

# Development of Individual Alcohol Inhalation Chambers for Mice: Validation in a Model of Prenatal Alcohol

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**Background:** The purpose of this work was first to develop a system of individual chambers through which controlled delivery of alcohol vapors allows us to target specific blood alcohol levels (BALs) in mice without requiring the administration of an alcohol dehydrogenase inhibitor. As a proof of concept, we demonstrated that this new system could be used to expose pregnant BALB/c or C57BL/6 mice to alcohol and that the hypothalamic-pituitary-adrenal (HPA) axis of their mature offspring exhibited the well-known hyperactivity that has been previously documented in rats.

**Methods:** A first series of experiments was designed to establish the parameters that resulted in specific BALs in nonpregnant adult male and female BALB/c as well as C57BL/6 mice that were exposed to various alcohol flow rates. Using information gathered from these experiments, we then chose a regimen of 6 hr of daily vapor exposure in pregnant mice to determine whether this regimen would alter the HPA axis activity of their mature offspring. Control dams were maintained in similar chambers but without alcohol. We first used control mice to assess plasma ACTH levels as a function of shock intensity as well as total duration of the shock session. The most suitable protocol was then used to measure shock-induced ACTH release in 2-month-old male and female offspring that were exposed to alcohol prenatally or not.

**Results:** BALs increased as a function of the alcohol flow rates and remained within an acceptable range of homogeneity, consistency, and reproducibility over the desired periods of time. There were no sex differences in BALs while vapors were delivered. However, there was a strain difference in that BALB/c mice displayed slightly higher BALs than C57BL/6. Female mice also exhibited a slightly more pronounced decrease in BALs, compared with male mice, once removed from the drug. Measurement of plasma ACTH levels as a function of the intensity and duration of the shock sessions indicated that 0.3 mA intensity, 1-sec duration shocks at the rate of 2 shocks/min for 20 min provided the most reliable protocol. We then used the alcohol model in pregnant mice. Alcohol exposure did not interfere with maternal weights during gestation. When offspring were tested at 8 to 9 weeks of age, male and female BALB/c as well as female C57BL/6 mice that were exposed to alcohol vapors prenatally exhibited significantly higher shock-induced plasma ACTH levels, compared with controls of the same strain.

**Conclusions:** Collectively, our results indicate that the individual alcohol chamber system that we have developed offers a reliable means of exposing mice to alcohol so that they reach predetermined BALs in the absence of the pharmacological manipulations often used to influence alcohol metabolism in this species. This system, which is compatible with normal weight gains, was used to provide evidence that as previously demonstrated in rats, adult murine offspring of alcohol-treated dams exhibit a hyperactive HPA axis. The development of protocols for use in mice offers the possibility of investigating the influence of alcohol in mutant animals with manipulations of specific genes of interest.

**Key Words:** Alcohol, Inhalation, Mice, Prenatal, ACTH.

**F**OLLOWING THE SEMINAL work of Taylor and colleagues (Taylor et al., 1981, 1986), the influence of prenatal alcohol on the hypothalamic-pituitary-adrenal (HPA) axis of adult offspring has been the object of intensive research by a number of laboratories (see, e.g., Aird et

al., 1997; Gabriel et al., 2000; Kim et al., 1999a,c; Lee et al., 2000b, 2003; Lee and Rivier, 1994; Osborn et al., 2000; Yirmiya et al., 1998). All concur to show that when exposed to neurogenic or systemic stressors, adult offspring display a hyperactive HPA axis that is characterized by increased peak ACTH/corticosterone levels and/or a delayed return to basal hormone levels once the stressor is discontinued. The mechanisms that are responsible for these responses include upregulated neuronal activity of the hypothalamic cells that synthesize corticotropin-releasing factor and vasopressin (Lee et al., 2000b), the two peptides that regulate the HPA axis; alterations in steroid feedback at the level of the brain (Kim et al., 1999b; Osborn et al., 1996); and increased catecholamine levels in the hypothalamus and hippocampus (Tran and Kelly, 1999).

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Although the rat has been the animal on which most of these studies have been based, the development and availability of mice with genetic mutations that target neurotransmitters of interest makes it imperative that systems be available for this species. A recent report provided information regarding the usefulness of a model of voluntary drinking in pregnant mice, which was based on the sucrose fading paradigm (Allan et al., 2003). Although no endocrine measures were reported, adult offspring of alcohol-exposed dams exhibited contextual decreased fear and increased time spent inspecting a novel object. However, the protocol used by these investigators (Allan et al., 2003) requires alcohol to be present before conception. This may more closely mimic models of human drinking, but it may also alter endocrine and other functions through genetically induced changes that are likely to be different from those caused by alcohol during specific stages of embryonic development. Also, with this method, alcohol is faded out only after the dams give birth. Although no differences were observed in terms of maternal care between controls and alcohol-treated dams once the drug was discontinued (Allan et al., 2003), the significant influence exerted by even subtle changes in dam-pup interactions on the adult offspring HPA axis (see Anisman et al., 1998; Levine, 2001; Meaney, 2001) suggested that this approach might not be suitable for our own endocrine studies. Finally, the sucrose-fading method is very time-consuming (involving 4 weeks of training) and, being based on voluntary drinking, can be accompanied by heterogeneity in the amount of alcohol consumed.

