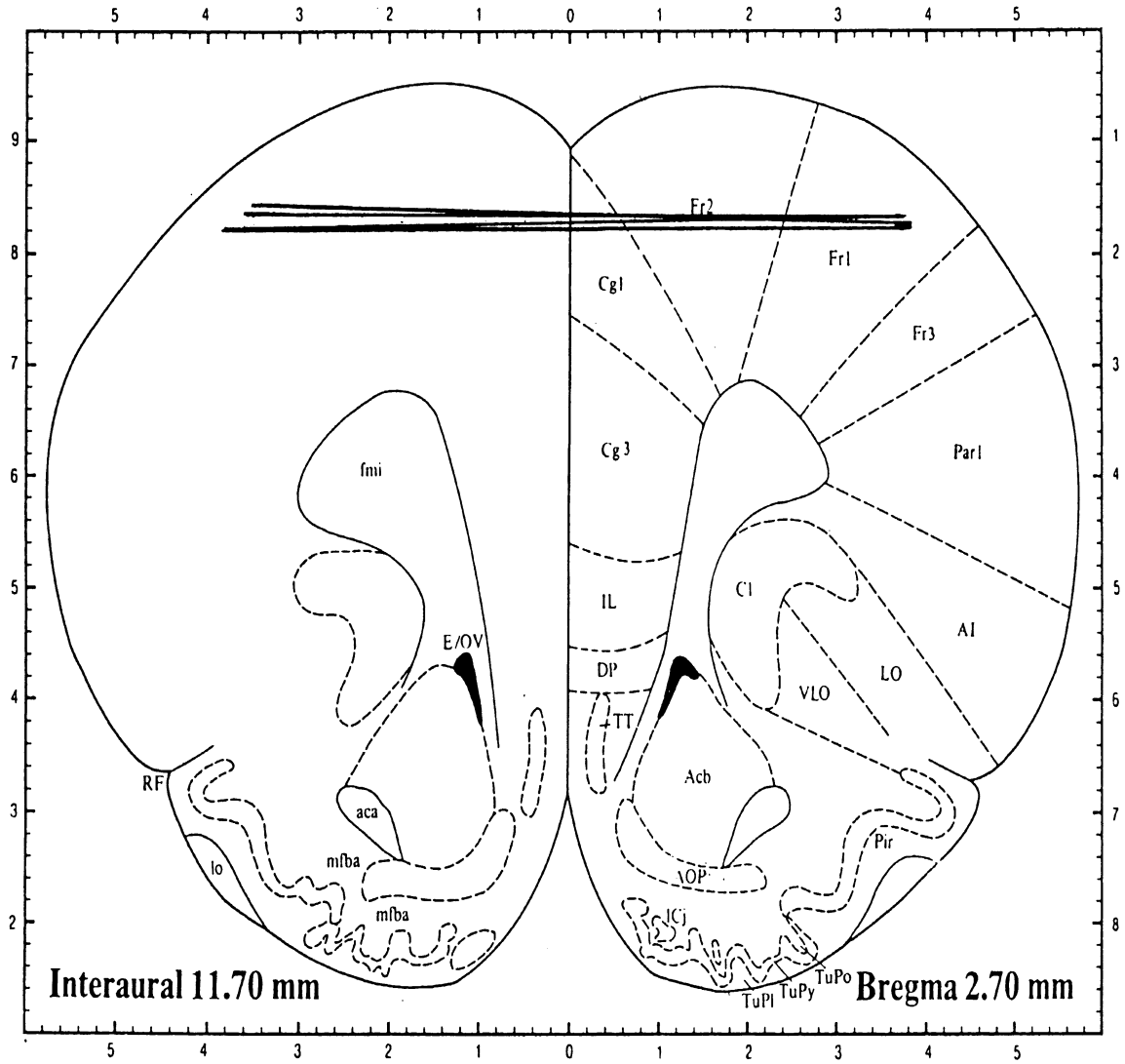

Figure 2. Cortical section, redrawing from Paxinos and Watson (1986), represents the track corresponding to the dialyzing portion of the probes implanted in the prefrontal cortex.

exactly located in the target area (Figure 2) were considered in the results.

Each experimental group consisted of the following number of animals: sNP-S = 5; sNP-A = 5; sP-S = 6; and sP-A = 4.

Statistical Analysis. Data were analysed by a three-way ANOVA for repeated measures followed by a post-hoc test (Dunnett's test).

