A Novel Method to Assess Initial Sensitivity and Acute Functional Tolerance to Hypnotic Effects of Ethanol

IGOR PONOMAREV and JOHN C. CRABBE

Department of Behavioral Neuroscience, Oregon Health & Science University, Portland Alcohol Research Center and Veterans Administration Medical Center, Portland, Oregon

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ABSTRACT

Loss of righting reflex (LRR) has traditionally been used to estimate hypnotic sensitivity to ethanol in rodents. Traditional methods of monitoring ethanol-induced sedation seem to lack accuracy in estimating blood ethanol concentration (BEC) at initial LRR, a measure of initial sensitivity. Herein, we present a novel method that improves detection of the onset of LRR by using a new apparatus and a loss-of-function criterion of 5 s. DBA/2J and C57BL/6J mice were placed in cylindrical restrainers after injection of 3 g/kg (20% v/v) ethanol. Restrainers were then turned until mice were no longer able to right themselves within 5 s from a position on their back, which represented the endpoint of the initial loss of righting reflex. Initial sensitivity and acute functional tolerance (AFT) to ethanol were assessed in the same group of mice by quantifying BEC at the initial loss and subsequent recoveries of righting reflex over four sequential injections [3 g/kg + (3 × 0.5 g/kg)]. Initial brain sensitivity was calculated from BEC at the first LRR, using the parameters of ethanol uptake kinetics. These values of initial sensitivity were similar for the two strains. On the other hand, DBA/2J mice recovered at higher BEC than C57BL/6J animals. AFT calculated as a difference between the maximum BEC at any recovery and the value of initial sensitivity was greater in DBA/2J mice. These results show that the novel method is a sensitive tool for the measurement of initial sensitivity and detection of AFT to the hypnotic effects of ethanol.

Evidence suggests that level of response or sensitivity to ethanol (EtOH) may play an important role in the development of alcoholism because subjects with a positive family history for alcoholism were less intoxicated than family history-negative controls, despite comparable blood ethanol concentrations (BECs) (Schuckit, 1980). Furthermore, the initial insensitivity in young men predicted alcoholism diagnoses later in life (Schuckit and Smith, 1996). When the level of response is measured, even if this occurs shortly after EtOH administration, it represents a combination of brain initial sensitivity (IS) and neuronal adaptation that rapidly develops after EtOH exposure. This phenomenon of rapid adaptation to a single dose of alcohol is referred as acute or within-session tolerance (Mellanby, 1919; Kalant et al., 1971). It is not clear how initial sensitivity and acute tolerance individually contribute to alcohol abuse. Human research faces a number of methodological and ethical problems that complicate dissociation of these two variables and investigation of their mechanisms independently. Therefore, animal models that offer reliably measured initial sensitivity and acute tolerance are desired. Because relationships between human behavior and its animal models are yet to be elucidated, it is important to study a variety of clearly defined measures of initial sensitivity and acute tolerance to different effects of EtOH.

Ethanol is a central nervous system depressant that produces sedative-hypnotic effects on behavior. Loss of righting reflex (LRR) has been used historically to assess sensitivity to ethanol’s hypnotic effects in mice (McClearn, 1962; Kakanha et al., 1966). LRR is a simple and reliably measured behavior. It has been widely used to study mechanisms of action of ethanol and other sedative drugs. Inbred mouse strains and mouse and rat lines selectively bred for differential sensitivity to the hypnotic effects of ethanol have served as a powerful tool to identify genetic (Markel et al., 1996; Zahniser et al., 1999; Deitrich et al., 2000), neurochemical (Velardo et al., 1998; Davies and Alkana, 2001), and electrophysiological (Palmer et al., 1987; Pearson et al., 1997; Hanaia et al., 2000) mechanisms underlying ethanol-induced sedation.

Traditionally, LRR is assessed by injecting the mouse i.p. with a hypnotic dose of ethanol and then placing the animal on its back in a V-shaped trough when it loses righting reflex.