We therefore chose to develop a different system that yielded consistent and predictable blood alcohol levels (BALs) in dams over a predetermined stage of pregnancy (days 7.5–17.5). To do so, we adapted for mice the alcohol vapor system that we had recently built for rats (Lee et al., 2000a). The objective of the present work thus was 3-fold: First, we established the parameters of the new system in terms of cage dimensions and drip rates such that BALs were stable, reliable, and reproducible. These experiments were carried out in BALB/c mice, which represents a commonly used outbred strain, and in mice of the genetically stable C57BL/6 strain, which forms the genetic background of many of the mutant mice that are currently used, for example, to investigate the influence of corticotropin-releasing factor and its receptors (see, e.g., Bale et al., 2002; Contarino et al., 1999; Jacobson et al., 2000; Preil et al., 2001; Turnbull et al., 1999). Second, we adapted this system to pregnant BALB/c and C57BL/6 mice such that predetermined BALs were reached and maintained in animals that rapidly gained weight. Third, we demonstrated the feasibility and usefulness of this system by showing that when exposed to shocks, adult offspring of mice that were exposed to alcohol during gestation released significantly more ACTH than controls.

## MATERIALS AND METHODS

### *Animals*

Adult male and female BALB/c and C57BL/6 mice (12 weeks old) that were purchased from Harlan Industries (Indianapolis, IN) were kept under a standard light regimen (12-hr light/12-hr dark, lights on at 6:30 AM) and fed a standard mouse diet and water ad libitum. When not in the alcohol chambers, nonpregnant animals were housed three to four per home cage. Pregnant mice were housed three per home cage until the end of the alcohol treatment, at which time they were housed individually and provided with nesting material. For experiments that were carried out in pregnant mice, virgin female mice were housed with a male at 10:00 PM, and the presence of plugs was checked in the morning. As mating was assumed to have taken place at approximately midnight, the day that a plug was found was considered day 0.5 of pregnancy. Once the mice were pregnant, they were fed a special breeder chow (Formulab Diet #5008; PMI Nutrition International, LLC, Brentwood, MO). The presence of pups was checked three times daily (8:00 AM, 2:00 PM, and 6:00 PM) from day 18 until delivery. All procedures were approved by the Salk Institute IACUC.

### *Alcohol Vapor System*

The mouse system was modified from the system that we had reported for rats (Lee et al., 2000a) and that is available from La Jolla Alcohol Research, La Jolla, CA (<http://www.ljari.com>). Basically, sets of four chambers are connected to a pump and a flask with four side arms, the bottom of which sits in a heater (see below). Alcohol flows from a large reservoir that contains 95% alcohol to a peristaltic pump (model QG-6; FMI Laboratory, Fluid Metering, Syosset, NY), from which it is delivered to a side-arm flask at a flow rate that can be regulated precisely. This round-bottom flask is placed inside the heater so that the drops of alcohol on the bottom are vaporized. Airflow controlled by a pressure gauge (adjusted to 5 psi for the mouse experiments) is delivered to the flask and serves to carry the alcohol vapors to the individual chambers through the tubes connected to the four side arms. Each tube also has its own pressure gauge that enables us to adjust evenly the conditions of each chamber. Alcohol vapors in the individual chambers are then drained out through tubes connected to a vacuum. This system was found to be air-tight, and the variability in the alcohol concentration among chambers is <10%.

### *Alcohol Chambers*

During initial experiments, we found that BALs of mice that were housed in rat chambers (Lee et al., 2000a) were heterogeneous, which we attributed to the chambers' being too large. The dimensions of the chamber (Allentown Caging Equipment Company, Allentown, NJ) were therefore modified to 7 in wide  $\times$  11 in deep  $\times$  5 in high, with an internal chamber area of 67 in<sup>2</sup> to accommodate the smaller size of the mice. This corresponds to the dimensions required by the Association for Assessment of Laboratory Animal Care for overnight housing. We further reduced variability in BALs by limiting the amount of bedding to only what was necessary to completely cover the floor and absorb urine. This was required because some mice tended to bury themselves under the bedding, which caused their BALs to be lower than those of mice that did not behave in that manner.

