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Scheduled access to ethanol results in motor impairment and tolerance in female C57BL/6J mice

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Abstract

We recently reported a method where water-restricted mice were given scheduled access to ethanol followed by access to water. C57BL/6J mice would repeatedly self-administer ethanol in amounts that produced high and stable blood ethanol concentrations (BEC) [Finn DA, Belknap JK, Cronise K, Yoneyama N, Murillo A, Crabbe JC. A procedure to produce high alcohol intake in mice. Psychopharmacol 2005;178:471–480]. The studies reported here demonstrate that behavioral signs of motor impairment result from these high alcohol intakes, and that there was some evidence of tolerance development across repeated sessions. Female C57BL/6J mice were allowed 30 min access to ethanol (5% v/v) followed by 2.5 h access to water either: every 3rd day for 12 days; every 2nd day for 28 days; or every 2nd day for 9 days. On intervening days, mice had 3 h access to water. A control group had daily access to water only. Mice consumed 2–2.5 g/kg ethanol in 30 min, resulting in BECs of 1.4–1.5 mg/ml. Motor impairment was assessed using the accelerating or fixed speed rotarod, balance beam or screen test. In all studies, mice were tested for motor impairment immediately after 30 min access to ethanol or water. In Experiment 1, ethanol-exposed mice had shorter latencies to fall from the fixed speed rotarod and more foot slips on the balance beam than the control group, indicating motor impairment. After drinking ethanol, mice also fell from a screen more quickly than during sober pretraining. In Experiment 2, mice tested (without prior training) for motor impairment and tolerance on the fixed speed rotarod at 6.5 and 10 RPM showed repeated motor impairment in the ethanol group, but did not develop tolerance. In Experiment 3, mice were first given rotarod training at 10 RPM. Following each fluid access period, performance was impaired in mice self-administering ethanol at 10, but not 15 RPM, when compared to control mice. There was no evidence of tolerance across days. However, on the last day, all mice were tested at both RPM following an i.p. injection of 2 g/kg ethanol. Ethanol-experienced mice were less impaired at both RPM than the ethanol-naïve mice, indicating tolerance development according to this between-groups index. These results suggest that C57BL/6J mice will repeatedly consume alcohol in amounts that produce motor impairment under these scheduled fluid access conditions, and that a modest degree of tolerance can be detected using appropriate tests.
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Keywords: Intoxication; Alcohol; Consumption; Tolerance; Motor incoordination; Self-administration; Mouse; Rotarod

1. Introduction

There are numerous animal models of alcohol consumption, yet very few of these models produce blood ethanol concentrations (BECs) that result in measurable intoxication, usually indexed as motor impairment [reviewed in (Falk and Tang, 1988; Grahame et al., 1999; McBride and Li, 1998; Spanagel and Holter, 1999)]. While animals may regularly consume rather large quantities of alcohol in protocols that offer them unlimited access to alcohol, the

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intake bouts are usually titrated over a 24-h period [reviewed in (McBride and Li, 1998)]. This pattern of consumption often results in high daily total intake levels and BECs that may be pharmacologically relevant at some points during the day, but no visible signs of motor impairment. Because repeated, high BECs may be important causes of some of the physical sequelae of alcohol abuse, whether or not they are important for the psychological motivation to continue abusive self-administration, we sought to develop a method where mice would repeatedly self-administer ethanol to the point of motor impairment.

C57BL/6J mice are genetically predisposed to self-administer ethanol solutions even when water is available as an alternative (McClearn and Rodgers, 1959). Although they self-administer significant quantities of alcohol, they may achieve significant BECs only transiently (Dole and Gentry, 1984). It had previously been shown that they will self-administer large amounts of ethanol under conditions where access to fluids is restricted. When water-deprived mice were given 90 min access to fluid daily for 2 days and then were offered a single bottle containing alcohol on the 3rd day, mice consumed as much as 3.2 g/kg alcohol in a 10-min access period (Belknap et al., 1978). However, these high levels, which led to motor impairment, were not maintained repeatedly. During a second, similar alcohol exposure, self-administered intakes declined significantly, suggesting that a taste aversion had likely occurred. Therefore, this method did not model the chronicity of excessive intake consistent with the clinical diagnosis. In recent studies, we have modified the above procedure to achieve both high and stable levels of alcohol self-administration in the C57BL/6J mouse (Finn et al., 2005). The total time allotted for fluid access was increased to 3 h daily, with 30 min access to alcohol followed by 2.5 h access to water every other day. Using this version in male and female mice from several strains, alcohol intakes averaged 2–2.5 g/kg during the 30 min session, with no decreases in average consumption on the second, third or fourth exposures. We found average BECs ranging from 0.60–2.34 mg/ml depending on alcohol concentration and strain of the mice (Finn et al., 2005). Because behavioral effects are seen after acute ethanol injections that produce BECs greater than 1.00 mg/ml (Crabbe et al., 2003b), the intakes and BECs achieved with this scheduled fluid access procedure should have been sufficient to produce motor impairment as well. Ascertaining whether such motor impairment had occurred was the first goal of the studies reported here.

