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ONTOGENY OF ETHANOL INTAKE IN ALCOHOL PREFERRING (P) AND ALCOHOL NON-PREFERRING (NP) RATS

Ricardo Marcos Pautassi^{a,c,*}, Michael E. Nizhnikov^b, Eric Truxell^b, Elena I Varlinskaya^b, and Norman E. Spear^b

^aInstituto de Investigación Médica M. y M. Ferreyra (INIMEC – CONICET), Córdoba, C.P 5000, Argentina

^bCenter for Development and Behavioral Neuroscience, Binghamton University, Binghamton, NY 13902-6000, USA

^cFacultad de Psicología, Univ. Nacional de Córdoba, Córdoba, C.P 5000, Argentina

Abstract

There is a scarcity of research on ethanol affinity in alcohol-preferring (P) rats before weaning and it is unknown if neonate P rats exhibit ethanol intake preferences comparable to those observed in adult P rats. This study examined ethanol intake in alcohol-preferring and non-preferring (NP) rats 3 hours after birth (Experiment 1, surrogate nipple test), at postnatal days (PD) 8, 12 and 18 (Experiment 2, consumption off the floor procedure) and at adolescence (Experiment 3, two-bottle choice test at PD32). The high-preference genotype was readily expressed three hours after birth. P neonates drank twice as much ethanol as their NP counterparts. This heightened ethanol preference transiently reversed at P8, reemerged as weaning approached (P18) and was fully expressed during adolescence. These results help clarify the ontogeny of genetic predisposition for ethanol. Genetic predisposition for higher ethanol intake in P than in NP rats seems to be present immediately following birth.

Keywords

P rats; ethanol intake; neonate; infant; adolescent

Early exposure to ethanol, whether in the womb, during infancy or in adolescence is a major predictive factor for future abuse of the drug (Spear and Molina 2005). Apart from early experience with alcohol, genetic factors also play a major role in the development of alcohol-related problems (Ducci and Goldman, 2008). Together these factors suggest that experience with ethanol early in life, especially for individuals genetically predisposed to alcoholism, is a major predictor for future alcohol abuse (Bonthius and West, 1990; Baer, Barr, Bookstein, Sampson and Streissguth, 1998; Baer, Sampson, Barr, Connor and Streissguth, 2003).

Rat lines selected through bidirectional breeding for either high or low ethanol acceptance have been successfully developed and are used as a model of genetic predisposition for alcohol abuse (Bell, Rodd, Lumeng, Murphy & McBride, 2006). Among several lines of these rats that have proven useful are the preferring (P) and the non-preferring (NP) rats bred

*Corresponding author: Instituto de Investigación Médica M. y M. Ferreyra (INIMEC – CONICET), Friuli 2434, Córdoba, C.P 5000, TE 54-351-4681465, FAX 54-3514695163; Argentina; rpautassi@gmail.com.
MEN and RMP equally contributed to this manuscript. ET now works at Research Institute on Addictions, 1021 Main Street Buffalo, NY 14203-1016.

at Indiana University Medical School (Bell, Rodd, Lumeng, et al., 2006; Grahame, 2000). Considering the association between early alcohol exposure and later alcohol-related problems, it is important to assess the drinking patterns of these rats soon after birth (when they have little or no experience with their postnatal environment), as well as during infancy and adolescence. It is not yet clear whether these animals express the genetic predisposition for high ethanol consumption right after birth or during later infancy. Does early ethanol exposure affect subsequent preference for ethanol more in animals bred for high ethanol acceptance than in those bred for low acceptance? To this end, research groups have begun to delineate the ontogeny of ethanol preference, acceptance and reinforcement in rat lines selected for high and low ethanol intake (McKinzie, Nowak, Murphy, Li and Lumeng, 1998; McBride, Cox, Murphy, Li, Lumeng and McBride, 1999). An important but largely ignored question is whether individual differences in ethanol acceptance and general risk for ethanol abuse are present from the moment of conception or emerge later through specific developmental processes.

McKinzie and colleagues (1999) assessed voluntary intake of ethanol through early infancy utilizing a procedure that allows preweanlings to ingest independently of the dam, the consumption off the floor (COF) paradigm. Starting on postnatal day (PD) 5 and up to PD20, rat pups were placed into a warmed chamber with kimwipes soaked in 15% ethanol or water, for 30 minutes. Body weight gain was used as an index of fluid intake (McKinzie et al., 1999). The results indicated that P rats had greater intake of ethanol relative to water only on PD15 and actually consumed more water than ethanol on PD5 and PD6. These results mimicked those seen in NP rats tested in an identical manner, with the exception that NP rats did not show any signs of ethanol preference on PD15. These results indicate that preference for ethanol is not observed during the first two weeks of life in the P line of rats (McKinzie et al., 1999). However, a methodological caveat acknowledged by the authors was that each subject experienced daily exposure to alcohol. Consequently the results on any given day, apart from the first day of testing, may have been influenced by the totality of previous experience with ethanol or lingering effects of ethanol intoxication experienced on the previous day.