### *Alcohol Treatment*

For conserving animals, not all experiments were conducted in both BALB/c and C57BL/6 mice, only those that we thought would provide relevant information regarding possible strain differences. In all experiments, the mice were put in the alcohol chambers (4/chamber) only for the duration of the treatments, then returned to their home cage at the end of the 6-hr session. As alcohol was delivered between 7:00 AM and 1:00 PM, which does not correspond to normal feeding time, the animals were not provided with food or water. For the experiment illustrated in Figs. 1 to 3, various parameters of flow rates of alcohol delivery and times over which

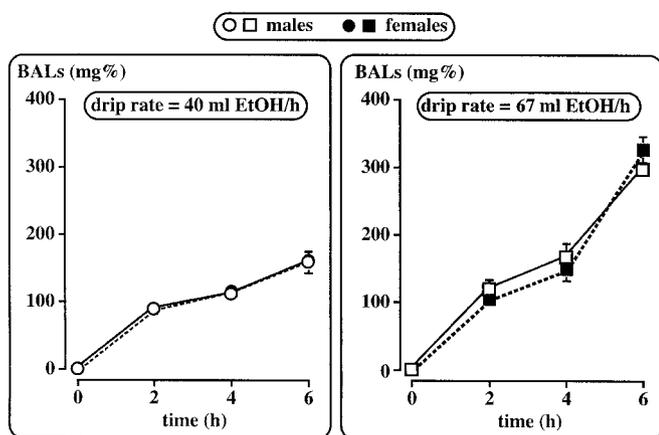


Fig. 1. Comparison between BALs measured in response to different alcohol flow rates as a function of sex. Each point represents the mean ± SEM of 8 to 15 adult male or female BALB/c mice.

the drug was given were used to provide information regarding BALs. Alcohol was vaporized at the rate of 40, 53, and 67 ml/hr to inhalation chambers that each housed four mice. For the prenatal alcohol experiments, plug-positive mice (12 weeks old) were randomly divided into controls or alcohol-exposed. Vapors were delivered at the rate of 40 ml/hr for 6 hr daily (7:00 AM to 1:00 PM) between gestational days 7.5 and 17.5.

BALs

BALs, taken from tail-vein samples, were measured in 5 µl of plasma using an Analox AM 1 analyzer (Analox Instruments, Lunenburg, MA). The reaction is based on the oxidation of alcohol by alcohol oxidase in the presence of molecular oxygen (alcohol + O<sub>2</sub> → acetaldehyde + H<sub>2</sub>O<sub>2</sub>) (Lee et al., 2000a). In the prenatal experiments, all mice were bled regardless of whether they were exposed to alcohol or not, and each animal was bled only once.

Plasma ACTH Levels

Plasma ACTH levels were determined by a commercially available two-site immunoradiometric assay (Allegro kit; Nichols Institute, San Juan Capistrano, CA) that we have validated for use in mice (Lee et al., 2001; Turnbull et al., 1999). Plasma was heated to 60°C for 30 min before incubation with the antibody. The lower detection limit and the intra-assay coefficient of variation were 15 pg/ml and <10%, respectively. Data were expressed in pg/ml of plasma.

Foot Shocks

Inescapable foot shocks were delivered to the paws according to a computer-driven schedule that generated randomly distributed shocks at a rate of 2 shocks/min for 20 min [Graphic State 2 (Version 2.101) & Precision Animal Shocker (Model-11R-TC-SF Modular Shock); Coulbourn Instruments, Allentown, PA]. Blood samples were obtained by rapid decapitation of nonanesthetized mice.

Statistical Analysis

The litter was used as the unit of analysis. Data were analyzed by two-way ANOVA and post hoc comparisons using the Newman-Keuls multiple comparison test or a linear regression test. In all statistical comparisons, a *p* < 0.05 was used to indicate significant difference.

RESULTS

BALs as a Function of Strains, Rate of Alcohol Delivery, and Sex in Nonpregnant Mice

BALs showed increases that were a function of time and flow rate in male and female BALB/c mice (Fig. 1). There were no sex differences. However, there were significant strain differences between female mice (Fig. 2), with animals of the BALB/c strain displaying higher BALs [*F*(1,56) = 13.767; *p* < 0.01] at the 6-hr time point for the two highest drip rates. We then measured BAL elimination rates, i.e., BALs measured once alcohol (67 ml/hr) was discontinued. As expected, BALs time-dependently decreased in both sexes (Fig. 3), but female BALB/c mice exhibited a slightly higher elimination rate, compared with male mice [sex × time, *F*(2,31) = 3.345; *p* < 0.05]. Two hours after cessation of the alcohol treatment, regression coefficients were 0.7768 in female mice and 0.7203 in male mice (*p* < 0.05 by ANOVA). Finally, we determined whether we could maintain constant BALs once predetermined values were reached. Thus, male BALB/c mice were exposed to three different flow rates, each lasting 2 hr. Although all four regimens tested provided a stabilization of BALs after the first 2 hr, only one (67, 33, 47 ml/hr) showed no statistically significant differences, measured