RESULTS

Blood Alcohol Levels (BALs)

The exposure to a 3% (v/v) alcohol solution, from Day 15 of pregnancy to Day 7 after parturition, resulted in equivalent BALs in sP and sNP dams. BALs were significantly increased on Day 7 after parturition relative to those found on Day 20 of pregnancy in both animal lines ($F_{times} = 17.38$, df = 1/28, $p < .0005$). Twenty-four hours after the end of perinatal exposure, alcohol was undetectable in the blood of both sP and sNP dams. Maternal BALs (mean mmol/l ± S.E.M.) were as follows: (i) Day 20 of gestation (sNP = 3.5 ± 0.7; sP = 4.3 ± 0.5); and (ii) Day 7 after parturition (sNP = 7.9 ± 1.4, $p < .01$ vs. sNP on Day 20 of gestation; sP = 8.2 ± 1/1; $p < .01$ vs. sP on Day 20 of gestation).

Fetal BALs (mean mmol/l ± S.E.M.) on Day 20 of gestation were equivalent to those exhibited by dams (sNP = 2.8 ± 0.4; sP = 3.6 ± 0.4). The neonatal BALs at postnatal Day 7 were as follows: sNP = 1.1 ± 0.2; sP = 1.3 ± 0.2. The statistical analysis of fetal and neonatal BALs showed no significant differences between the two lines. Twenty-four hours after termination of maternal alcohol exposure, alcohol was undetectable in the blood of both sP and sNP pups.

Behavioral Studies

Homing Behavior and Locomotor Activity. Perinatal alcohol exposure did not significantly affect homing behavior or locomotor activity in sP and sNP offspring (Figures 3A and 3B). A two-way ANOVA of orientation scores revealed no significant differences among groups ($F_{rat\ lines} = 2.22$, df = 1/28, n.s.; $F_{treatments} = 4.73$, df = 1/28, $p < .05$; $F_{rat\ lines \times\ treatments} = 3.79$, df = 1/28, n.s.). As far as locomotor activity, a two-way ANOVA of the number of crossings showed the following results: $F_{rat\ lines} = 2.80$, df = 1/28, n.s.; $F_{treatments} = 2.60$, df = 1.28, n.s.; $F_{rat\ lines \times\ treatments} = 2.18$, df = 1/28, n.s.

Elevated Zero-Maze Test. % TIME OPEN (TO). The data in Figure 4A show that sP-S rats spent significantly less time on the open arms than sNP-S rats at either 90 or 180 days of age. Perinatal exposure to alcohol significantly increased the percentage of time spent on the open arms by 90 days old sP and sNP rats.

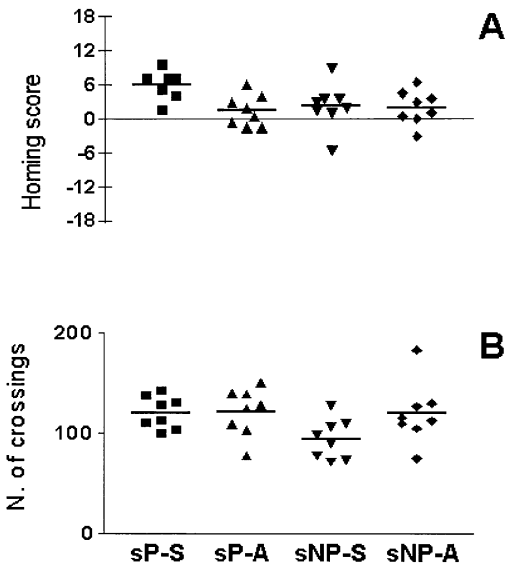

Figure 3. Effects of perinatal exposure to alcohol on homing behavior **(*Panel A*)** and locomotor activity **(*Panel B*)** of 10-day old male pups. Each point represents an individual observation. Horizontal lines indicate the mean.

At 180 days of age, sP-A rats spent significantly more time on the open arms than sP-S animals, whereas sNP-S offspring did not exhibit any significant difference with respect to sNP-A ones. A two-way ANOVA of % TO for each age showed the following differences: (i) 90 days: $F_{rat\ lines} = 229.7$; df = 1/36, $p < .001$; $F_{treatments} = 42.6$; df = 1/36, $p < .001$; $F_{rat\ lines \times\ treatments} = 5.68$, df = 1/36, $p < .05$; (ii) 180 days: $F_{rat\ lines} = 107.5$; df = 1/36, $p < .001$; $F_{treatments} = 10.8$; df = 1/36, $p < .005$; $F_{rat\ lines \times\ treatments} = 4.25$, df = 1/36, $p < .05$.