Impairment of motor coordination is often used as an indicator of alcohol sensitivity in humans and rodents. Furthermore, such responses differentiate individuals with a positive family history for alcoholism from those without, as some studies have suggested that family history positive individuals show decreased basal body sway and are less sensitive to the acute effects of alcohol on body sway (Schuckit, 1985; Schuckit and Gold, 1988). While other studies suggest that during the rising phase of the blood alcohol curve, family history positive individuals were more sensitive to the effects of alcohol on body sway (Newlin and Thomson, 1990), sensitivity to ethanol intoxication is clearly influenced by genetics in humans (Schuckit et al., 2001; Wilhelmsen et al., 2003). Numerous studies in mice have shown that several behavioral assays related to ethanol's effects on motor coordination are strongly influenced by genotype, although the domain of "ethanol-induced ataxia" is complex (Crabbe et al., 2005). Therefore, we selected several different measures of motor performance in our effort to detect self-intoxication using the scheduled fluid access method. We elected to use C57BL/6J female mice, as they self-administer more ethanol than males under most conditions, although the sexes achieve comparable BECs with this procedure (Finn et al., 2005).

Our second objective was to assess the possible development of tolerance within our high alcohol intake model. Tolerance to the impairing effects of ethanol has been hypothesized to lead to escalations of intake, which then in turn may result in some of the neurotoxic effects of chronic alcohol abuse (Hoffman and Tabakoff, 1996). Improvement in motor coordination with repeated stable levels of alcohol exposure could signify that tolerance had developed. Alternatively, tolerance could manifest as an increased sensitivity to a challenge with ethanol after prior experience (Kalant et al., 1971). Numerous studies with humans and rodents have shown that tolerance can develop to the motor incoordinating effects of alcohol during chronic consumption (Boulouard et al., 2002; Darbra et al., 2002; Erwin et al., 1992; Gatto et al., 1987; Middaugh et al., 2003; Sdao-Jarvie and Vogel-Sprott, 1992; Zack and Vogel-Sprott, 1993) or when it was administered repeatedly by injection (Rustay et al., 2001; White et al., 2002). Thus, we tested motor responses repeatedly after successive drinking bouts to determine whether the high alcohol intake procedure produced tolerance.

2. Materials and methods

2.1. Animals and husbandry

Female C57BL/6J mice ($n=107$) were used. Mice were bred at the Oregon Health & Science University Department of Comparative Medicine from stock purchased from The Jackson Laboratory (Bar Harbor, ME). Until the time of testing, mice were housed 4 per cage in clear polycarbonate or polysulfone cages ($28 \times 18 \times 13$ cm) on corncob bedding (Bed-o-cob), changed twice weekly. Rodent block food (Purina 5001) was available ad libitum, ambient temperature was maintained at 21 ± 2 °C, and fluorescent lighting was on from 0600 to 1800 h daily. All procedures followed USDA and NIH Guidelines for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee. Mice were allowed to acclimate to the testing room for one week prior to testing and ranged

from 60 to 80 days old at the start of testing. The day before the onset of water deprivation, mice were individually housed with one water bottle (25 ml graduated cylinder with sipper tube).

2.2. Scheduled fluid access procedure

2.2.1. Experiment 1. Motor impairment

Our scheduled fluid access procedures have been described elsewhere (Finn et al., 2005). On Day 0, mice were weighed and the water bottle was removed from each cage at 12:30 p.m. On Days 1 and 2 at 12:30 p.m., mice were weighed and water was returned to the cage in a graduated 25 ml cylinder with sipper tube for 3 h. Water volume was recorded at the start and end of the access period. On Day 3, mice were weighed and a graduated cylinder with ethanol (Pharmco Products, Inc., Brookfield CT, 5% v/v in tap water) was placed on the cage for 30 min. Immediately following the alcohol access period, the alcohol tube was removed and mice were allowed 2.5 h access to water. Control animals received water only for the full 3 h. Volumes were recorded at the start and end of each access period. Water was returned to the control group at this point. Food was available ad libitum throughout the experiment. For the alcohol group, this 3 day cycle of fluid access was repeated 4 times (12 days total) with 4 alcohol access days.

2.2.2. Experiments 2 and 3. Motor impairment and tolerance

The scheduled access procedure was generally the same as in Experiment 1 but these studies differed in the number and timing of the alcohol access periods. In both studies, mice were given access to ethanol every other day, rather than every 3rd day as in Experiment 1. In Experiment 2, mice were given a total of 14 alcohol access periods. In Experiment 3, after a total of 4 alcohol access periods followed by an extra water access period (Day 9), mice were then given an ethanol injection on the challenge day (Day 10). In both studies, there were separate control groups of animals that received only water access. However, in Experiments 2 and 3, the control groups were maintained on the same 3-hr daily total fluid access schedule as the alcohol groups.

2.3. Behavioral testing

All mice were given training trials on their assigned test of motor performance prior to the onset of alcohol exposure. On test days, mice were then retested immediately after each alcohol or water access period.