Clearly, however, the preference for ethanol in P rats develops prior to adolescence, when P rats consumed significantly more ethanol than NP rats in a two bottle free choice test (McKinzie et al., 1998). Both P and NP rat lines in this experiment were induced to drink ethanol by having access to the drug (10%) as their only source of fluid from PD29 to PD35 and, as in the McKinzie et al. (1999) study, ethanol drinking was repeatedly measured in the same animal. This leaves open the question of ontogeny-dependent expression of ethanol preference in these lines of rats independent of effects of prior exposure to ethanol. Moreover, it is still unknown if P rats tested earlier than P5 (the youngest rats tested by McKinzie et al., [1999]) will exhibit ethanol preferences comparable to those observed in adult P rats.

The goal of the current set of experiments was to extend this previous research on the ontogeny of ethanol intake by P and NP rats. Experiment 1 examined the ethanol intake of P and NP newborns just 3 hours after cesarean delivery. Neonates were tested by means of the surrogate nipple procedure, which has proven successful to examine determinants of early ethanol affinity (Nizhnikov, Molina, Varlinskaya and Spear, 2006). Not having any experience with maternal care these animals represent an ideal opportunity for assessing genetic predisposition for ethanol acceptance in these rat lines. In Experiment 2 consumption of ethanol from the floor in 8, 12 and 18 day old rat pups was examined. The rationale for using the COF is the same as that of McKinzie et al. (1998; 1999) -- rats of these ages do not readily drink from standard tubes. In the final experiment adolescent (PD32) acceptance of ethanol was examined, using a two-bottle choice test. Aside from the

important issue of testing P and NP neonates, the primary additions to the fine experiments by McKinzie et al. (1998, 1999) were that in the present study, all animals were naïve to alcohol prior to each test and a wider range of ages and ethanol concentrations was tested. The overarching objective is to help clarify the ontogeny of genetic predisposition for ethanol in this selectively bred line of rats.

General Methods

Experimental Subjects

A total of 621 rats were employed. Ninety-four neonates (P rats $n=48$, NP rats $n=46$), representative of 19 litters (9 P litters and 10 NP litters), were used in Experiment 1. Ethanol intake measurements in infancy employed 200 P rats and 240 NP animals, derived from 44 litters (20 P litters and 24 NP litters). Eighty-seven adolescents (31 P rats and 56 NP rats), derived from 11 litters (4 P litters and 7 NP litters) were employed in Experiment 3. For breeding, adult P or NP rats (obtained from Indiana University Medical School, with permission of Dr. T-K Li) were housed for 4 days in groups of two females and one male in plastic, opaque cages (45 cm long $\times$ 23 cm wide $\times$ 20 cm high) that were partially filled with pine shavings. Vaginal smears were collected daily for the detection of sperm. The day sperm was detected was considered embryonic day (E) 0. After 4 days the male was removed from the cage. Pregnant females were housed together (2 in a cage) until E19, when they were separated and placed into individual cages. All animals were housed in a temperature-controlled (22°C) vivarium maintained on 12-hr light / dark cycle (lights on at 0700) with ad libitum access to food and water.

The neonates employed in Experiment 1 were delivered by cesarean section (for details, Cheslock, Varlinskaya, High and Spear, 2003). Pups were placed in a plastic container (12 cm long $\times$ 12 cm wide $\times$ 6 cm high) lined with moistened paper towels. The temperature at the bottom of the plastic container was maintained at 35.0° C using a heating pad. The entire procedure was completed within 6–8 min. Pups were stroked gently to promote independent respiration. They were then transferred to an incubator maintained at 34.5° C $\pm$ 0.5° C with 90% humidity, where they remained for 3 – 3.5 hours until the beginning of the experiment. From birth and throughout Experiment 1, neonates were maintained in temperature- and humidity-controlled environments with conditions that simulate the natural nest (Nizhnikov et al., 2006; Nizhnikov, Varlinskaya and Spear, 2009).

To avoid confounding effects of experimental treatment with litter variance, no more than two animals per litter (one male and one female) were assigned to any particular experimental condition (Holson & Pearce, 1992).

Dams assigned to Experiments 2 and 3 were checked for births daily at 0800. Upon discovery of birth, rat pups were assigned an age of PD0. At PD1 litters were culled to five males and five females. P and NP litters employed in Experiment 3 were weaned at PD21. Four male and four female rats from each of the 11 litters were used.

At all times, animals were treated in accordance with the guidelines for animal care established by the National Institutes of Health on the Guide for the Care and Use of Laboratory Animals (National Research Council, 1996).