HEAD DIPS (HDIPS). A two-way ANOVA for HDIPS in 90-days old offspring showed the following differences: $F_{rat\ lines} = 205.65$; df = 1/36, $p < .0001$; $F_{treatments} = 47.31$; df = 1/36, $p < .0001$; $F_{rat\ lines \times\ treatments} = 0.71$, df = 1/36, n.s. In the absence of significant interaction between treatment and line, selective post-hoc tests (Tukey's test) performed on the basis of data inspection as suggested by Wilcox (1987), provided evidence that sP-S rats exhibit a significantly lower number of HDIPS with respect to sNP-S rats. Moreover, 90-days old sP-A and sNP-A rats performed a number of HDIPS significantly higher than controls (Figure 4B).

A two-way ANOVA for HDIPS in 180-days old offspring showed the following differences: $F_{rat\ lines} = 175.1$; df = 1/36, $p < .0001$; $F_{treatments} = 7.86$; df = 1/36, $p < .01$; $F_{rat\ lines \times\ treatments} = 7.0$, df = 1/36, $p < .05$. Figure 4B shows a significantly lower number of HDIPS in sP-S than sNP-S rats. Moreover, a significant increase in the number of HDIPS was observed in 180-days old sP-A but not in sNP-A rats.

STRETCHED-ATTEND POSTURES (SAP). A two-way ANOVA for SAP in 90-days old offspring showed the