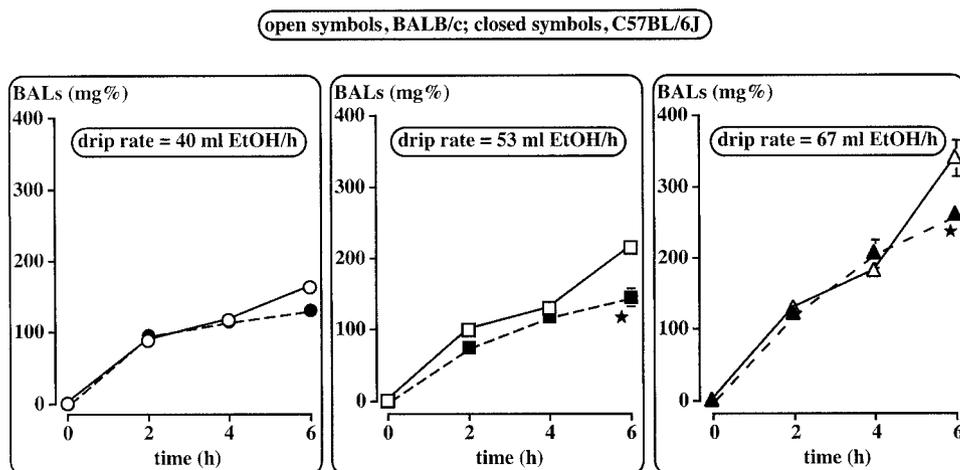
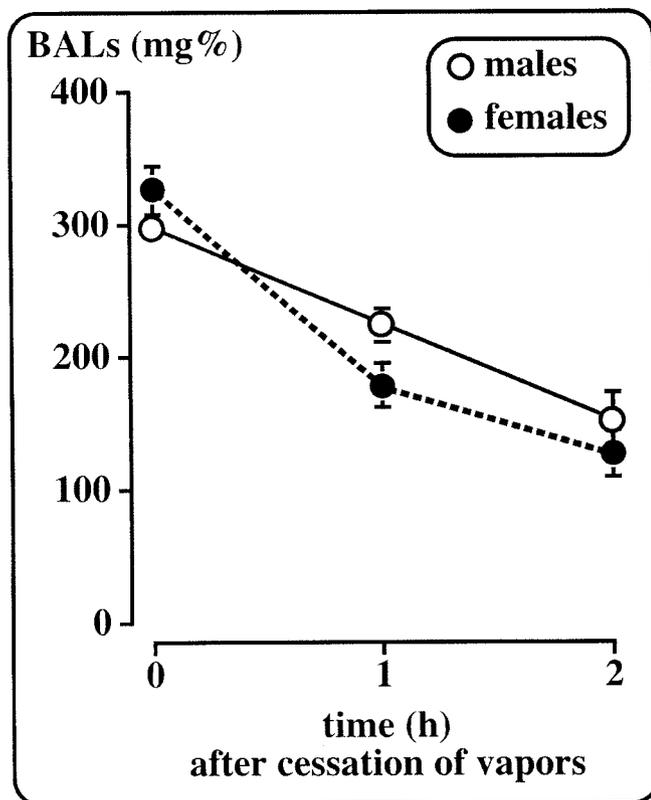


Fig. 2. Comparison between BALs measured in response to different alcohol flow rates as a function of the strain. Each point corresponds to the mean ± SEM of five to eight female BALB/c or C57BL/6 mice; \**p* < 0.05.

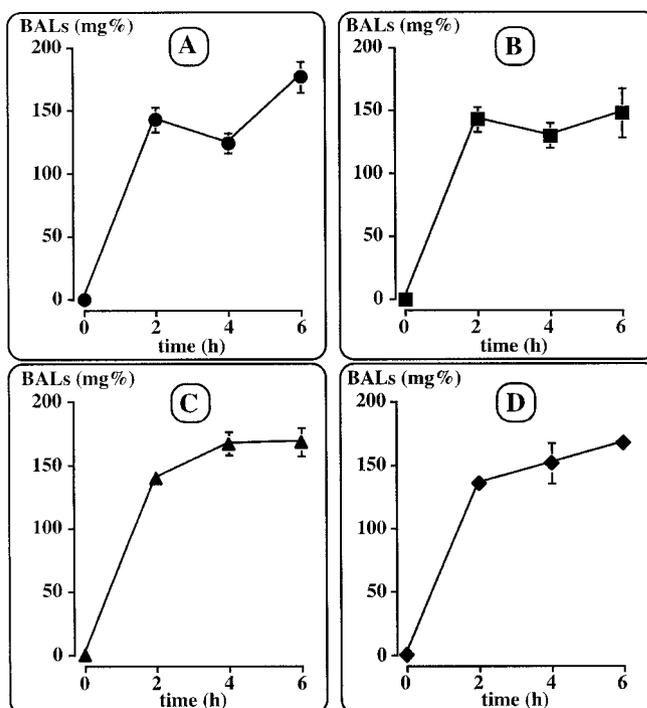


**Fig. 3.** Decreases in BALs after a 6-hr exposure to vapors (60 ml ethanol/hr) in adult BALB/c mice. The animals were returned to their home cage at the end of the 6-hr session. Each point represents the mean  $\pm$  SEM of five to six mice.

with the Newman-Keuls test, between BALs at the 2-, 4-, and 6-hr time points (Fig. 4).