ABBREVIATIONS: EtOH, ethanol; BEC, blood ethanol concentration; IS, initial sensitivity; LRR, loss of righting reflex; BrEC, brain ethanol concentration; AFT, acute functional tolerance; ANOVA, analysis of variance; iBEC, initial blood ethanol concentration; iBrEC, initial brain ethanol concentration.
The mouse stays in the trough until it regains its righting reflex. The criterion for the loss of righting is failure to right twice within a 30-s period. Similarly, the animal is considered to have regained righting reflex when it could right itself twice within a 30-s period. BEC and brain ethanol concentration (BrEC) at initial LRR are often used as measures of initial sensitivity, with loss of function at lower BEC or BrEC indicating higher sensitivity. The difference between the initial BEC and BEC at recovery from hypnosis, or between recoveries after different doses, is used to estimate acute functional tolerance (AFT) (Tabakoff and Ritzmann, 1979; Tabakoff et al., 1980; Keir and Deitrich, 1990).

Several studies that used the traditional LRR method failed to observe AFT in a variety of mouse genotypes (Tabakoff et al., 1980; Crabbe and Kosobud, 1986), whereas others have seen AFT only in some strains (Tabakoff and Ritzmann, 1979; Ritzmann and Tabakoff, 1980). It is important to point out that behavioral neuroadaptation can be assessed to a full extent only when initial brain sensitivity is accurately measured. Keir and Deitrich (1990) outlined two potential problems with detection of IS, and hence AFT, using the traditional procedure. First, blood alcohol level is rising very rapidly after an i.p. injection, which makes it very difficult to determine BEC at the precise moment of the LRR when it is defined by the relatively long 30-s criterion. Second, blood and brain ethanol do not reach equilibrium in mice for at least 5 min after an i.p. dose of EtOH (Smolen and Smolen, 1989). Therefore, BEC cannot be used as a reliable estimate of initial sensitivity unless both blood and brain ethanol uptake kinetics are studied, and BrEC can be reliably predicted from a blood sample.

We present a method that seems to overcome the aforementioned problems by using a new apparatus and procedure that allows us to establish LRR more rapidly and by taking into account parameters of ethanol uptake kinetics. After estimating initial sensitivity, we used a modification of a serial recovery method previously described by Gallaher et al. (1982). BEC was repeatedly measured to monitor development of acute functional tolerance in the same groups of mice. The objective of these studies was to demonstrate this novel technique that monitors ethanol-induced sedation and tolerance, and to characterize DBA/2J and C57BL/6J mice, the two inbred strains most commonly used in alcohol research.

**Materials and Methods**

**Animals**

Male mice from the C57BL/6J and DBA/2J strains were obtained from The Jackson Laboratory (Bar Harbor, ME) at the age of 49 to 56 days and were 60 to 78 days of age at the time of testing. Upon arrival, mice were maintained on a 12-h light/dark cycle and were allowed free access to food and water. All experiments were initiated and completed between 8:30 AM and 5:00 PM.

**Apparatus**

Animals were tested in polycarbonate cylindrical restrainers of our design, manufactured by a local company (Flair Plastic Products, Inc., Portland, OR). The restrainer (Fig. 1) is a hollow cylinder permanently attached to a squared base at one end and open at the other. After a mouse is placed inside the cylinder, the open end is shut with a sliding door through a gap located on the cylinder 6 mm from the open end. Both the squared base and the door contain round holes for ventilation. An adjustable plastic screw is located on the upper part of the door to tighten the door to the wall of the cylinder if necessary.

Restrainers of two sizes were used for bigger and smaller animals. Mice with a body weight of 25 g or heavier were tested in restrainers with the following parameters: cylinder length (between base and door), 100 mm; inner diameter of the cylinder, 44 mm; and base side, 60 mm. Parameters for lighter mice were as follows: cylinder length, 100 mm; inner diameter of the cylinder, 38 mm; and base side, 55 mm. When placed in the restrainer, mice could easily turn around and had enough space to take one or two steps.