2.3.1. Experiment 1

Three groups of mice were given access to alcohol. One group ($n=13$) was tested on both the accelerating and fixed speed rotarods. Separate groups of alcohol-exposed mice (n's=11–12) were tested on the balance beam and screen tests. A control group ($n=4$) also was tested on the accelerating and fixed speed rotarod and on the balance beam (see below). Since animals were tested for initial competence on the screen test (see below), the alcohol group served as its own control on this task.

2.3.1.1. Rotarod. A modified AccuRotor Rota Rod (Accuscan Instruments, Columbus, OH) was used for both the accelerating and fixed speed rotarod tests using procedures discussed elsewhere (Rustay et al., 2003a,b). The rotating rod was 6.5 cm in diameter, covered with sandpaper to prevent slippage, and elevated 63 cm above bedding. A comparison of training regimens for the accelerating and fixed speed rotarod tests across all experiments is shown in Table 1. For Experiment 1, on Day 1, immediately following water consumption, mice were trained on the accelerating rotarod which started at 0 RPM and then accelerated at a constant rate of 20 RPM/min until the mouse fell from the rod. Mice were given 10 training trials with a 30 s rest between each trial, and latency to fall was recorded for each trial After all mice were trained, these same mice were then tested on the apparatus at a fixed speed of 6.5 RPM. Mice were given 1 training trial on the fixed speed rotarod during which they were allowed to remain on the rotarod for up to 3 min. Latencies to fall prior to reaching the 3 min maximum were recorded. Mice were then re-tested on the rotarod after each alcohol or water access period, i.e. on days 3, 6, 9, and 12. During these tests, mice were given 1 trial on the accelerating followed immediately by 1 trial on the fixed speed rotarod, and latencies to fall were recorded.

2.3.1.2. Balance beam. Apparatus and procedures are described in detail elsewhere (Crabbe et al., 2003b). On Day 1, mice were given one training trial in which they were required to traverse the 12.7 mm beam 4 times, preferably without any encouragement (however, it was acceptable to "coach" the animals with a light tail touch or by placing food under their noses). We have found that after this pretraining, mice tested the next day will readily run the entire length of the beam without any encouragement (Crabbe et al., 2003b). In this task, hind foot slips were recorded as the measure of motor impairment. Mice were then tested on the balance beam following each alcohol or water access period as for the rotarod testing. We had

Table 1
Training protocol for the accelerating (AR) and fixed-speed (FR) rotarods in experiments 1, 2, and 3

Experiment	AR	# 3 min trials		
		FR 6.5	FR 10	FR 15
1	10 trials	1 trial	–	–
2	–	4 trials	4 trials	–
3	1 trial	–	6, 30-s trials followed by 3–16, 3-min trials[a]	1 trial

[a] Mice were given a maximum of 16 FR trials at 10 RPM in Experiment 3 to reach the criterion of 3, 3-min trials without falling.

presumed that C57BL/6J mice, who perform well on this task, would show virtually no foot slip errors under control conditions (Crabbe et al., 2003b). However, after the first testing session, it was unclear whether the performance of the ethanol group (average of about 4 foot slips) represented substantial motor impairment. Therefore, we added a control group ($n=4$), which we pretrained on the following water day (Day 4) and then added for comparison during the second, third and fourth testing sessions. During all test sessions, mice were required to traverse the beam only once.

2.3.1.3. Screen test. The screen test apparatus and protocol have been discussed elsewhere (Crabbe et al., 2003a). On Day 1, mice were given one trial to determine their ability to perform the task while sober. They were placed on the horizontal screen, the screen was rotated vertically and mice were required to remain on the screen for 2 min. Only 1 mouse did not reach the 2 min criterion. The testing was the same following each alcohol or water access period, and latencies to fall were recorded.

2.3.2. Experiment 2

On Day 1, 4 groups of mice ($n=7–8$ per group, two ethanol and two control) were given 4 training trials on the fixed speed rotarod, half the groups at 6.5 RPM and half at 10 RPM, and were allowed to remain on the rotarod for up to 3 min (Table 1). While the training trials served to familiarize the mice with the apparatus, it was not required that mice remain on the rod for 3 min (i.e. mice were not trained to a specific criterion for this study). Thus, no mice were excluded based on initial performance. It was expected that ethanol would impair performance initially, but that as tolerance developed, performance would improve in the alcohol self-administering groups, albeit more slowly than in the control groups. Mice were then retested on the fixed speed rotarod following alcohol or water access periods on days 2, 4, 6, 8, 10, 12, 14, and 16. A blood sample was taken immediately following this test on Day 16. On each test day, mice were given 3 trials on the rod at either 6.5 or 10 RPM and the latencies to fall were recorded and averaged for each day. Mice were not tested following alcohol or water access periods on days 18, 20, 22, 24, 26 or 28. On Day 29, mice were assessed for tolerance using a method that assesses tolerance after it has developed completely. All mice (ethanol self-administering and control groups) were given a challenge injection of 2 g/kg alcohol (20% v/v in saline, ip) and tested 30 min after injection on the rotarod at their assigned RPM, and another blood sample was taken.