#### *ACTH Release as a Function of Shock Intensity in Control Mice*

Restraint has traditionally been used as a stressor in mice, but we thought it important to use a model that allowed us to modify the intensity of the stimulus to ensure that it did not induce maximum ACTH release, thereby obscuring potential differences between groups. We therefore used a shock system specifically designed for mice, and because to our knowledge this system has not been fully characterized, we started by establishing the dose relationship between shock intensity and ACTH release in control, nonpregnant BALB/c animals. After a 20-min shock session, plasma ACTH levels increased between 0.1 and 0.5 mA, and this response did not show sex differences (Fig. 5). To ascertain that the 20-min shock session that we chose provided peak ACTH responses, we then extended the session to 60 min, using the 0.3-mA shock protocol. As illustrated in Fig. 6, there were no further increases in plasma ACTH levels between the 20- and 60-min time points and no sex differences at any time. Finally, we determined whether this shock regimen was adequate in C57BL/6 mice. We carried out one experiment in which female mice of this strain were exposed to 20-min shocks at



**Fig. 4.** Effect of alternating alcohol flow rates on the BALs of adult male BALB/c mice. Each drip rate was administered for 2 hr, and BALs were measured at the end of each 2-hr session. (A) 67, 33, and 53 ml/hr. (B) 67, 33, and 47 ml/hr. (C) 67, 47, and 33 ml/hr. (D) 67, 40, and 47 ml/hr. Each point represents the mean  $\pm$  SEM of five to eight mice.

0.3 mA. Their stress-induced ACTH response ( $449 \pm 58$  pg ACTH/ml) was comparable to that of BALB/c female mice ( $558 \pm 52$  pg ACTH/ml;  $p > 0.05$ ). Basal ACTH levels remained below 60 pg/ml in both sexes.

#### *Effect of Alcohol in Pregnant Dams*

In order to avoid repeated stress, tail bleeds were obtained in different groups of pregnant mice ( $n = 5$ ) on each of the 3 days illustrated below. Control mice ( $n = 5$  each time) were bled according to the same schedule. BALs, measured at the end of the 6-hr vapor session in different animals on days 9.5, 13.5, and 17.5, were  $180.3 \pm 7.6$ ,  $203.5 \pm 15.7$ , and  $202.2 \pm 5.8$  mg %, respectively. Alcohol did not significantly alter the rate of weight gain of the pregnant dams (Fig. 7), the weight of their offspring from birth to 4 weeks of age (Table 1), or the number of live pups per dam (control,  $5.3 \pm 0.40$ ; alcohol-exposed,  $4.5 \pm 0.65$ ;  $p > 0.05$ ). There was no dam mortality, but some of the mice displayed cannibalism toward their newborn pups, a phenomenon that is not uncommon during murine first gestations.

#### *Shock-Induced ACTH Release in Mice That Were Exposed Prenatally to Alcohol*

In view of the data presented in Figs. 5 and 6, we used a 20-min shock session (0.3 mA) to determine whether prenatal alcohol upregulated the ACTH response to this stressor. Basal ACTH levels were higher in female mice of each

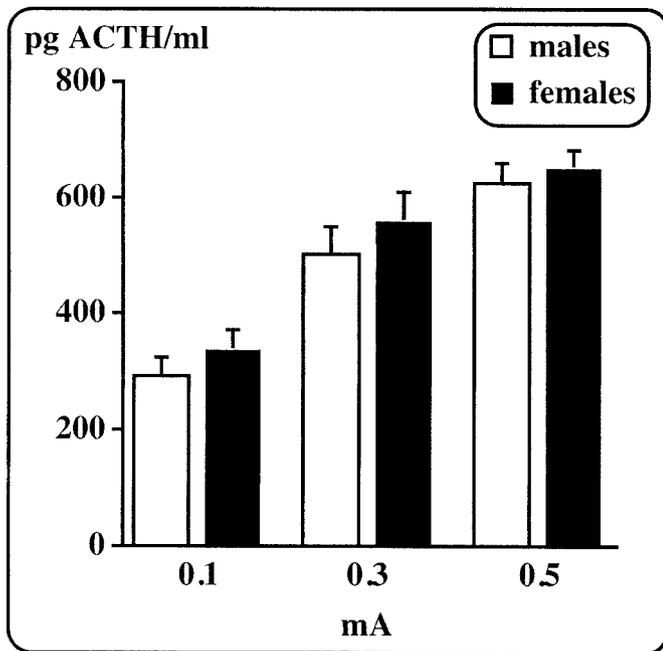


Fig. 5. ACTH release as a function of shock intensity (0.1, 0.3, and 0.5 mA, 1-sec duration, 2 shocks/min, for 20 min) in adult male and female BALB/c mice. Each bar point represents the mean  $\pm$  SEM of six animals.