Ethanol intake assessment at PD1: the surrogate nipple technique

The surrogate nipple technique has been widely employed to assess self-administration of ethanol, milk and other nutritive or non-nutritive fluids, shortly after birth (Petrov, Nizhnikov and Smotherman, 2000; Petrov, Varlinskaya and Spear, 2003). It has also been successfully used to assess ethanol-mediated conditioning (Abate, Varlinskaya, Cheslock,

Spear and Molina, 2002; Nizhnikov et al., 2006). The surrogate nipple was cast from latex rubber (AMACO rubber latex, Indianapolis, IN) and shaped into a conical form to measure 12 mm in length with 1 mm diameter at the rounded tip and 2.5 mm diameter at the base. A circular piece of vinyl, measuring 5 mm in diameter, was positioned 6 mm from the tip of the nipple to provide a point of contact for the pup's snout during oral grasping of the surrogate nipple. The base of the nipple was attached to the end of an angled dental probe to facilitate presentation by the experimenter. A length of PE 10 tubing extended through the length of the surrogate nipple and ended flush with the rounded tip of the nipple.

In these circumstances pups actively extract water or a given concentration of ethanol during suckling due to their application of slight negative pressure on the nipple. This was accomplished by the use of a 1 mm diameter hole through the wall of the syringe that permitted the movement of water through the tubing. The syringe was placed in a holder in the test chamber and was positioned to be level with the experimental subject's snout. In this position, the hole in the wall of the syringe was on the upper surface of the body of the syringe and in front of the piston. The PE 10 tubing and the syringe, when filled with the fluids, represented an open hydraulic system with two ends: the end of the PE 10 tubing located at the tip of the nipple and the hole located on the upper surface of the body of the syringe. The small diameter of the tubing, along with surface tension of the fluid, prevented its spontaneous effusion from the end located at the tip of the nipple. Slight negative pressure produced by the pups while attached to the nipple was necessary and sufficient to extract water or a given ethanol concentration from the surrogate nipple.

Pups were exposed to the nipple in a plastic container (5 cm long $\times$ 5 cm wide $\times$ 2 cm high) placed in a transparent glove box (63 cm long $\times$ 50 cm wide $\times$ 25 cm high). The bottom of the plastic container was maintained at $35.5^{\circ}\text{C} \pm 0.5^{\circ}\text{C}$ and the inside of the glove box at $28.0^{\circ}\text{C} \pm 1.0^{\circ}\text{C}$ by two temperature controllers (Model 40–90-8B, Frederick Haer, Inc., Brunswick, ME). Exposure to the surrogate nipple involved gentle contact between the tip of the nipple and the oral area of the test subject. No attempt was made to force the tip of the nipple into the mouth of the pup. The subject was completely free to grasp and capture the nipple or disengage from it. The procedure excluded any compulsion from the experimenter, since an attempt to force the nipple into the oral cavity aside from active grasping and voluntary oral capture evokes vigorous nipple rejection and inevitably results in choking and asphyxiation of the experimental subject.

During nipple presentation, in order to facilitate nipple exposure and minimize individual differences in gross body movement, each rat pup was strapped and buckled into a "vest" made from ultra-thin, elastic rubber. The vest was designed to hold the pup in a semi-supine posture. This simulated the natural position of neonatal rats suckling at the maternal nipple. It also prevented pups from righting in the beginning of the testing procedure. The subsequent oral grasping of the nipple in the supine posture involved active suppression of righting. The vest did not otherwise restrict the pup's spontaneous motor activity and produced no apparent discomfort or behavioral incompetence.

Before exposure to the surrogate nipple, the anogenital region of the pup was stroked gently with a cotton ball moistened in distilled water to stimulate urination and defecation. Pups were weighed to the nearest 0.01 g and placed into the test container. At the end of the test session, pups were removed from the testing container, dried with Kimwipes, and again weighed to the nearest 0.01 g.

Ethanol Intake at PD8, PD12 and PD18: the consumption-off-the floor procedure

We closely followed the procedures described in Truxell, Molina and Spear (2007). At the start of the session, animals were removed from the maternity tub and placed into a warmed

holding tub lined with wood shavings. They were then voided by a gentle stroking of the genital region, weighed to the nearest 0.01 g, and placed in pairs into the experimental chambers. The rationale for assessing ethanol intake in pairs was to reduce social isolation stress which, by itself, can alter ethanol intake. The experimental chambers were five glass terrariums with slate bottoms (31×16×20 cm). Each chamber was sectioned in half so that each pair of subjects was contained in a space measuring 15.5 × 16 cm. Chamber temperature was maintained at 32°C via 50w heating pads and was measured continuously with the temperature probe of an exterior thermometer placed directly onto the chamber floor. Kim wipes were used to line the floor and 30 ml of the appropriate ethanol solution [0 (i.e., distilled water), 5, 10, 15 or 20% v/v] was poured into the chamber. When all the pups were weighed and the chambers reached the appropriate temperature, one male and one female from a given litter were placed together in each of the five chambers. After 20 min all pups were removed and placed back into a warmed holding tub, dried, and weighed.

Ethanol Intake at PD32: two-bottle choice procedure

To familiarize rats to the ball-bearing drinking tubes employed at test, subjects were provided two water bottles from PD21 until PD26, one with the same standard drinking tube available in the home cage with the dam, and one with the ball-bearing tube. After PD26 the water bottle with the standard tube was removed.