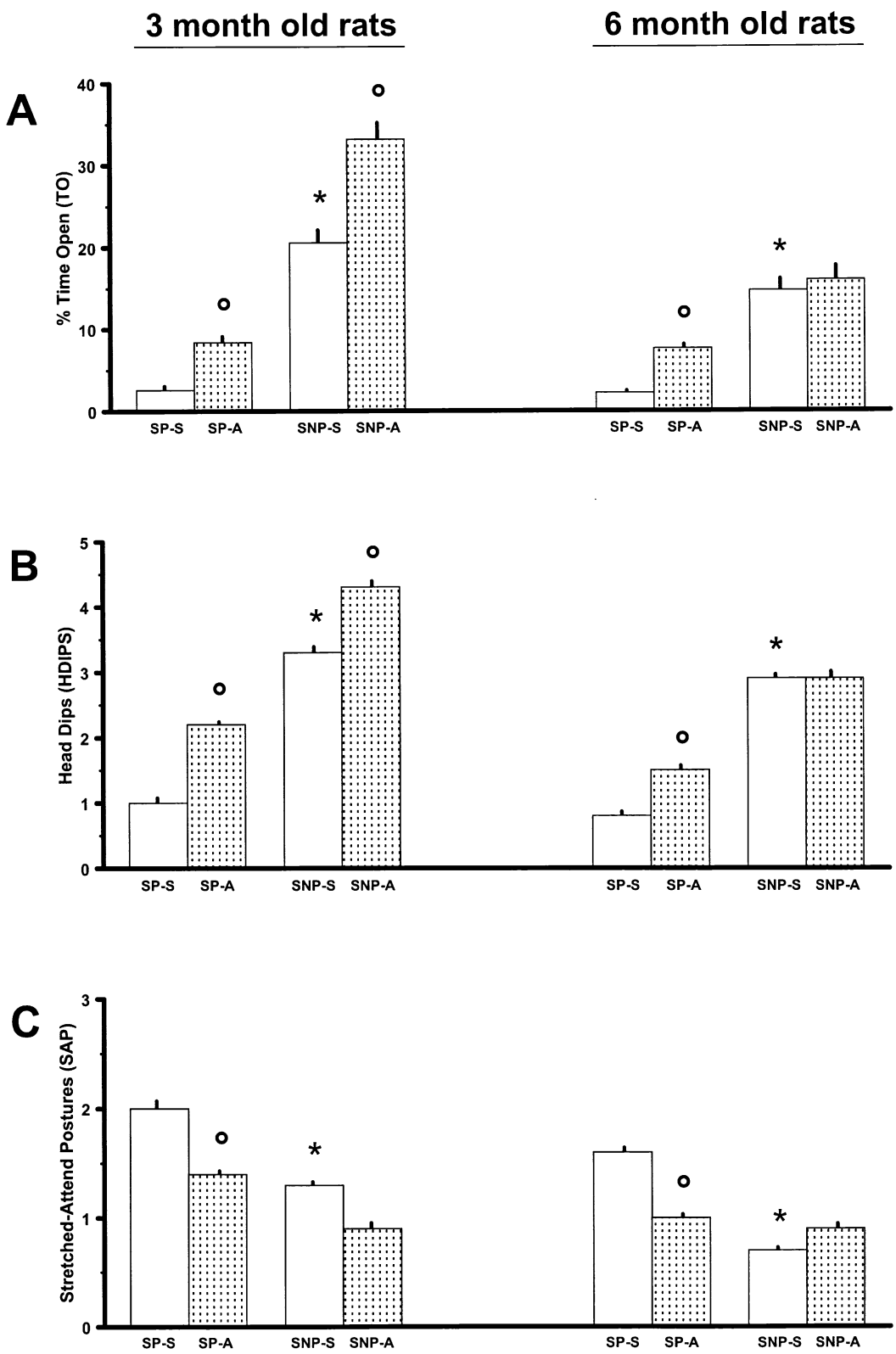

Figure 4. Effects of perinatal exposure to alcohol on elevated zero-maze test. ***Panel A*:** Data represent the mean ± S.E.M. of percent time spent on the open arms (TO). *$p < .05$ vs. sP-S; °$p < .05$ vs. the respective control line (sP-S or sNP-S); ***Panel B*:** Data represent the transformed mean ± S.E.M. of the number of HDIPS (square root). *$p < .05$ vs. sP-S; °$p < .05$ vs. the respective control line (sP-S or sNP-S); ***Panel C*:** Data represent the transformed mean ± S.E.M. of the number of SAP (square root). *$p < .05$ vs. sP-S; °$p < .05$ vs. the respective control line (sP-S or sNP-S) (Duncan's Multiple Comparison test and Tukey's test)).

following differences: $F_{rat\ lines} = 14.57$; df = 1/36, $p < .001$; $F_{treatments} = 8.62$; df = 1/36, $p < .01$; $F_{rat\ lines \times treatments} = 0.68$, df = 1/36, n.s. In the absence of significant interaction between treatment and line, selective post-hoc tests (Tukey's test) performed on the basis of data inspection as suggested by Wilcox (1987), provided evidence of a higher number of SAP in sP-S than in sNP-S rats. SAP was clearly reduced by perinatal alcohol exposure in the former line, while alcohol did not affect this endpoint in sNP rats.

A two-way ANOVA for SAP in 180-day old offspring showed the following differences: $F_{rat\ lines} = 5.70$; df = 1/36, $p < .05$; $F_{treatments} = 0.74$; df = 1/36, n.s.; $F_{rat\ lines \times treatments} = 4.17$, df = 1/36, $p < .05$. sP-S rats exhibited a higher number of SAP with respect to the sNP line. Perinatal alcohol exposure significantly reduced SAP in sP rats but not in sNP offspring (Figure 4C).

As concerns the number of transitions from the closed to open arms, the data (not reported) failed to show any difference related to line or alcohol exposure, which was confirmed by a two-way ANOVA, which did not reveal significant main or interaction effects.

Sexual Behavior and Ultrasonic Emission. Perinatal exposure to alcohol notably altered copulatory activity of sP offspring, while it did not affect sexual behavior of sNP animals. As far as the latency to the first intromission, Kruskal Wallis ANOVA showed significant differences among groups in the third 10-min session (H = 16.11, df = 3, $p < .005$). Individual comparisons (Dunn's Multiple Comparison test) indicated that the latency to the first intromission was significantly lower in sP-S rats than in sNP-S animals. Perinatal alcohol exposure significantly increased the latency to the first intromission in sP offspring whereas it did not affect this behavioral end-point in sNP rats (Figure 5). The percentage of rats exhibiting at least one intromission in the fourth 30-min session was significantly lower ($p < .05$, Fisher's exact test) in sP-A group with respect to the control group (sP-S = 100%; sP-A = 50%; sNP-S = 70%; sNP-A = 60%).

A trend towards a decrease in the percentage of sP-A achieving ejaculation with respect to sP-S ones was observed in the first two 10-min sessions. This tendency reached statistical significance in the third 10-min session (Figure 6).

As far as ultrasonic emission during sexual encounters, an overall Kruskal Wallis ANOVA of 50 kHz call latencies showed the following significant difference (fourth 30-min session): H = 13.35, df = 3, $p < .005$. Individual comparisons indicate that the latency to the first 50 kHz call was significantly lower in sP-S rats than in sNP-S animals. Perinatal alcohol did not affect this end-point in both sP and sNP offspring (Figure 7).