**LRR Test**

An animal was placed in a cylindrical restrainer immediately after injection of 3 g/kg (20% v/v) EtOH i.p. The restrainer was then gently turned 90 degrees every 2 to 3 s. For the first few iterations of this procedure, mice immediately righted themselves. However, after 20 to 40 such tests, mice would remain on their back after two successive 90-degree turns. Thus, the mouse was considered to have lost its righting reflex if it was no longer able to right itself within 5 s from a supine position. When that happened, the experimenter immediately started the timer. Latency to LRR was calculated as a time interval between onset of the injection and the end of the 5-s cutoff period. A peri-orbital sinus blood sample (20 μl) was obtained as rapidly as possible for an estimate of initial sensitivity and the mouse was placed back in the restrainer. After practice, it was possible to obtain a sinus sample by 10 s after LRR, and thereafter, efforts were made to keep this interval as regular as possible in all further studies. Mice remained in the restrainers throughout the experimental session.

Animals were then tested for the recovery of righting reflex at 3-to-10-min intervals. Testing for the recovery was similar to the procedure described above for the loss of righting reflex. Every testing episode began with the mouse being placed in upright position. The restrainer was then again rotated 90 degrees every 2 to 3 s. Some of the mice could be placed on their back within the first two
turns, whereas the others were able to right themselves each time after a single 90-degree turn. Animals were considered to have regained righting reflex if they could either right themselves from a supine position within a 5-s period or could not be placed on their backs after eight successive 90-degree turns of the restrainer.

**Brain and Blood Ethanol Uptake Kinetics**

Mice of both genotypes were injected with 3 g/kg EtOH (20% v/v). BEC and BrEC were measured in separate groups of mice at 30, 60, 90, 120, 180, 240, and 300 s ($n = 6–12/strain/tissue/time point$). To obtain more data points for curve-fitting analysis, some BECs were measured twice from the same mice ($n = 6–8/group$) at one of the first four and one of the last three time points.

Because of the large variability of ethanol concentrations at each time point (Fig. 2, A and B), sampling distributions at each time point were tested for normality. Before testing for normality, data were analyzed with a three-way (strain $\times$ tissue $\times$ time) ANOVA with the exploratory goal of determining whether there were substantial differences between strains or tissues. Because two basic ANOVA assumptions of normality and homoscedasticity were violated, the results of this analysis were not interpreted beyond the normality test. The only significant effect detected by the analysis was a main effect of time, indicating a similarity of ethanol concentrations in brain and sinus blood for the two strains at any given time point during the first 5 min. Therefore, normality was tested for the data sets collapsed across strains and tissues at each time point.

All distributions except for the 30-s time point failed the Kolmogorov-Smirnov normality test with Lilliefors probabilities (Statistica for Windows; StatSoft, Tulsa, OK) and were either clearly binomial (90 and 120 s) or negatively skewed (all other time points). The binomial nature of the distributions suggested different absorption rates for two different sample classes. These data suggest that the lower ethanol concentration values at each time point represent injections that did not completely reach the intraperitoneal cavity.

To minimize the probability of including other than i.p. injections in further analysis, the lower concentration values were trimmed until ethanol concentrations at each time point were normally distributed. The trimmed data points ($\sim 15\%$) were excluded from further analysis.

**Curve-Fitting Procedure.** To determine the absorption rates and maximum ethanol concentration in blood and brain, BEC and BrEC values were separately fit for each strain (Table 1), and for a combined data set collapsed across strains (Fig. 2C; Table 1) to the following three-parameter equation (for details, see Ponomarev and Crabbe, 1999):

$$Y = C_{\text{max}} - C_{\text{max}} [1 + (\text{time}/T_{\text{ip}})]^b$$

where $C_{\text{max}}$ is the maximal ethanol concentration, $T_{\text{ip}}$ is the time point, in seconds, at which ethanol concentration equals 50% of $C_{\text{max}}$, and $b$ is the slope of the function.