The subjects' home cages served as the experimental chambers. During the test at PD32 the chamber was divided in half by a wire mesh screen. Each side of the cage held two 100 ml graduated ball-bearing tubes, one contained water, and the other contained the appropriate ethanol solution. The bottom of the tub was lined with wood shavings and the animals had ad lib access to food throughout testing. Animals could see and smell each other, thus reducing isolation stress, but could not disturb each other's fluid bottles.

Testing started at 1730, when animals were weighed and placed into half of the home cage with two bottles, one containing ethanol (5, 10, 15 or 20% v/v) and one containing water (i.e., 0 % v/v). At 0730 (14 hrs later, 10 hrs of dark starting at 1900) of the following day the amount of fluid consumed from the graduated bottles was recorded.

Data analysis

Appropriate measures were taken to check whether the data set met the requirements for parametric tests. Homogeneity of variance was analyzed through Levene's test and the Kolmogorov-Smirnov test assessed normality of the distribution.

Body weights at testing were analyzed using two- or three-way analysis of variance (ANOVA; sex × rat line, Experiments 1 and 3; sex × rat line × age; Exp. 2).

In Experiments 1 (neonates) and 2 (infants), the main dependent variable for ethanol consumption was the percent body weight gain (% BWG) achieved at test. This variable was calculated as follows: [(posttest weight - pretest weight)/ pretest weight × 100]. Percent body weight gain was analyzed using a factorial ANOVA that included sex (male or female), ethanol concentration offered (for neonates: 0, 5, 10, or 15% ethanol; for infants: 0, 5, 10 15 or 20% ethanol) and rat line (P or NP). In Experiment 2 the between factor age (PD8, 12 or 18) was also included. Absolute ethanol intake (g/kg) scores were calculated as a function of body weight gain during the training session and in accordance with the concentration of the ethanol solution used. These scores were analyzed through a factorial ANOVA that included sex, ethanol concentration offered, rat line and, in Experiment 2, age of testing (PD8, 12 or 18).

In Experiment 3 adolescents had access to water and varying ethanol solutions. During this two-bottle choice intake test, water and ethanol drinking was measured from the graduated tubes. Water consumption scores (ml/100 g) were analyzed using a factorial ANOVA. Sex (male or female), ethanol concentration offered (5, 10, 15 or 20% ethanol), and rat line (P or NP) were the between-group factors. A similar ANOVA was employed to analyze overall liquid scores (ml/100 g). Absolute ethanol intake was expressed in terms of absolute grams of ethanol per kilogram of body weight (g/kg). These scores were analyzed through a factorial ANOVA that included sex, ethanol concentration and rat line. Percent selection scores ([consumption of ethanol/overall liquid ingestion] $\times$ 100) exhibited neither homogeneity of variance ($F_{15,71} = 4.66$, $p < 0.0001$) nor an approximately normal distribution ($d = 0.15$, $p < 0.05$). Consequently, differences in percent selection between P and NP rats were assessed for each ethanol concentration offered (5, 10, 15 and 20% ethanol) through the Mann-Whitney test.

The loci of significant main effects or interactions were further examined using follow-up ANOVAs and pair-wise comparisons (Fisher's LSD *post hoc* tests). For all analyses, values of $p < 0.05$ were considered statistically significant.

Results

Experiment 1: Neonate ethanol consumption (PD1)

The 2 (rat line) $\times$ 2 (sex) ANOVA for body weights revealed significant main effects of rat line and sex; $F_{1,90} = 37.23$, $F_{1,90} = 23.78$, respectively, $p < 0.001$. Males had greater body weight than females (7.42 ± 0.13 g and 6.65 ± 0.14 g, respectively) and P rats were significantly heavier than NP rats (7.51 ± 0.13 g and 6.54 ± 0.12 g, respectively).

The results of Experiment 1, as indexed by %BWG at test, are summarized in Fig. 1, upper panel. Acceptance of water or 5% ethanol through the surrogate nipple was not affected by the genetic background of the neonates. On the other hand, alcohol-preferring P rats ingested more of the higher ethanol concentrations (10 and 15%) than their NP counterparts. The inferential analysis of the data confirmed these observations. The 2 (rat line) $\times$ 4 (ethanol concentration) $\times$ 2 (sex) ANOVA on %BWG indicated a significant main effect of line as well as a significant rat line $\times$ ethanol concentration interaction; $F_{1,78} = 15.58$, $F_{3,78} = 3.09$, respectively; $p < 0.05$. Post-hoc tests indicated that acceptance of 0% (water) and 5% ethanol was similar across P and NP rats. However, intake of 10% and 15% ethanol was higher in P than in NP neonates. Moreover, NP rats given access to 10% and 15% ethanol drank significantly less than their counterparts given only water (0 %). P rats given 5%, 10% or 15% ethanol, on the other hand, drank the same amounts as their pertinent vehicle controls. Sex exerted no significant main effect on percent body weight gain nor significantly interacted with the remaining variables.