2.3.3. Experiment 3

The results from Experiment 2 suggested that pretraining on the rotarod may be necessary to see robust tolerance after a challenge injection (see Discussion). Therefore, on Day 1, all mice were trained to criterion on the fixed speed rotarod at 10 RPM (see Table 1). The training procedure was as follows. First, mice were given one trial on the rod, accelerating from 0 RPM at 20 RPM/min until they fell. Next, mice were given six, 30-s trials on the fixed speed rotarod at 10 RPM with 30 s intervals between trials. Finally, mice had to remain on the rod at 10 RPM for three, 3-min trials. Latencies to fall were recorded. A maximum of 16 training trials for the final 3×3-min criterion was set a priori, but all mice reached this criterion within 7 trials and no mouse had to be dropped from the study based on performance. Following training at 10 RPM, mice were then given 1 trial on the rod at a fixed speed of 15 RPM to a maximum of 3 min, but were not trained to any criterion. Mice were then tested following each alcohol ($n=15$) or water ($n=11$) access period on days 2, 4, 6, and 8 at both 10 and 15 RPM. They were given 3 trials (1 trial at 10 RPM and 2 at 15 RPM) with a ceiling of 3 min each. Latencies to fall were recorded at each RPM, and latencies were averaged in the 15 RPM condition to derive daily performance. On day 10, all mice were injected ip with 2 g/kg ethanol and given 2 trials on the fixed speed rotarod at each RPM starting 30 min after the injection.

2.4. Blood ethanol concentrations (BEC)

For all studies, 20 ul tail blood samples were taken on specified days by nicking the tail vein immediately following testing. In Experiment 1, samples were taken on Day 12. For Experiment 2, samples were taken on Day 16 and then again following the testing after challenge injection on Day 29. For Experiment 3, blood samples were taken following testing on the challenge injection day (Day 10). Samples were assayed by gas chromatography using a previously published method (Terdal and Crabbe, 1994).

2.5. Statistical analyses

Analysis of variance was used to analyze consumption of water or alcohol across days, behavioral measures of motor impairment and body weight. Tukey's HSD post hoc comparisons were used to assess main effects. Simple main effects were conducted to examine significant interactions. ANOVA was used to compare BECs of the alcohol self-administering and control groups in response to a challenge injection of ethanol in Experiments 2 and 3.

3. Results

3.1. Experiment 1

3.1.1. Consumption

Water and alcohol self-administration means are given in Fig. 1. Alcohol consumption (g/kg) ranged between about 2.0–2.5 g/kg/30 min session, and varied significantly across sessions [$F(3,106)=5.02$, $p<01$]. Post hoc analyses revealed that mice drank less during the alcohol session on

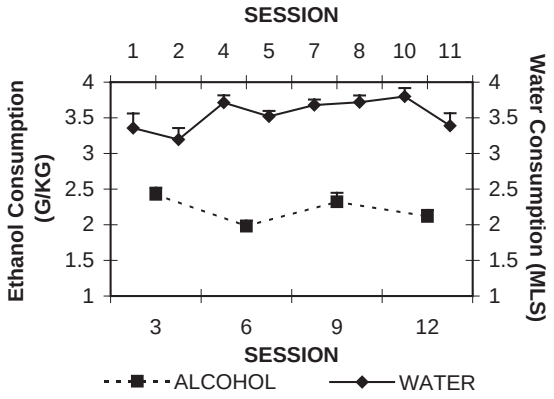

Fig. 1. *Experiment 1*: *Mean ± SEM alcohol* (*g/kg*) *and water consumption* (*ml*). Alcohol consumption on session 6 was significantly less than sessions 3 and 9 ($p < 0.05$). Alcohol and water groups did not differ across sessions for water consumption; thus, the water intake data shown above are collapsed across group.

day 6 than during the sessions on days 3 or 9. Water consumption in both groups remained stable across sessions.

Blood Ethanol Concentrations and Body Weight. Alcohol consumption resulted in mean BECs of 1.49 ± 0.04 mg/ml on Day 12 with a range of 0.89 to 1.98 mg/ml. All but one mouse had BECs that were >1 mg/ml. For body weight, there was a significant main effect of group [$F(1,39) = 8.50$, $p < 0.01$], as the alcohol group weighed significantly less (17.2 ± 0.2 g) than the water only control group (19.0 ± 0.4 g) on Day 12. The effect of session (day) and the day by group interaction were not significant. After one day of water restriction, all mice showed a weight reduction of approximately 15% (2.6 ± 0.2 g), and groups did not differ [$F(1,39) < 1$]. This weight reduction was maintained throughout the remainder of the paradigm in the alcohol-administering group, while the water control group returned to baseline weights by the third session.

3.1.2. Accelerating rotarod

Results are given in Table 2. Mice learned the accelerating rotarod task during training, as the average of the last 3 trials was greater than that of the first three trials [$F(1,15) = 23.5$, $p < 0.001$). However, groups did not differ or learn at different rates (both $F < 1$). The latencies to fall from the accelerating rotarod after drinking also did not differ between groups or across days (both $F < 1$). Despite the numerical difference between groups on test session 1, there was also no significant group X day interaction [$F(3,45) = 2.08$, $p > 0.10$].