For all other sexual and ultrasonic parameters, statistical analysis did not show any significant difference between rat lines and between sucrose- and alcohol-exposed rats (data not shown).

Sleep Time. A two-way AVOVA for onset of the righting reflex loss revealed no significant differences among groups $F_{rat\ lines} = 1.59$; df = 1/27, n.s.; $F_{treatments} = 0.18$; df = 1/27, n.s.; $F_{rat\ lines \times treatments} = 0.01$, df = 1/27, n.s.), even though a trend towards a decrease in onset of sleep time was observed in sP rats with respect to sNP ones (Figure 8A). Nevertheless, sP rats were significantly slower in recover of the righting reflex than sNP rats (Figure 8B). Perinatal alcohol exposure did not significantly affect either onset or duration of sleep time. A two-way ANOVA for sleep time showed the following differences: $F_{rat\ lines} = 86.64$; df = 1/27, $p < .0001$; $F_{treatments} = 0.21$; df = 1/27, n.s.; $F_{rat\ lines \times treatments} = 1.62$, df = 1/27, n.s.

Neurochemical Studies

Effects of Amphetamine Challenge on Extracellular DA Concentrations in the Prefrontal Cortex of Perinatally Sucrose- and Alcohol-Exposed sP and sNP Rats. A three-way ANOVA for repeated measures of basal DA concentrations (two consecutive samples collected before amphetamine challenge) indicated that basal DA concentrations (Figure 9) did not significantly differ in both sucrose- and alcohol-treated sP and sNP rats ($F_{rat\ lines} = 0.18$; df = 1/16, n.s.; $F_{treatments} = 0.40$; df = 1/16, n.s.; $F_{times} = 0.15$, df = 1/16, n.s.; $F_{rat\ lines \times treatments} = 0.28$, df = 1/16, n.s.; $F_{rat\ lines \times times} = 0.26$, df = 1/16, n.s.; $F_{treatments \times times} = 0.23$; df = 1/16, n.s.; $F_{rat\ lines \times treatments \times times} = 1.08$, df = 1/16, n.s.).

Furthermore, a three-way ANOVA for repeated measures of changes in extracellular DA concentrations (actual values) elicited by a challenge dose of amphetamine (last basal value and six consecutive samples after amphetamine challenge) gave the following differences: ($F_{rat\ lines} = 1.41$; df = 1/16, n.s.; $F_{treatments} = 3.10$; df = 1/16, n.s.; $F_{times} = 50.97$, df = 6/96, $p < .0001$; $F_{rat\ lines \times treatments} = 0.20$, df = 1/16, n.s.; $F_{rat\ lines \times times} = 2.22$, df = 6/96, n.s.; $F_{treatments \times times} = 3.71$; df = 6/96, $p < .01$; $F_{rat\ lines \times treatments \times times} = 1.41$, df = 6/96, n.s.

Within-group comparisons (Dunnett's test) showed that amphetamine administration induced a significant increase in extracellular DA concentrations (actual values) with respect to the last basal sample in both alcohol- and sucrose-treated sP and sNP animals.

DISCUSSION

The present findings show that developmental exposure to alcohol, at doses not associated with tolerance, dependence, withdrawal symptoms, or overt signs of toxicity (Battaini et al. 1998; Cagiano et al. 1998a; Govoni et al. 1994), produces different long-lasting behav-

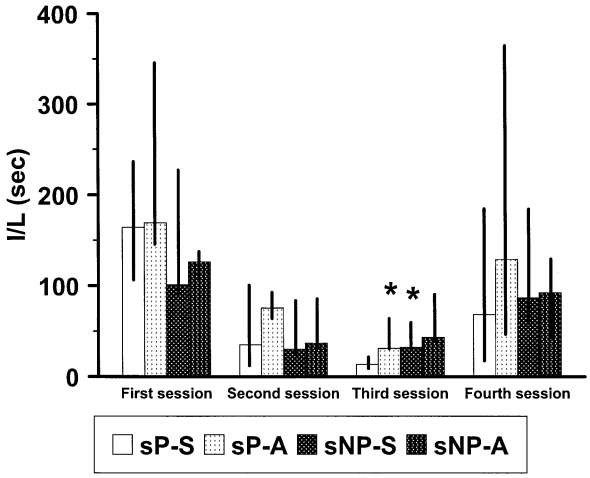

Figure 5. Effects of perinatal exposure to alcohol on Intromission Latency (I/L). Data are expressed as median values and interquartiles (—). *$p < .05$ vs. sP-S (Dunn's Multiple Comparison test).