The 2 (rat line) $\times$ 3 (ethanol concentration: 5, 10, and 15%) $\times$ 2 (sex) ANOVA for ethanol intake, as measured by grams of ethanol per kilogram of body weight, revealed significant main effects of rat line and ethanol concentration, $F_{1,58} = 23.32$, $F_{2,58} = 8.49$, respectively; $p < 0.001$. The interaction between these factors was also significant, $F_{2,58} = 3.03$, $p < 0.05$. Post-hoc tests indicated the absence of significant differences between P and NP lines in intake of 5% ethanol. However, P neonates given 10 and 15% ethanol achieved higher g/kg than any other group. P rats given 10 and 15% ethanol had similar ethanol intake levels. These results have been depicted in Figure 1, lower panel.

Experiment 2: Infantile ethanol consumption (PD8, PD12 and PD18)

Body weights are presented in Table 1. The ANOVA on body weights yielded significant main effects of sex, age and rat line, $F_{1,424} = 30.98$, $F_{2,424} = 2296.77$, $F_{1,424} = 577.04$,

respectively; $ps < 0.001$. The age $\times$ rat line interaction also achieved significance, $F_{2,424} = 672.6$, $p < 0.05$. As expected, males had higher body weights than females. Moreover, alcohol-preferring P rats were heavier than alcohol non-preferring NP rats at each of the ages tested, a difference that became more pronounced at PD18.

Figure 2 depicts ethanol consumption during the COF test, as expressed through %BWG. From a descriptive perspective the concentration of ethanol offered to the preweanlings affected intake levels across ages and rat lines. There was a u-shaped relationship between concentration and consumption, with the highest intake observed for the 10% concentration, and the lowest for 0% (water) and 20% solutions. Perhaps more important, genetic background did not affect ethanol intake at PD12 but apparently did so at PD8, when NP rats ingested significantly more 10% and 15% ethanol than P rats. On the other hand, on PD18 P rats took in considerably more 20% ethanol than their NP counterparts. These impressions were confirmed by the ANOVAs.

The 2 (sex) $\times$ 2 (rat line) $\times$ 3 (age) $\times$ 5 (ethanol concentration) ANOVA revealed significant main effects of age, rat line and ethanol concentration, $F_{2,376} = 9.13$, $F_{1,376} = 4.49$, $F_{4,376} = 16.10$, respectively; $ps < 0.05$. The following interactions also achieved significance: age $\times$ line, $F_{2,376} = 6.75$; line $\times$ ethanol concentration, $F_{8,376} = 3.27$; and age $\times$ line $\times$ ethanol concentration, $F_{8,376} = 2.10$; respectively; $ps < 0.05$. There were no significant main or interactive effects of sex.

To better understand the loci of the multiple interactions, follow-up ANOVAs (age $\times$ rat line) were conducted for each ethanol concentration. The ANOVAs for 0% and 5% ethanol consumption only yielded a significant main effect of age, $F_{2,82} = 8.27$, $F_{2,82} = 8.65$, respectively; $ps < 0.0001$. Post-hoc tests indicated that, at both concentrations, fluid consumption was significantly higher at PD8 than at PD12. Twelve-day old P and NP rats, in turn, ingested more 0% and 5% ethanol than 18-day old rats.

In the case of the intermediate ethanol concentrations (10% and 15%), the corresponding ANOVAs yielded a significant age $\times$ rat line interaction, $F_{2,82} = 5.18$, $F_{2,82} = 3.74$, respectively; $ps < 0.05$. As depicted in Figure 2 and confirmed by post-hocs tests, %BWG in P and NP rats exposed to 10% or 15% ethanol were fairly similar at PD12 and PD18, but not at PD8. Eight-day old NP rats ingested significantly more 10% and 15% ethanol than P rats. Ethanol-preferring rats, however, drank more of 20% ethanol, although only when tested at postnatal day 18 (significant age $\times$ rat line interaction, $F_{2,82} = 2.78$; $p < 0.05$).

The ANOVA for g/kg yielded a significant main effect of ethanol concentration, $F_{3,300} = 55.83$, $F_{2,376} = 4.16$, $p < 0.001$. As expected from previous studies, g/kg ethanol intake increased with ethanol concentration. The mean and SEM in terms of ethanol intake (g/kg) for each concentration was as follows: 5% ethanol, 0.76 ± 0.91 ; 10% ethanol, 1.70 ± 0.91 ; 15% ethanol, 2.03 ± 0.91 ; 20% ethanol, 2.36 ± 0.91 . Moreover, the age $\times$ line interaction achieved significance, $F_{2,376} = 4.16$; $p < 0.005$. The post-hoc tests revealed that there were no significant differences across rat lines at P12. That was not the case when testing occurred at PD8 or PD18, with NP rats ingesting more ethanol than P rats at PD8. At PD18, however, ethanol intake (g/kg) was significantly greater in alcohol-preferring (P) rats than in their non-preferring (NP) counterparts. This significant interaction has been depicted in Figure 3.