Table 2
Latencies to fall (seconds) from the accelerating rotarod in experiment 1

Session	Acquisition		After Drinking			
	1st 3 trials	Last 3 trials	1	2	3	4
Alcohol	45 ± 2.5	61 ± 2.4	48 ± 4.6	57 ± 6.1	62 ± 4.3	65 ± 9.3
Water	39 ± 8.5	60 ± 3.6	73 ± 4.9	68 ± 5.4	59 ± 5.9	61 ± 5.7

Means ± SEM are shown for alcohol or water consumption sessions. There were no significant effects either before or after drinking.

3.1.3. Fixed speed rotarod

Results are shown in Fig. 2A. There were no differences between groups in latencies to fall on the training day (session 0: 180s ± 0 control, 160 ± 14 alcohol). In a separate analysis of post-drinking sessions 1–4, rotarod performance

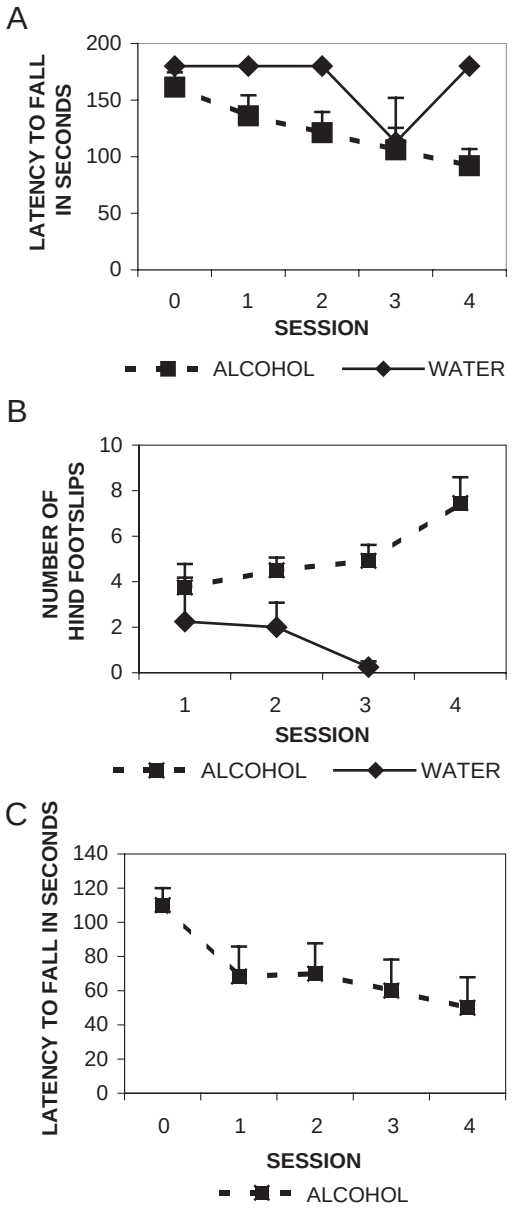

Fig. 2. *Experiment 1*: *A) Mean ± SEM latencies to fall from the fixed speed rotarod at 6.5 RPM for the training and alcohol test sessions*. There were no differences between groups on training session (session 0). For the remaining sessions, there was a non-significant tendency toward a main effect of group ($p = 0.08$). SEM was 0 for the water group, except on day 3. *B) Mean ± SEM hind foot slips on the balance beam*. Alcohol self-administering mice made more hind foot slips than the water mice ($p < 0.05$). Control mice were started one session later and therefore did not undergo a 4th test session. *C) Mean ± SEM latencies to fall from the vertical screen for the training and alcohol test sessions*. After drinking alcohol, mice had shorter latencies to fall from the screen following each alcohol test session (1, 2, 3, and 4) than they did during the initial baseline test session (0) ($p < 0.05$).

varied significantly across sessions [$F(3,45)=2.86$, $p<0.05$], but there was only a trend for performance to be lower in mice drinking alcohol than in mice drinking water [$F(1,15)=3.57$, $p=0.08$], and no significant interaction was seen.

3.1.4. Balance beam

Data for the first 3 post-drinking tests were analyzed. (see Fig. 2B). The alcohol group was more impaired (made more foot slips) than the water group [$F(1,14)=22.91$, $p<0.01$]. The effect of day and the day by group interaction were not significant.

3.1.5. Screen test

Given that all mice in the alcohol group were trained to a criterion test and passed, these mice served as their own control group. A one-way mixed ANOVA showed that there was a main effect of session [$F(4,44)=5.75$, $p<0.01$]. Post hoc analysis demonstrated that on each post-alcohol test session, performance was worse than during the initial drug-free training session (Fig. 2C). That post-alcohol days did not differ from each other suggests that it was unlikely that the effects seen were a result of repeated testing.