Equations derived from this curve-fitting procedure were later used to estimate BrEC from a blood sample taken at the initial loss of righting reflex. In addition, parameters of the equations were compared pairwise between strains and tissues using a $t$ test.

**Initial Sensitivity Measurements**

To measure initial sensitivity to the hypnotic effects of ethanol, a separate group of mice of each strain ($n = 19$ for DBA2J, 16 for C57BL/6J) was injected with 3 g/kg (20% v/v) EtOH and immediately tested for LRR as described above. Failure to lose righting reflex was registered. Initial brain sensitivity was calculated using BEC at the initial LRR (iBEC). First, we determined where an iBEC value was located relative to the BEC curve. A BEC residual was calculated as the difference between the iBEC and the value on the BEC curve corresponding to the same time point at which the initial blood sample was taken [blood]. This residual shows the deviation of the individual iBEC from an estimated value on the BEC curve. Given the similarity of changes in BrEC and BEC over time (Fig. 2C), we assumed that the brain concentration of this subject would also deviate from the BrEC curve to the same extent. Therefore, the BEC residual was subtracted from the value on the BrEC curve at the time of initial LRR [brain] to obtain the estimate of brain’s initial sensitivity, iBrEC.
Acute Functional Tolerance Measurements

We used a modification of the method of Gallaher et al. (1982). When a mouse passed its first recovery test, a peri-orbital blood sample was taken, and a “booster” injection of 0.5 g/kg i.p. EtOH (20% v/v) was given to induce loss of righting again. The mouse was tested again until recovery from the second dose and another blood sample was taken. This procedure was repeated for a total of four injections (total dose of 4.5 g/kg). Mice remained in the restrainers throughout the whole experiment. The average recovery time after initial and all subsequent EtOH injections was 60 to 80 min. At these later time points, BEC at any recovery time point can be used as a reliable estimate of BrEC because concentrations of EtOH have equilibrated in these two tissues by 60 min after i.p. injection. AFT was calculated as the difference between the maximum BEC at any recovery and the estimated iBrEC. Differences between strains were determined by 2-way ANOVA. In addition, strain AFT values were compared for each strain and for data sets collapsed across strains.

Ethanol Assays

Procedures used to determine EtOH concentration in blood and brain were described in detail by Gallaher et al. (1996). Briefly, assays were carried out using a modification of the method of Roach and Creaven (1968). Brains were weighed and homogenized in ice-cold mixture of ZnSO4 (150 μl; 0.3 N), and water (brain weight × 1.5–300 μl). The homogenate was centrifuged at 12,000 rpm for 10 min, and the supernatant was assayed using a gas chromatograph. A blood sample (20 μl) was added to a tube containing 50 μl of ZnSO4. An additional 50 μl of BuOH1 and 300 μl of water were added to the tube, followed by centrifugation at 12,000 rpm for 5 min. The supernatant was also assayed using gas chromatography. Sample peak area was referred to a standard curve derived from duplicates of four concentrations of ethanol in values bracketing the expected range. BrEC values were expressed as milligrams of EtOH per gram of brain tissue, whereas BEC values had milligrams of EtOH per milliliter of blood units.