Experiment 3: Adolescent Ethanol Consumption (PD32)

P adolescent rats were heavier than NP rats and males had higher body weights than females. These differences were more pronounced in alcohol-preferring rats. The ANOVA for body weights revealed significant main effects of sex and rat line, $F_{1,83} = 49.83$, $F_{1,84} =$

98.81, $p < 0.001$. The age $\times$ rat line interaction achieved significance as well, $F_{1,83} = 7.16$, $p < 0.005$. Descriptive data (mean and SEM) for body weights in adolescent animals can be found in table 1.

Water intake and overall liquid consumption (ml/100 g) were similar across P and NP male and female adolescents. These scores were not affected by the ethanol concentration offered during the two-bottle choice tests. The ANOVA for both variables indicated a lack of significant main effects or significant interactions. Overall intake values and water consumption scores can be seen in Table 2.

In terms of absolute ethanol ingestion (g/kg) the ANOVA showed a significant main effect of rat line, $F_{1,71} = 6.08$, $p < 0.05$. The main effect of concentration approached but did not reach significance, $F_{3,71} = 2.62$, $p = 0.058$. Animals offered 20% ethanol had somewhat higher ingestion scores than the remaining subjects. More importantly, as depicted in Figure 4 and confirmed by the post-hocs, P adolescents drank more ethanol, as expressed through g/kg, than their NP counterparts. Although this effect appeared to be greater for the 5 and 20% ethanol concentrations (see Fig. 4), the two-way interaction (line $\times$ ethanol concentration) did not achieve significance ($p = 0.33$).

Percent selection of ethanol as a function of rat line was assessed separately for each ethanol concentration through the Mann-Whitney tests. These analyses revealed no difference in percent selection among P and NP rats when employing 10%, 15% or 20% ethanol. ($U = 54$, $U = 53$, $U = 38$; respectively, all p 's > 0.20). In sharp contrast, in adolescents exposed to 5% ethanol there were significant differences between rat lines ($U = 21.5$, $p < 0.05$). Mean and SEM in P and NP animals for each ethanol concentration can be found in Table 3.

Discussion

The present study provides new information towards understanding the ontogeny of ethanol intake in P and NP rats. Yet care must be taken in the discussion of these results because the experiments were conducted sequentially and employed different models for testing ethanol intake across ages. So, the differential pattern of ethanol preference observed across ages and conceivably could be due to differences in test sensitivity. Aside from obvious age-related differences in weight, size and thermal condition of the subjects, the rationale for choosing the specific test at a given age was provided by previous research indicating that these age-appropriate tests are sensitive to differences in ethanol intake from birth through adolescence after a variety of experimental manipulations (Truxell et al., 2007; Sanders and Spear, 2007; Petrov, Varlinskaya, and Spear, 2001). After accepting this caution, it seems prudent to affirm that the overall finding was that relative intake of ethanol in P and NP rats changes with ontogeny and ethanol concentration. The high-preference genotype was readily expressed very soon (3 h) after birth. This heightened preference seemed to be transient and reversed as the animals entered their second week of life (PD8). However, when weaning approached (PD18), the expected pattern of ethanol affinity across lines reemerged and was expressed during adolescence (PD32).

In more detail, when three-hour old cesarean delivered P and NP rat pups were allowed to ingest water or a low concentration of ethanol (5%) through a surrogate nipple, no differences in intake across lines were apparent. On the other hand, when given the opportunity to ingest higher concentrations of ethanol (10% or 15%) P rats ingested significantly more, twice as much as their NP counterparts in terms of grams per kilogram ethanol intake. Moreover, acceptance of ethanol decreased in NP rats as a function of ethanol concentration: NP rats given 10% and 15% ethanol exhibited significantly less fluid intake than NP animals given water. These results suggest that the genetic predisposition for

higher intake of ethanol in the P rat line is present immediately following birth. Two factors may have helped the apparent clarity of these results. First, animals were tested using the surrogate nipple technique, an age-appropriate test that minimizes intervening postnatal environmental factors that could alter expression of the genetic predisposition to ingest ethanol. Second, perhaps the fact that animals were challenged with increasingly concentrated ethanol solutions facilitated the expression of ethanol preference in P rats. Most if not all heterogeneous, non-selected rats, such as Wistar (the strain from which P/NP rats originated; Ponce, Pautassi, Spear and Molina, 2004; 2008; Pepino, Abate, Spear and Molina, 2004) and Long–Evans hooded rats (Youngentob, Molina, Spear and Youngentob, 2007), are highly reluctant to drink ethanol, particularly when its concentration reaches or exceeds 7% v/v. To our knowledge, this is the first time that ingestion of ethanol by P and NP rats has been compared at such a young age, just hours after birth.