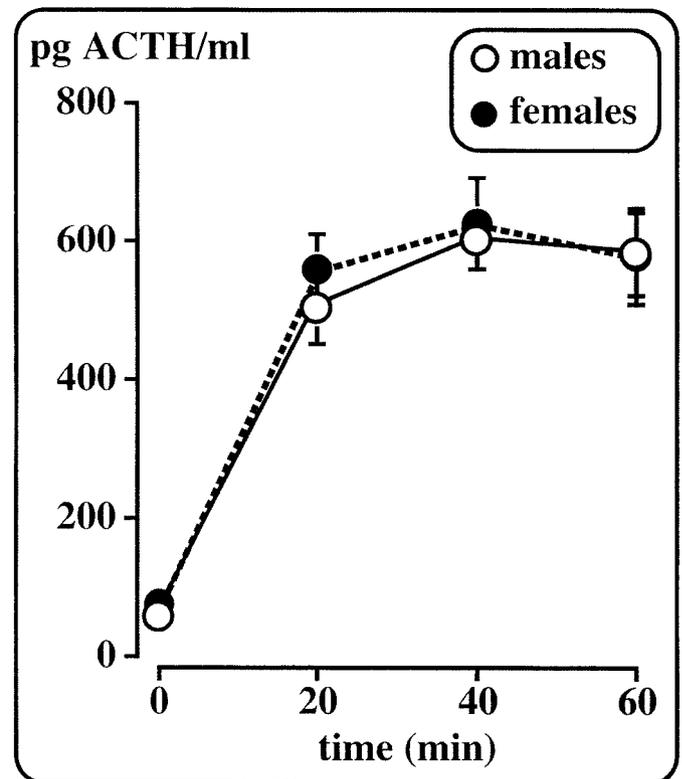


Fig. 6. Effect of the exposure to a 60-min session of foot electroshocks (0.3 mA, 1-sec duration, 2 shocks/min) on plasma ACTH levels in control adult male and female BALB/c mice. Each bar represents the mean  $\pm$  SEM of six animals.

strain, compared with male mice, but this difference reached statistical significance ( $p < 0.01$ ) only in C57BL/6 mice (Fig. 8). There was no difference between control and alcohol-pretreated animals. Shocks induced significant increases in plasma ACTH levels of both control and prenatally treated offspring in both strains [BALB/c:  $F(1,80) = 187.4, p < 0.01$ ; C57BL/6:  $F(1,72) = 231.0, p < 0.01$ ]. ACTH release measured at the end of the 20-min shock sessions was significantly higher in BALB/c male and female mice, as well as in C57BL/6 animals that were exposed prenatally to alcohol [ $F(1,80) = 13.069, p < 0.01$  for BALB/c mice and  $F(1,72) = 5.114, p < 0.05$  for C57BL/6 mice]. Finally, when ACTH levels were measured 20 min after cessation of the shocks (an experiment that was carried out only in BALB/c mice), decline rates were statistically comparable among groups (Fig. 8).

DISCUSSION

We report here the development of a new system of individual cages through which alcohol vapors are circulated and whose parameters have been adapted to mice. In the past, the maintenance of constant BALs in mice has been hampered by the fact that these levels tend to increase suddenly and abruptly at alcohol concentrations that are not always predictable. This can result in alcohol-induced coma that is obviously inadequate for research purposes. To circumvent this problem, investigators have resorted to blockers of alcohol metabolism such as the alcohol dehydrogenase inhibitor pyrazole hydrochloride to stabilize BALs (Finn and Crabbe, 1999; Terdal and Crabbe, 1994).

One of the purposes of the development of the new mouse chamber system was to circumvent this problem while allowing us to target specific BALs. We show here that this aim was achieved and that the BALs that we measured showed consistent and progressive increases that could be controlled easily as a function of time and/or drip rates. Once reached, specific levels could be maintained by alternating lower and higher drip rates. After the mice were removed from the alcohol chambers, BALs decreased over time similarly to what we observed in rats (Lee et al., 2000a). It is interesting that the sex differences that we observed in nonpregnant mice were not in agreement with those that we had reported in rats (Rivier, 1993). Namely, in the present work, there were no sex differences in BALs that were measured during alcohol exposure, but female mice showed a slightly quicker elimination rate. Finally, C57BL/6 mice displayed slightly lower BALs than BALB/c mice, which is in keeping with strain differences reported earlier by Crabbe et al. (1983).