Although BrEC and BEC were expressed in different units, absolute values of ethanol concentration in these tissues should be comparable. One milliliter of blood weighs approximately 1 g (density of blood is just slightly greater than 1). Therefore, BEC values could also be expressed in milligrams per gram. In addition, water content in brain is similar to that in blood (approximately 79–81%). It is believed that EtOH is mainly distributed to tissues with higher water content (Loeppky et al., 1977; Goldstein, 1983; Faulkner et al., 1990). Thus, ethanol concentrations in brain and blood are expected to be similar when tissue equilibrium is reached.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Cmax (mg/g brain or mg/ml blood)</th>
<th>T50 (s)</th>
<th>b</th>
<th>Variance Accounted for by Sigmoidal Fit</th>
</tr>
</thead>
<tbody>
<tr>
<td>C57BL/6J</td>
<td>[Brain] 4.16 ± 0.13; Blood 4.33 ± 0.11</td>
<td>[Brain] 55.55 ± 2.80; Blood 61.37 ± 2.71</td>
<td>3.6 ± 0.6</td>
<td>85</td>
</tr>
<tr>
<td>DBA/2J</td>
<td>[Brain] 4.73 ± 0.14; Blood 4.53 ± 0.13</td>
<td>[Brain] 61.25 ± 2.54; Blood 64.58 ± 2.63</td>
<td>2.8 ± 0.3</td>
<td>90</td>
</tr>
<tr>
<td>C57BL/6J</td>
<td>[Brain] 4.44 ± 0.10; Blood 4.45 ± 0.09</td>
<td>[Brain] 58.19 ± 1.92; Blood 63.62 ± 1.96</td>
<td>3.1 ± 0.3</td>
<td>87</td>
</tr>
</tbody>
</table>

Results

Ethanol Absorption Curves. Ethanol concentration data are shown in Fig. 2. All data are shown in Fig. 2, A and B, which illustrates the bimodality of ethanol concentration values after 30 s. The curves on Fig. 2C indicate the best sigmoidal fits to the i.p. samples after removal of the low values presumed to reflect non-i.p. injections (data collapsed across strains). BrEC and BEC curve parameters (mean ± S.E.) for each strain and for data sets collapsed across strains are shown in Table 1. Each best-fit curve accounted for more than 85% of the observed variability.

Although maximum ethanol concentration (Cmax) in the Brain of DBA/2J mice was significantly higher than brain Cmax of the C57BL/6J strain \( t(95) = 3.0; p < 0.01 \), BrEC and BEC curves for each strain and for the data set collapsed across strains reached similar plateaus \( C_{max} \), an expected result after tissue equilibration (Goldstein, 1983). Compared with the BEC curve, the BrEC curve was shifted slightly to the left (Fig. 2C), indicating that BrEC was higher than BEC during the fast absorption phase (first 90 s). A two-way ANOVA on a subset of the data that included 30-, 60-, and 90-s time points confirmed this observation, showing a main effect of tissue, with BrEC being higher than BEC \( F(1,82) = 4.57; p < 0.04 \). In addition, the value of the \( T_{50} \) parameter for the [blood] curve was significantly greater than the similar value for the [brain] equation \( (t(210) = 1.98; p < 0.05) \), indicating a delay in absorption of ethanol in peri-orbital venous blood compared with concentration in the brain.

Initial Sensitivity. Separate groups of mice were tested for initial sensitivity and acute functional tolerance. Figure 3 shows BEC at the loss of righting reflex, plotted versus latency to lose righting reflex (data collapsed across strains). Based on the kinetics data derived from Fig. 2C, we assumed that all mice that received an appropriate i.p. injection should have reached a near maximum BrEC by 120 s after injection and lose righting reflex within this period. Data samples shown as filled circles represent such animals. Only these data were further analyzed to predict iBrEC at LRR.

\[
iBrEC = \text{[brain]} - ([\text{blood}] - \text{iBEC})
\]
The present study introduces a novel method to assess the hypnotic sensitivity to EtOH. According to the traditional method, mice are manually restrained and observed for at least 30 s after placing them on their backs (Tabakoff and Ritzmann, 1979). This makes it difficult to detect the precise moment of the initial loss of function (Gill and Deitrich, 1998). Furthermore, the 30-s criterion pushes the initial blood sampling closer to the alcohol concentration plateau, thus diminishing chances to detect possible subsequent development of AFT. Our alternative of placing animals in the restrainers and slowly rotating them enables clear detection of the initial loss of upright posture and allows a shorter criterion for establishing LRR. The shorter criterion, in turn, results in lower and more sensitive values of initial brain response, which allows us to assess the range of neuroadaptation characterizing AFT more fully.