Previously, McKinzie et al. (1999) assessed intake of ethanol or water in P and NP rats between postnatal days 5 and 20, using a test similar to that of the present study. There were few, if any signs of greater intake of ethanol than water in either line of rats (McKinzie et al., 1999) and the ethanol drinking pattern of P and NP rats was fairly similar across the 16-day repeated exposure test. As interesting as these results are, the authors themselves recognized a caveat in their study. The substantial amount of repeated exposure could have led to a myriad of effects, such as animals developing aversion towards the taste or odor of ethanol after reaching maximal amounts of ethanol consumption (Ponce et al., 2004; Hunt, Molina, Rajachandran, Spear and Spear, 1993). Lingering effects of ethanol, such as withdrawal (Spiers and Fusco, 1992) or memory impairment (Hunt, Levillain, Spector and Kostelnik, 2009) could also have affected the results on any given day. The present study avoided these confounds by comparison with naïve animals in each analysis. Among infant rats tested at PD8, PD12 or PD18, we observed that NP rats ingested significantly more 10% and 15% ethanol than P rats at postnatal day 8. This difference disappeared by PD12. By postnatal day 18, however, P rats consumed more ethanol than their NP counterparts, albeit only at the highest ethanol concentration (20%).

What neurobiological mechanisms could underlie this differential pattern of ethanol preference in P and NP rats across the second and third weeks of life? One avenue worth exploring involves the changes in function of neurotransmitter and hormonal systems associated with ethanol intake. The kappa opioid system seems to change from appetitive to aversive during early infancy (e.g., compare Barr, Wang and Carden, 1994 and Petrov et al., 2006). Also, around PD10–PD14 (depending on sex and area of brain) GABA receptors switch from excitatory to inhibitory function (Rivera et al., 1999). Another possible mechanism relates to the well-known stress hypo-responsive period (Sapolsky and Meaney, 1986). From PD4 to PD14 rats show a blunted corticosterone (CORT) response to stressors. Alcohol intake is known to be affected by stress and by corresponding CORT levels and is itself a mediator of CORT release (Kiefer and Wiedeman, 2004). None of these possibilities have been explored in infant P and NP rats. Thus, any relationship between these phenomena and those found in the present study are just hypothetical and require further assessment.

Previous studies have shown that the adult pattern of increased alcohol consumption in P compared to NP rats is not present between PD36 to PD41. After substantial exposure to oral ethanol, however, P adolescents show greater ethanol intake than NP adolescents and this difference invariably continues up to adulthood (PD71, McKinzie et al., 1998). It should be noted that the latter study employed extended ethanol pre-exposure (7 days of forced access to 10% ethanol just prior to test) for facilitation of ethanol drinking. Therefore, the possibility that these animals developed dependence or withdrawal cannot be discounted. Our study generally agrees with the conclusion of McKinzie et al (1998), however. We

observed higher ethanol intake in naïve P than in NP adolescent rats. When focusing on g/kg ethanol intake, this effect was not only observed for the ethanol concentration with which this line of rats was selected genetically, but also across a fairly wide range of concentrations. In terms of percent selection, P rats exhibited greater scores than their NP counterparts only for the lowest concentration of ethanol offered (5% v/v). It should be noted that, when offered this relatively low ethanol concentration, adolescent P rats achieved a strikingly high relative preference for ethanol (91.8 %). These results indicate that there is no need for substantial exposure to the drug for the genetic predisposition for ethanol to be expressed during adolescence. Furthermore, the acute nature of ethanol assessment in the present study makes alternative explanations for this effect less likely. For instance, it has been shown that P rats are less sensitive to ethanol withdrawal, as measured by a startle response, than NP animals (Jones et al., 2000; Chester, Blose and Froehlich, 2003). Although we cannot preclude the possibility of intra-trial withdrawal, this seems less likely in the acute assessment employed in the present study.

Our results suggest differential sensitivity to ethanol's motivational effects across P and NP lines. Although yet to be tested in infancy and adolescence, it has been shown that P rats are less sensitive than NP rats to the aversive motivational effects of ethanol, as measured in conditioned place preference and conditioned taste aversion tests (Froehlich, Harts, Lumeng and Li, 1988; Stewart, Murphy, McBride, Lumeng and Li, 1996). P rats also show heightened ethanol-induced stimulatory responding and less sensitivity to the motor depressant effects of ethanol than their NP counterparts (Murphy et al., 2002). The aversive and motor depressant effects of alcohol are thought to function as a deterrent for further escalation in alcohol consumption (Spear, 2004). Another possibility is that P rats found the flavor of ethanol more palatable than NP rats. Bice and Kiefer (1990), however, found no differences in acute responding to the taste of ethanol in naïve P and NP rats.

A previous study (Bell, Rodd, Sable, et al. 2006) found that adolescent female P rats consumed more water than adolescent male P rats. In contrast, in the present study there were no differences between male and female alcohol-preferring rats in the amount of water or alcohol intake at any of the developmental stages assessed. This difference might relate to procedural differences across studies. The present study assessed intake mainly during the dark period and through ball-bearing containing ball-point drinking tubes, whereas Bell, Rodd, Sable, et al. (2006) employed a 24-h access to open-ended sipper tubes. These factors significantly affect the outcome of self-administration studies (Vetter, Doremus-Fitzwater and Spear, 2007).