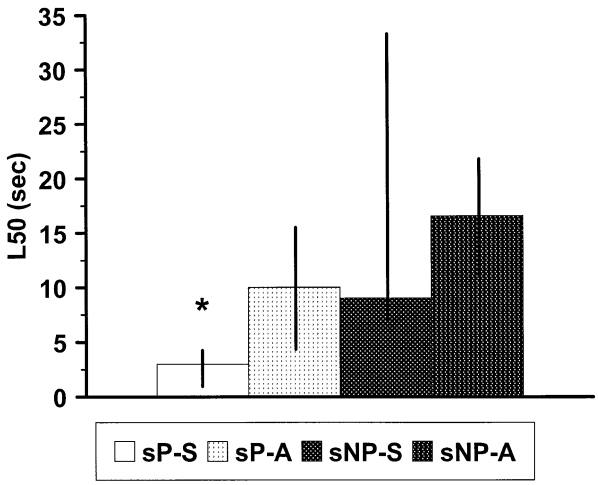

Figure 7. Effects of perinatal exposure to alcohol on the latency to the first 50 kHz call in the fourth 30-min session. Data are expressed as median values and interquartiles (—). *$p < .05$ vs. sNP-S (Dunn's Multiple Comparison test).

ioral changes in the offspring of two rat lines (sP and sNP rats) selectively bred for opposite alcohol preference and consumption. In agreement with our recent findings (Tattoli et al. 2001), the present results, showing a selective differential sensitivity (end-point specific) of sP and sNP lines to developmental low alcohol exposure, suggest that genetic factors may confer specific susceptibility to ARNDs.

In particular, the present findings have shown that perinatal exposure to low doses of alcohol did not affect homing performance of both sP and sNP offspring. This test exploits the strong tendency of the immature pup to maintain body contact with the dam and the siblings, which requires adequate sensory, olfactory, motor and ultrasonic communication capabilities as well as adequate associative and discriminative capabilities that allow the pup to become imprinted by the mother's odour, to remember it, and to recognize it among others (Bignami 1996). The lack of changes in this simple form of learning is in agreement with recent findings (Tattoli et al. 2001) showing that perinatal exposure to low doses of alcohol did not affect the acquisition of an active avoidance task and hippocampal Long-Term Potentiation (a cellular and molecular model for learning

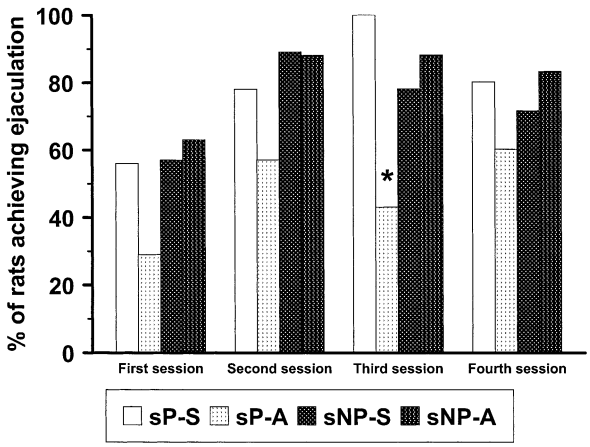

Figure 6. Effects of perinatal exposure to alcohol on ejaculation. *$p < .05$ vs. sP-S (Fisher's exact test).

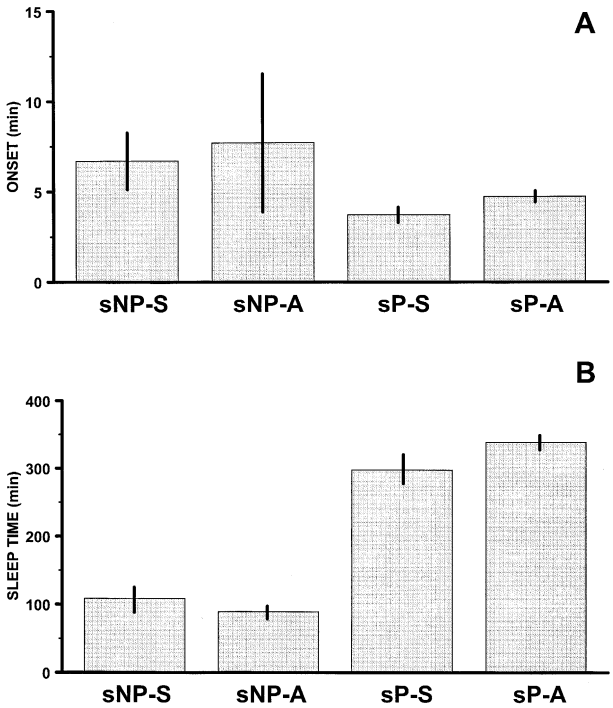

Figure 8. Effects of perinatal exposure to alcohol on time for loss (onset, ***Panel A***) and regaining (sleep time, ***Panel B***) of the righting reflex after a challenge dose of alcohol (3 g/kg, i.p.). Each column is the mean ± S.E.M..