3.2. Experiment 2

3.2.1. Consumption

There were no differences in alcohol intake between groups of mice tested on the fixed speed rotarod at 6.5 or 10 RPM. Therefore, consumption data were collapsed across test condition, as depicted in Fig. 3. There was a significant main effect of session [$F(13,265)=2.75$, $p<0.01$], with post hoc analysis revealing that mice drank less alcohol on day 22 than during alcohol sessions on days 18, 20, 24 and 26. Water consumption remained stable across sessions and resembled the levels seen in Experiment 1. Table 3A shows water consumption in the ethanol and the water only groups on the odd numbered days (when only water was offered to both groups). Average group intakes ranged from 2.16 to 3.77 ml across the experiment, and each group averaged

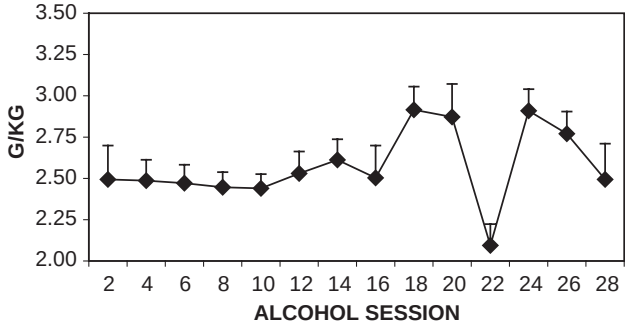

Fig. 3. *Experiment 2*: *Mean±SEM alcohol consumption in g/kg*. Alcohol consumption on session 22 was significantly less than on session 20, 24, 26 and 28 ($p<0.05$). As mice in the 6.5 and 10 RPM testing groups for fixed speed rotarod did not differ in consumption, intakes shown are collapsed across groups.

Table 3A
Water consumption days in experiment 2

Day	Group	
	ETOH	Water
1	2.60±0.53	2.16±0.49
3	3.03±0.63	3.04±0.26
5	3.46±0.59	3.03±0.62
7	3.61±0.63	3.04±0.52
9	3.23±0.58	2.85±0.80
11	3.54±0.44	3.21±0.36
13	3.66±0.82	2.95±0.54
15	3.49±0.54	3.20±0.48
17	3.19±1.02	3.03±0.53
19	2.94±0.47	2.67±0.38
21	3.23±1.23	2.51±0.93
23	3.63±0.55	2.96±0.55
25	3.36±0.51	2.66±0.45
27	3.77±0.53	3.20±0.81
29	3.11±0.58	2.98±0.30

Mean water intakes in ml/mouse (±SEM) for the ETOH and Water Only (Control) groups on the Water Only days. Means represent intakes for the full 3 h exposure.

about 3 ml in 3 h. There was a main effect of group [$F(1,42)=24.70$, $p<0.0001$]: the alcohol group consumed more water (3.33±0.04) than the water control group (2.90±0.03). There was also a main effect of session [$F(14,573)=9.23$, $p<0.0001$]. Post hoc analysis showed that there were lower intakes on session 1 than on all other sessions, as well as some other small differences among sessions ($p<0.05$). The interaction was not significant ($F<1$).

Table 3B shows that on the alternate days, the ethanol group averaged 1.20±0.02 ml/mouse during the 30 min ethanol access period (average daily range from 1.00 to 1.43), while the water group drank between 1.30 and 1.81 ml of water on those days, averaging 1.59±0.02) ml [$F(1,42)=42.78$, $p<0.001$]. There were some fluctuations

Table 3B
ETOH consumption days in experiment 2

Day	Group	
	ETOH	Water
2	1.00±0.46	1.76±.31
4	1.11±.28	1.81±.55
6	1.14±.26	1.66±.36
8	1.14±.22	1.59±.33
10	1.18±.27	1.57±.34
12	1.18±.32	1.67±.35
14	1.23±.29	1.66±.34
16	1.16±.44	1.63±.27
18	1.34±.27	1.68±.38
20	1.34±.47	1.72±.31
22	1.01±.31	1.30±.89
24	1.37±.28	1.44±.27
26	1.43±.61	1.53±.38
28	1.13±.36	1.30±.34

Mean ethanol intakes in ml/mouse (±SEM) for the ETOH and Water Only (Control) groups on the ETOH consumption days. Means represent intakes for the first 30 min.