In summary, the major finding of the present study is that the genetic predisposition for higher ethanol intake in P than in NP rats is present immediately following birth. Another new finding is there is a discontinuity in ethanol preference during the beginning of the second week of life. Specifically, 8-day old NP rats drank more ethanol than P animals. This pattern, however, was transient. The expected pattern of high ethanol preference in P rats was once again observed as weaning approached and was reliably observed at adolescence.

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Figure 1.

Ethanol intake in cesarean-delivered alcohol preferring (P) and alcohol non-preferring (NP) neonate rats. Top Panel: intake of water (0%), 5% ethanol, 10% ethanol or 15% ethanol available from the surrogate nipple, expressed as the percentage of body weight gain (%BWG) in P and NP neonates tested on Postnatal Day 1 (PD1). Bottom Panel: Absolute ethanol intake (g/kg) from the surrogate nipple in neonate rats as a function of the ethanol solution available (5, 10 or 15% v/v) and rat line (P or NP). Vertical lines represent standard errors of the mean.

Figure 2.

Ethanol intake during a 20-min consumption-off-the floor (COF) test, as expressed through percent body weight gain (%BWG) in preweanling rats as a function of age (postnatal days 8, 12 or 18; PD8, PD12 and PD18, respectively), ethanol concentration offered at test (0% [i.e., water vehicle], 5%, 10%, 15% or 20%) and rat line (alcohol preferring [P] or alcohol non-preferring [NP] rats). Vertical lines represent standard errors of the mean.

Figure 3.

Absolute ethanol intake (g/kg) in preweanling rats during a 20-min consumption-off-the floor (COF) test, as a function of age (postnatal days 8, 12 or 18; PD8, PD12 and PD18, respectively) and rat line (alcohol preferring [P] or alcohol non-preferring [NP] rats). Data has been collapsed across ethanol concentration offered to the preweanling. According to the ANOVA, ethanol concentration exerted a significant main effect (i.e., the higher the ethanol concentration, the greater the g/kg achieved by the infants) but did not significantly interact with the remaining factors. Vertical lines represent standard errors of the mean.

Figure 4.

Absolute ethanol intake (g/kg) in adolescent rats during a 14-h 2-bottle preference test as a function of ethanol concentration offered at test (5%, 10%, 15% or 20%) and rat line (alcohol preferring [P] or alcohol non-preferring [NP] rats). Vertical lines represent standard errors of the mean.

Table 1

Body weights across experiments

Body weight of male and female neonate, infant and adolescent rats tested for ethanol intake. Neonates were assessed at postnatal day 1 (PD1), infants were assessed at PD8, PD12 or PD18. Adolescent testing took place at PD32. Data is presented as a function of sex (male or female) and rat line (alcohol preferring [P] or alcohol non-prefering [NP]). Values represent mean $\pm$ SEMs.

		Age at testing									
		P1		P8		P12		P18		P32	
Rat line		♂	♀	♂	♀	♂	♀	♂	♀	♂	♀
P rats	7.95	7.07	18.45	17.29	28.43	26.68	43.02	42.72	120.00	99.94	
	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	
	0.16	0.16	0.28	0.29	0.51	0.54	0.52	0.73	3.89	2.90	
NP rats	6.87	6.2	16.74	15.29	22.98	22.03	33.47	30.96	94.00	84.96	
	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	
	0.12	0.18	0.26	0.23	0.35	0.29	0.36	0.44	1.24	0.95	

Table 2
Overall liquid and water consumption (ml/100g)

Overall intake and water intake values (both variables expressed as ml/100g of body weight) in adolescent rats during a 14-h 2-bottle preference test, as a function of sex (male or female) and rat line (alcohol preferring [P] or alcohol non-preferring [NP] rats). Overall liquid and water consumption scores were not affected by the ethanol concentration offered during the two-bottle choice tests. Values represent mean $\pm$ SEMs.

Rat Line	Overall liquid consumption		Water consumption	
	♂	♀	♂	♀
P rats	12.11 $\pm$ 0.35	12.65 $\pm$ 1.06	7.38 $\pm$ 0.80	5.62 $\pm$ 1.34
NP Rats	11.85 $\pm$ 0.80	10.92 $\pm$ 0.90	7.51 $\pm$ 1.18	6.58 $\pm$ 1.20

Table 3
Percent selection of ethanol in adolescent rats

Percent selection of ethanol in adolescent rats during a 14-h 2-bottle preference test as a function of ethanol concentration offered at test (5%, 10%, 15% or 20%) and rat line (alcohol preferring [P] or alcohol non-preferring [NP] rats).

Ethanol concentration	5%	10%	15%	20%
P rats	91.80 +/- 1.91	47.16 +/- 10.10	26.33 +/- 5.5	48.20 +/- 11.37
NP rats	55.87 +/- 10.76	51.25 +/- 10.25	42.55 +/- 10.85	40.05 +/- 10.65