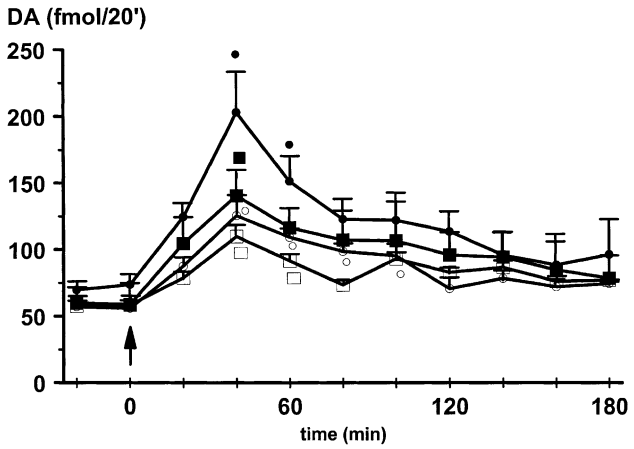

Figure 9. Effects of amphetamine challenge (0.5 mg/kg, s.c., ↑) on extracellular DA concentrations in the prefrontal cortex of 90-day old sP and sNP rats perinatally exposed to sucrose (○-○ sP-S; □-□ sNP-S) or alcohol (●-● sP-A; ■-■ sNP-A). Data are mean ± S.E.M. Significant differences: sP-S (○), sP-A (●), sNP-S (□), sNP-A (■), $p < .05$ vs. last basal sample (Dunnett's test).

and memory) in either adult sP or sNP offspring. Furthermore, locomotor activity of both sP and sNP rats was not influenced by perinatal alcohol exposure. By contrast, developmental alcohol exposure differentially affected the performance of the two lines in the elevated zero-maze.

This test, based on the natural aversion and avoidance of rodents for open spaces, is a modification of the elevated plus-maze model of anxiety which incorporates both traditional (percent of time in the open arms) and novel ethological measures (number of head dipping over the edge and stretched-attend postures from the closed to the open quadrant) in the analysis of treatment effects. The present design comprises an elevated annular platform with two opposite enclosed quadrants and two open ones, removing any ambiguity in the interpretation of time spent on the central square of the traditional design and allowing uninterrupted exploration (Shepherd et al. 1994). The results have shown that sP-S rats behaved differently from sNP-S rats. Both 90- and 180-days old sP-S rats spent less time in the open arms and made fewer head dips over the edge of the platform than sNP-S rats, while the number of SAP made by sP-S was higher with respect to sNP-S. These data parallel those reported by Colombo and coworkers (1995) suggesting a higher degree of anxiety in sP than sNP rats.

As far as perinatal alcohol treatment is concerned, both 90- and 180-days old sP-A rats exhibited an increase in % TO on the open arms and HDIPS as well as a decrease in SAP. This profile is similar to that elicited by the acute administration of benzodiazepine derivatives in adult rats (Shepherd et al. 1994). Conversely, SAP was not affected by perinatal alcohol exposure in either 90- or 180-days old sNP offspring. Unlike sP-A rats, the increase in both % TO on the open arms and HDIPS exhibited by sNP-A was reversible. These data indicate that the two rat lines display different effects on emotional reactivity in response to developmental alcohol exposure. Interestingly, the present results are in agreement with previous findings showing that perinatal alcohol exposure significantly decreased the rate of ultrasonic emission in sP pups, whereas it did not influence this indicator of emotional reactivity in sNP neonates (Tattoli et al. 2001).