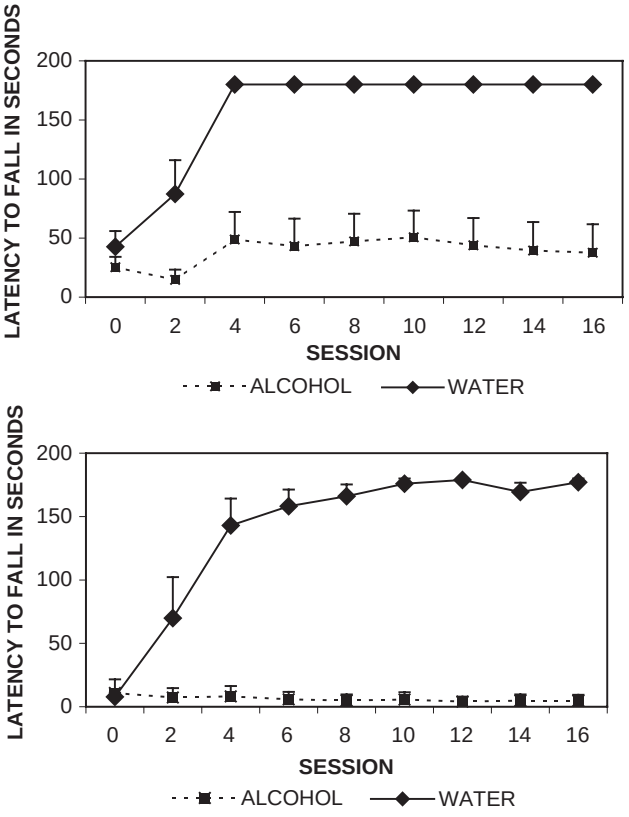

Fig. 4. *Experiment 2*: *Mean ± SEM latencies to fall from the fixed speed rotarod at 6.5 (top) and 10 (bottom) RPM for the training (session 0) and test sessions following consumption of alcohol or water*. On the training session there were no differences between groups. For the remaining sessions, the water control group tended to improve across session while the alcohol group showed no improvement ($p < 0.01$).

across sessions [$F(13,540) = 3.35$, $p < 0.0001$] as well as a significant group X session interaction [$F(13,540) = 2.82$, $p < 0.0005$]. Analysis of simple main effects showed that the water exposed group consumed more fluid than the alcohol exposed group on sessions 2 through 20 (Table 3B).

3.2.2. BECs and body weight

BECs were taken following alcohol consumption and testing on session 16 and again following the challenge injection and testing on session 29. The average BEC for consumption session 16 was 1.36 ± 0.09 mg/ml with 86% of the mice having BECs greater than 1.0 mg/ml. The BECs in response to challenge injections on session 29 differed in alcohol experienced versus alcohol naïve mice. For the 6.5 RPM groups, the alcohol experienced mice had a lower BEC than the alcohol naïve mice (1.42 ± 0.07 mg/ml vs 1.82 ± 0.08 mg/ml, respectively), with all mice having 1.0 mg/ml or greater [$F(1,12) = 11.79$, $p < 0.005$]. For the 10 RPM groups, BEC's were 1.48 ± 0.07 mg/ml and 1.65 ± 0.05 mg/ml for the alcohol experienced and alcohol naïve mice, respectively, a non-significant difference. Body weights did not differ between the water and alcohol-experienced groups in this experiment (data not shown).

3.2.3. Fixed speed rotarod acquisition

The 6.5 and 10 RPM groups were each compared only to their own water control group in 2 separate tests (Fig. 4). There was no difference between groups during training on session 0 for either RPM. For sessions 2 through 16, there were group X session interactions at both RPM [$F(7,84) = 2.44$, $p < 0.02$] and [$F(7,84) = 6.86$, $p < 0.0001$, respectively], and the water groups performed better than the alcohol self-administering groups.

3.2.4. Fixed speed rotarod following challenge injections

Results are shown in Fig. 5. There were no differences between naive and alcohol-experienced groups at either RPM, although there was a non-significant tendency for the water group to be more impaired at 10 RPM ($p = 0.12$).

3.3. Experiment 3

3.3.1. Consumption

The alcohol and water consumption for each session can be seen in Fig. 6. Alcohol consumption remained high and relatively stable, and there were no significant differences across days. Considering both groups together, there tended to be a main effect of session on water consumption [$F(4,96) = 2.23$, $p < 0.09$)].

3.3.2. BECs and body weight

BECs on the challenge injection day were 1.89 ± 0.07 mg/ml and 1.99 ± 0.05 mg/ml for the alcohol-experienced and alcohol-naïve mice, respectively. These concentrations did not differ significantly between groups. Body weights also did not differ between the groups (data not shown).

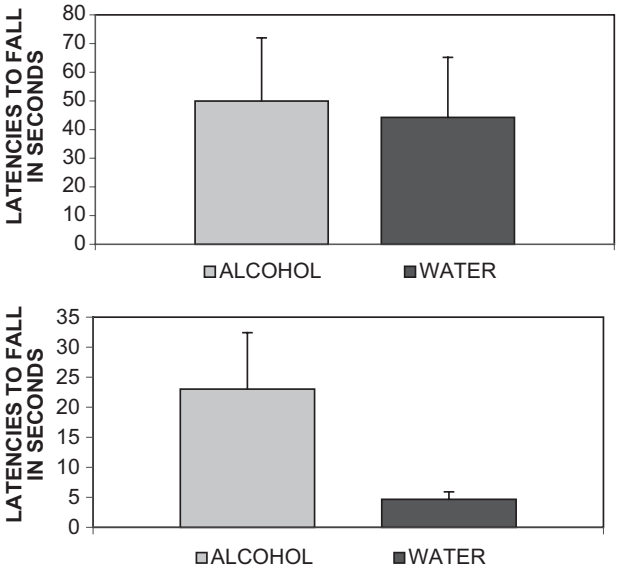

Fig. 5. *Experiment 2*: *Mean ± SEM latencies to fall from the fixed speed rotarod at 6.5 (top) and 10 (bottom) RPM during the challenge injection session*. There were no differences between groups at 6.5 RPM. At 10 RPM, there was a non-significant tendency for the ethanol group to have longer latencies than did the water control group ($p = 0.12$).