In pregnant dams, we also wanted a method that allowed us to target a more specific period of gestation than is possible through the sucrose-fading method (Allan et al., 2003) and to avoid the potentially confounding problem of alcohol being present while the dams were nursing. Voluntary alcohol drinking has been shown to influence reward circuits that are different from those present in models of experimenter-administered alcohol (Moolten and

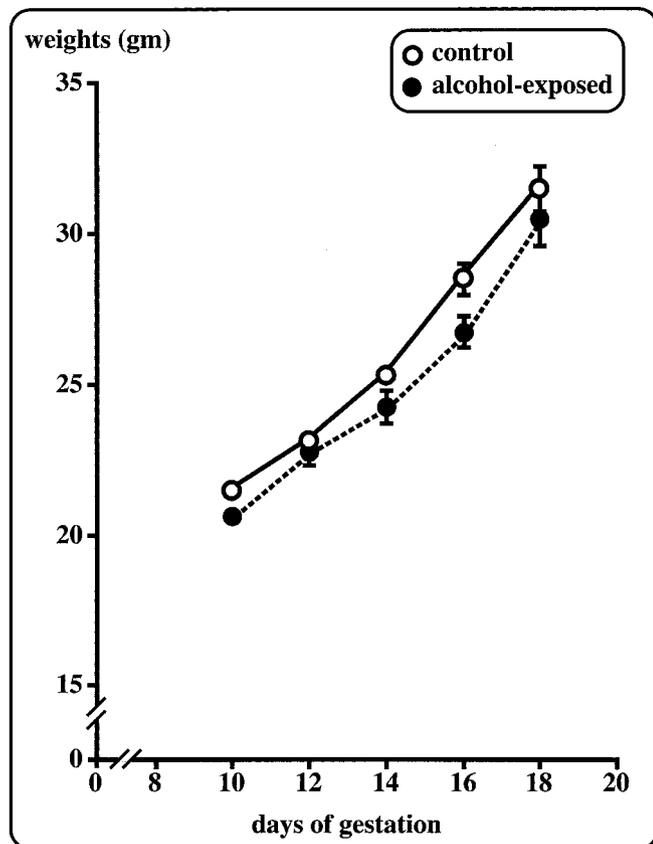


Fig. 7. Effect of alcohol on the weight of pregnant BALB/c mice. Each point corresponds to the mean  $\pm$  SEM of 8 to 10 animals.

**Table 1.** Weights of Pups at Birth and on Postnatal days 7 and 28

Age (days)	Controls	Alcohol-treated
0	1.30 $\pm$ 0.02	1.27 $\pm$ 0.02
7	4.38 $\pm$ 0.07	4.00 $\pm$ 0.08
28	15.05 $\pm$ 0.28 (males)	14.99 $\pm$ 0.51 (males)
	13.57 $\pm$ 0.16 (females)	13.34 $\pm$ 0.39 (females)

Data, presented in grams, represent the mean  $\pm$  SEM of 80-100 pups/group when genders were not taken into consideration (postnatal days 0 and 7), and 19-28 when randomly chosen offspring of each experimental group were weighted at 38 days of age.

Kornetsky, 1990). Furthermore, there may be differences in HPA axis activation between contingent and noncontingent administration of alcohol (Ogilvie and Rivier, 1997a) and other drugs (Galici et al., 2000). Nevertheless, the influence of prenatal alcohol exposure on the HPA axis of offspring has been found repeatedly to be comparable between rat models in which alcohol was self-administered (Aird et al., 1997; Gabriel et al., 2000; Kim et al., 1999a,c; Osborn et al., 2000; Yirmiya et al., 1998) and those in which dams were exposed to alcohol through vapors (Lee et al., 1990, 2000b, 2003). Thus, although the gastric route undoubtedly mimics human patterns of alcohol ingestion, the endocrine consequences of different models of drug delivery do not seem to be significantly different. We therefore used the recently developed mouse inhalation chambers to determine whether prenatal alcohol exposure would alter HPA axis activity of adult offspring similarly to what we had