The present experiments have shown subtle differences between adult sP-S and sNP-S male rats in sexual behavior. Specifically, the intromission latency was significantly lower in the former than in the latter. Decreased pre-ejaculatory 50 kHz call latencies were observed in sP-S rats with respect to sNP-S line. Since these two endpoints reflect rat sexual motivation (McIntosh and Barfield 1980; Cagiano et al. 1989a), it could be hypothesized that sP rats are characterized by higher levels of sexual arousal than sNP animals. Between lines differences have also been observed in alcohol-related sexual alterations. In particular, sP-A male rats exhibited a different profile of copulatory activity with respect to sNP-A offspring. Specifically, sP-A rats showed a higher intromission latency than sNP-A ones and the percentage of sP-A rats exhibiting at least one intromission or achieving ejaculation was significantly lower than that of sNP-A offspring.

The differences between the two lines in alcohol effects on copulatory activity are interesting in view of evidence indicating that pharmacologically induced changes in anxiety may alter the expression of male sexual behavior; in particular, it has been shown that a benzodiazepine derivative (diazepam) produces an inhibition of copulatory activity in rats (Fernandez-Guasti et al. 1990). Since sP-A rats, unlike sNP-A rats, exhibited a benzodiazepine-like profile when tested in the elevated zero-maze model of anxiety, alcohol-induced changes in sexual behavior of sP offspring could be partly linked to the effects of perinatal exposure to this drug of abuse on their emotional reactivity.

The results obtained in the present study are in agreement with both preclinical and clinical data (see Kelly et al. 2000 for references) showing alterations of sexual behavior in alcohol-exposed humans and animal models of FAS. According to Kelly and coworkers (2000), there is enough evidence to indicate that, in addition to cognitive deficits, important indicators of CNS dysfunction in FAS are problems in the social domain, including sexual endpoints.

The present experiments have demonstrated that adult sP-S rats were significantly slower in recovery of the righting reflex than sNP-S rats in response to the acute administration of a challenge dose of alcohol.

These results confirm recent findings of Colombo and coworkers (2000) who have shown that sP rats treated with a challenge dose of ethanol regained the righting reflex at significantly lower BALs than sNP rats; this suggests that the longer sleep time recorded in sP rats was indeed the consequence of a higher neurosensitivity to ethanol and not due to a reduced rate of ethanol metabolism.

However, perinatal alcohol exposure did not affect the responsiveness of either line to the acute administration of this drug of abuse.

As far as the dopaminergic system is concerned, the present data have shown that basal levels of DA in prefrontal cortex did not significantly differ either in sucrose- or alcohol-treated sP and sNP animals. In addition, the administration of a challenge dose of amphetamine induced a similar significant increase in extracellular DA concentrations with respect to the last basal sample in both sucrose- and alcohol-treated sP and sNP rats.

Our recent findings have demonstrated that basal levels of DA in the nucleus accumbens (NAC) were significantly higher in sP rats than sNP animals. These differences were present in both alcohol- and sucrose-treated offspring. Moreover, perinatal alcohol exposure did not affect basal DA levels nor the size and temporal pattern of amphetamine-induced increase in DA concentrations in the NAC of both rat lines (Tattoli et al. 2001).

DA inputs to the NAC arise from cell bodies in the ventral tegmental area. The frontal cortex receives dopaminergic inputs from both the ventral tegmental area and the substantia nigra (Bjorklund and Lindvall 1984). The higher DA levels found in the NAC of sP rats may reflect a difference from the sNP line in this distinct group of DA neurons. Conversely, the demonstration that extracellular concentrations of DA in the prefrontal cortex of sP and sNP rats are similar could suggest that there is no difference between sP and sNP lines in cortical DA innervation.

It should be recalled, however, that differences in opioid, serotonergic and GABAergic functions have been observed between sP and sNP rats (Devoto et al. 1998; Fadda et al. 1999; Saba et al. 2001), which might be indirectly responsible for selective modification of dopaminergic activity in particular brain areas.

Finally, the present data indicate that developmental alcohol exposure produced equivalent BALs in the sP and sNP lines, excluding the possibility that differential alcohol effects were due to differences between lines in the rate of alcohol metabolism. Alcohol was undetectable in the blood of dams and male pups 24 h after the end of the exposure and, therefore, the behavioral alterations observed in the offspring do not seem to be attributable to direct actions of this compound. Since dam and male pup weight gain as well as dam food consumption were not affected by the exposure to this drug of abuse, nutritional deficits were not responsible for neurofunctional abnormalities observed in this study.

The present experiments, confirming that animal models may provide a valuable tool for assessing genetic influences on sensitivity to alcohol neurobehavioral teratogenicity, suggest that genetic factors (although not necessarily those responsible for differences in alcohol preference) could contribute to the variability in outcomes observed after gestational intake of this drug of abuse (West 1989).

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