The ethanol uptake kinetics data introduce several observations that should be of interest when acute effects of ethanol are studied. First, during the period of rapid absorption, a V-shaped trough was used to assess the loss and recovery of righting reflex. According to the traditional method, mice are more sensitive to the hypnotic effects of EtOH. There was no significant difference in initial sensitivity between C57BL/6J and DBA/2J mice. Circles connected with a solid line represent BEC at four successive recoveries of righting reflex. DBA/2J mice recovered at higher BEC than C57BL/6J animals. B, acute functional tolerance, calculated as a difference between the maximum BEC at any recovery (milligrams per milliliter) and the iBEC value, was greater in the DBA/2J strain.

The initial sensitivity values for each strain are shown in Fig. 4A as circles at time 0. The iBrECs of the two strains were compared using one-way ANOVA. No significant strain difference in initial sensitivity was found \( F(1,27) = 1.63\); \( p < 0.01 \). Each animal’s iBrEC was calculated using the curve parameters for its corresponding strain (Table 1). Given that each blood sample was taken approximately 10 s after initial loss of righting reflex, the exact formulas for iBrEC were as follows:

\[
\text{C57BL/6J:iBrEC} = 4.16 - 4.16(1 + (\text{time}/55.5)^{2.3}) - \text{iBEC}
\]

\[
\text{DBA/2J:iBrEC} = 4.73 - 4.73/(1 + (\text{time}/61.2)^{2.8}) - ((4.53 - 4.53/(1 + (\text{time} + 10)/64.6)^{2.3}) - \text{iBEC})
\]

The lack of a strain difference at time 0, the significant interaction indicated differential development of AFT for the two strains. AFT calculated as a difference between the maximum BEC at any recovery and the iBrEC (Fig. 4B) was greater in DBA/2J mice \( F(1,27) = 7.1\); \( p < 0.001 \). Animals of both strains reached nearly maximum tolerance after the initial 3-g/kg dose.

**Discussion**

The present study introduces a novel method to assess the hypnotic sensitivity to EtOH. The major differences between this technique and the traditionally used method are a new apparatus and the manner in which animals are handled and scored for loss of function. A cylindrical restrainer instead of
peri-orbital sinus BEC closely paralleled BrEC. Both retro-orbital BEC and BrEC reached equilibrium and plateaued between the 2nd and 3rd min after injection of 3 g/kg EtOH (20% v/v). In general, these results were consistent with previous findings of Gill and Deitrich (1998) and Smolen and Smolen (1989) who studied ethanol absorption after a number of doses ranging from 2 to 5 g/kg (20% w/v). Data from our laboratory also provide evidence that the close relationship between BrEC and retro-orbital BEC could be seen for a variety of EtOH doses. We calculated the correlation between retro-orbital BEC and BrEC measured in the same mouse (genetically heterogeneous population) 30 s apart at different time points during the first 4 min after an i.p. injection of ethanol. For ethanol doses of 2, 3, and 4 g/kg, the highly significant correlation value ranged from 0.94 to 0.96 for groups of 38 to 40 mice. These results suggest that even during the rapid absorption phase BrEC can be reliably predicted from a retro-orbital blood sample.