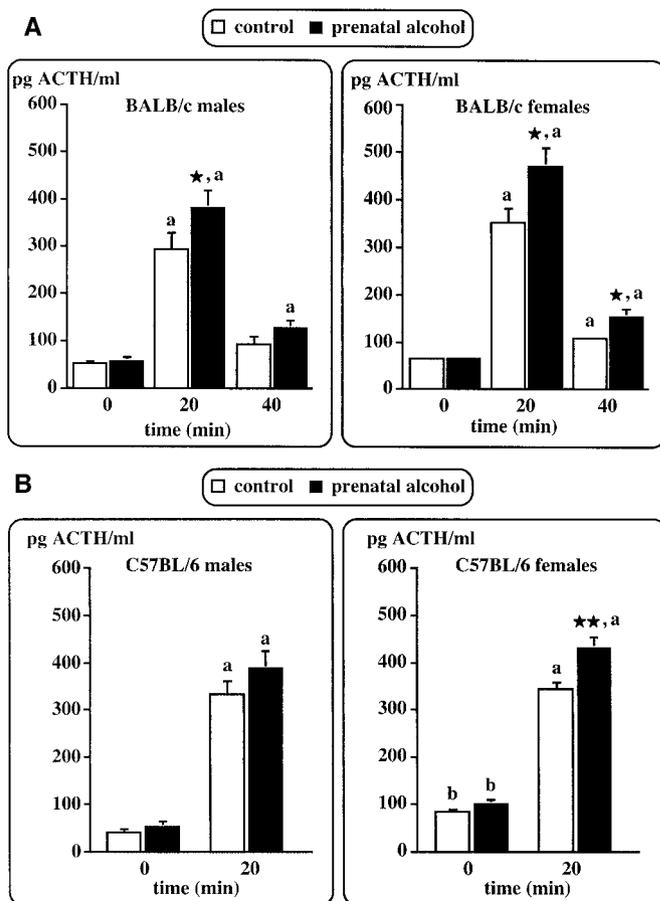


Fig. 8. Plasma ACTH levels in offspring that were exposed to foot shocks: Male and female mice (8-9 week old) of the BALB/c (A) or C57BL/6 (B) strain that were born to control dams or dams that were exposed to alcohol during gestation were exposed to a 20-min session of foot electroshocks (0.3 mA, 1-sec duration, 2 shocks/min). The mice were decapitated at the end of the shock session (20-min time point) or, in Balb/c animals, after a 20-min recovery (40-min time point). One group was decapitated under nonstress conditions (basal ACTH levels,  $t = 0$ ). Each bar represents the mean  $\pm$  SEM of six (controls) or eight (shocked) animals. \* $p < 0.05$  and \*\* $p < 0.01$  versus controls at the corresponding time point; <sup>a</sup> $p < 0.01$  versus basal levels of the corresponding sex; <sup>b</sup> $p < 0.01$  versus basal levels in male mice.

reported in rats using a comparable individual chamber system (Lee et al., 2000b, 2003). One significant advantage of this method was that alcohol exposure was compatible with normal weight gains during pregnancy, which was an important feature of the new model. We did not measure maternal plasma ACTH and corticosterone levels in these experiments, but it seems safe to assume that the procedure that we used did not cause a stress strong enough to interfere with normal food intake. Indeed, the concept that alcohol inhalation is compatible with adequate nutrition is also supported by the finding that exposing neonate mice to these vapors did not affect weight gains in the pups or the dams (Pal and Alkana, 1997). Finally, from an endocrine point of view, we report here that after prenatal alcohol treatment, male and female offspring of the BALB/c, as well as female mice of the C57BL/6 strain, indeed displayed significantly higher ACTH responses to shocks, which is in

agreement with findings previously reported in rats. Some investigators have reported that after exposure to a stressor, corticosterone levels of rats that were exposed prenatally to alcohol displayed a slower return to basal concentrations compared with controls (see, e.g., Weinberg, 1992). We therefore measured ACTH levels in BALC/c mice that were exposed to shocks for 20 min then sampled 20 min after cessation of this stimulus. As indicated in Fig. 8, no such influence of alcohol was observed in this model.

In conclusion, we have developed an easy-to-use and reliable method of exposing mice to alcohol. This method allows the investigator to target a wide range of BALs and to deliver alcohol over precisely controlled periods of time. Using this approach, we have found that adult murine offspring of dams that are exposed to the drug display the HPA axis hyper responsiveness that has been well characterized in rats. To our knowledge, this represents the first demonstration that prenatal alcohol exposure up-regulates stressor-induced ACTH release of mature mice. Importantly, the influence of prenatal alcohol was observed in both the BALB/c strain and the genetically stable inbred C57BL/6 strain, which indicates that it is applicable to mutant (transgenic or knock-out) mice. The model that we described is consistent with appropriate nutrition of the pregnant dams (as measured by weight gains) and does not interfere with infant–dam interactions because the drug is discontinued 2 to 3 days before parturition. This also avoids the requirement for cross-fostering, which in itself alters the HPA axis of offspring (Meaney, 2001; Ogilvie and Rivier, 1997b). We therefore believe that this newly developed system for mice will be useful for a wide variety of experiments in which alcohol delivery must be controlled accurately while avoiding other potentially confounding factors.

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