Second, the BEC curve is shifted to the right compared with the BrEC curve. This finding is also consistent with previous literature. There is a significant arteriovenous difference in alcohol concentration in the rising portion of the blood curve, with venous BEC being generally lower than arterial concentration (Kalant, 1998). Therefore, an early venous sample taken from the peri-orbital sinus would underestimate the ethanol concentration in the arterial blood, which is in equilibrium with the concentration in the brain (Smolen and Smolen, 1989; Kalant, 1998). The current investigation showed that during the rapid absorption phase, BEC reaches the BrEC level with a 5- to 7-s delay (Fig. 2C). Thus, a peri-orbital blood sample taken shortly after the initial LRR should provide a fair estimate of the brain’s initial sensitivity. For example, the use of iBEC instead of iBrEC values in our study did not change any of the statistical outcomes despite slightly reducing AFT magnitude (I. Ponomarev and J. C. Crabbe, unpublished data). Even if the sampling occurs 15 to 20 s after the loss of function, a good estimate of BrEC can be calculated using the formula in the text. Different mouse genotypes (DBA/2J, C57BL/6J, WSP, and WSR) seem not to differ dramatically in the rate of initial ethanol absorption after an i.p. injection (I. Ponomarev and J. C. Crabbe, unpublished data). Therefore, this formula can probably be used for a variety of strains.

We found that 15% of the ethanol concentration samples in our study deviated from a normal distribution at their corresponding time points. We hypothesize that these injections did not reach the i.p. cavity completely. An early study that investigated errors of intraperitoneal injections in rats reported that about 20% of all i.p. injections partially or entirely missed the intraperitoneal cavity (Lewis et al., 1966). Therefore, the percentage of errors in our study is not unexpected. It is not clear how different routes of administration affect brain’s initial sensitivity measured by ethanol concentration at the loss of function. However, the slower rates of absorption in these deviant samples resulted in longer latencies to the initial functional impairment, which confounds this variable by increasing the error of measurement variance. The slower absorption may also result in a lower maximum BrEC after a given dose (I. Ponomarev and J. C. Crabbe, unpublished data), which may, in turn, affect the development of APT. To overcome the problem of “bad” injections, we suggest that those mice that do not lose righting reflex within 2 min after i.p. injection with 20% (v/v) ethanol should be rejected from the experiment. Any mouse with a “clean” i.p. injection will have reached a near maximum BrEC by this time (Fig. 2B). Based on LRR data collected from 20 inbred mouse strains (Ponomarev and Crabbe, 2002; I. Ponomarev and J. C. Crabbe, unpublished data), we anticipate that mice of most genotypes will lose righting reflex before the 2 min cutoff after a 3-g/kg (20% v/v) dose. Although we did not try to determine whether all booster injections reached the i.p. cavity, exclusion of bad booster injections does not seem to affect the recovery level of either strain because both strains showed nearly maximum recovery BEC after the initial 3-g/kg injection.

To summarize the pharmacokinetic portion of our study, these experiments resulted in two recommendations for future studies that plan to investigate mechanisms of IS and AFT to ethanol-induced sedation. First, for accurate estimates of initial brain sensitivity, blood from retro-orbital sinus should be taken within 10 s after initial loss of function. If blood sampling occurs 20+ s after LRR, using the formula in the text for calculation of IS is suggested. Partial AFT could also be compared among groups without calculation of IS only if the interval between initial LRR and blood sampling is kept the same for all groups. Second, to eliminate possibility of “contaminant” non-i.p. injections, those mice that do not lose righting reflex within 2 min after the injection should be excluded from the experiment. It should also be noted that stress of blood sampling at LRR does not seem to affect either duration of LRR or BEC at the recovery of righting because mice sampled both at the initial LRR and at recovery did not differ in either variable from mice sampled at the recovery only (I. Ponomarev and J. C. Crabbe, unpublished data).

One of the goals of the present study was to test DBA/2J and C57BL/6J mice using the novel procedure and then compare our data with existing literature on the traditional LRR method. Investigation of the hypnotic sensitivity in these strains has a long history. Two variables have traditionally been used to assess ethanol-induced narcosis, the duration of LRR or “sleep time” and BEC or BrEC at awakening (McClearn, 1962; Kakahana et al., 1966; Belknap et al., 1972; Damjanovich and Maclnnes, 1973; Tabakoff and Ritzmann, 1979). None of the studies that measured both duration and an alcohol level reported significant strain differences in BEC or BrEC at the recovery of the righting reflex, despite some inconsistency in the sleep time findings. The duration differences might result from the use of different substrains: DBA/2J > C57BL/6J (Damjanovich and Maclnnes, 1973; Tabakoff and Ritzmann, 1979), DBA/2Abg > C57BL/6Abg (Dudek and Phillips, 1990), DBA/2J = C57BL/6J (Alkana et al., 1988; Browman and Crabbe, 2000), and DBA/2N < C57BL/6N (Crabbe, 1983). No strain difference in initial sensitivity measured as BrEC at the initial loss of righting reflex was found (Tabakoff and Ritzmann, 1979). The two strains also had similar values for another initial sensitivity measure, the ethanol dose required to cause initial LRR in 50% of mice (ED50) (Crabbe et al., 1994). None of the aforementioned studies except for Tabakoff and Ritzmann (1979) were designed to investigate AFT to the hypnotic effects of ethanol in DBA/2J and C57BL/6J mice. The authors of the 1979 article reported that AFT developed to a measurable degree in C57BL/6J but not in DBA/2J animals. To summarize the
findings based on the traditional approach to measuring LRR, DBA/2 mice did not differ consistently from C57BL/6 animals in the hypnotic sensitivity to ethanol, whereas one instance of differential development of APT was reported (C57BL/6J > DBA/2J).

The results of the present study are in agreement with previously published data on initial hypnotic sensitivity but contradict the observations on recovery from hypnotis and development of APT in DBA/2J and C57BL/6J mice because our DBA/2J mice had similar IS values, shorter duration of LRR, recovered at higher BEC, and developed greater APT, compared with the C57BL/6J animals. These findings have been recently replicated in our laboratory (Ponomarev and Crabbe, 2002) One most likely reason for the differential results is the different behavioral task used in our study to assess the ethanol-induced LRR. Although based on the traditional LRR approach, our novel technique uses a new apparatus and a substantially shorter criterion for establishing LRR. It is possible that, compared with the original criterion, the new behavioral endpoint is controlled by some different neuronal circuits, which may contribute to the genetic differences in the development of tolerance to ethanol. Recent publications have reported that genetic differences in sensitivity and tolerance to ethanol-induced incoordination depend on the specific behavioral tasks used (Boehm et al., 2000; Deitrich et al., 2000). Further investigation is required to elucidate how different behavioral assays that are thought to assess the same effects of ethanol are related.

It is not clear how alcohol-related behaviors in rodents are related to their prototypes monitored in human alcoholics. Therefore, to understand basic mechanisms of alcohol actions, it is beneficial to study a wide range of ethanol phenotypes. The purpose of the present article was to introduce a novel behavioral test that allowed us to assess accurately initial sensitivity and acute functional tolerance to ethanol sedation. This method uses a new apparatus and can detect the onset of LRR within very short latencies. Estimation of initial brain sensitivity from a blood sample using ethanol absorption parameters allows measurement of initial sensitivity and acute functional tolerance to the hypnotic effects of ethanol in the same group of mice. This technique should be useful to study pharmacological and genetic mechanisms underlying the development of AFT and should be broadly applicable to other sedative hypnotics.

References
Gill K and Deitrich RA (1998) Acute tolerance to the ataxic effects of ethanol in short-sleep (SS) and long-sleep (LS) mice. Pharmacology Biochemistry and Pharmacology 13691–98.
Mellany E (1910) Alcohol: its absorption into and disappearance from the blood under different conditions, National Research Council (Great Britain) Special Report Series No 31.

Address correspondence to: Igor Ponomarev, VA Medical Center (R&D-12), 3710 SW U.S. Veterans Hospital Rd., Portland, OR 97201. E-mail: ponomare@ohsu.edu