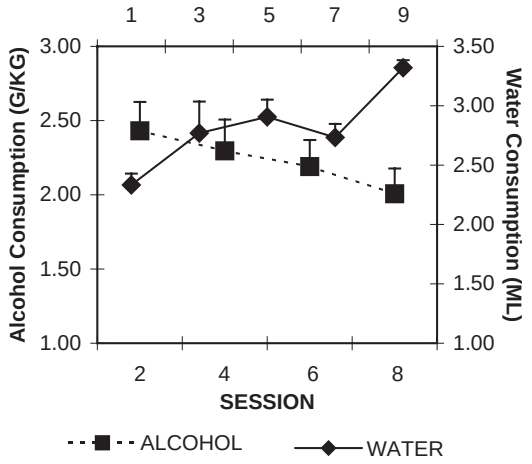

Fig. 6. *Experiment 3*: *Mean ± SEM alcohol (g/kg) and water consumption (ml)*. Alcohol consumption remained stable across sessions. There was a main effect of session for water intake that approached significance ($p < 0.09$). However, water intake did not differ between the alcohol or water drinking groups, so water intake data shown are collapsed across group.

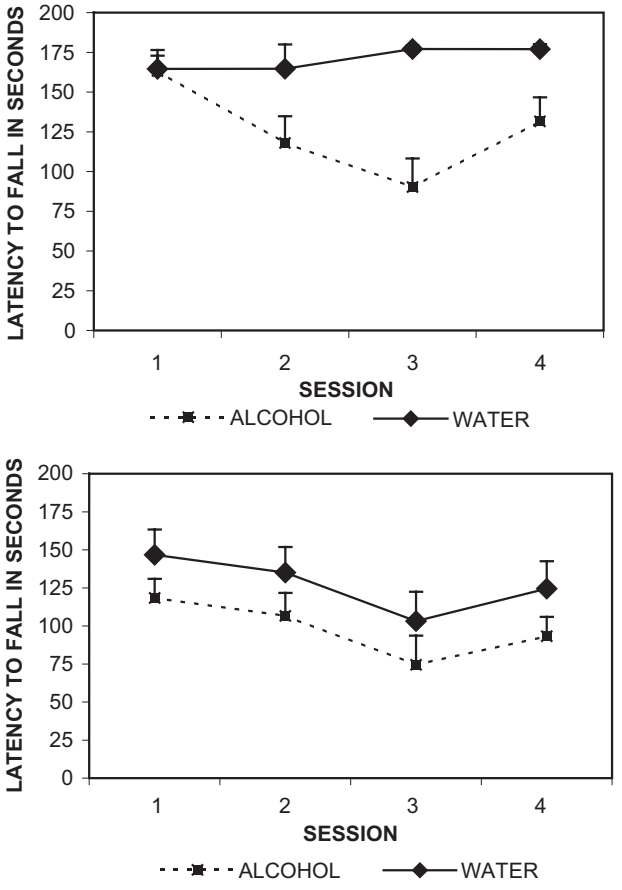

Fig. 7. *Experiment 3*: *Mean ± SEM latencies to fall from the fixed speed rotarod at 10 (top) and 15 (bottom) RPM*. At 10 RPM, the alcohol group was intoxicated as compared to the water control group on sessions 2, 3 and 4 ($p < 0.02$). At 15 RPM, there were no differences between groups. The water control group did not show significant improvement across sessions at either RPM.

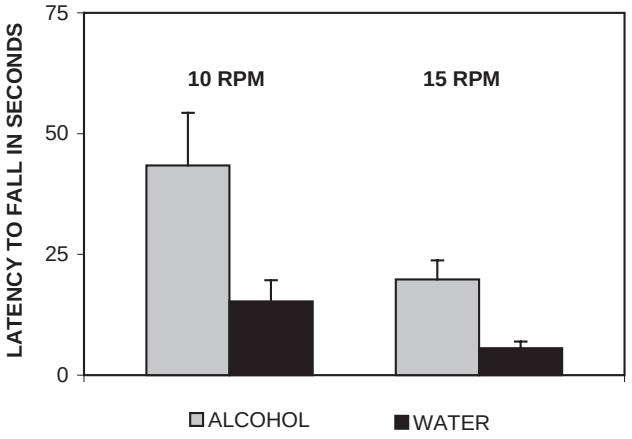

Fig. 8. *Experiment 3*: *Mean ± SEM latencies to fall from the fixed speed rotarod at 10 and 15 RPM during the challenge injection session*. At both RPM's, the alcohol-experienced group was tolerant to the challenge injection as compared to the alcohol-naïve (water) group ($p < 0.05$ and $p < 0.001$, respectively).

3.3.3. Fixed speed rotarod acquisition

There were no differences between groups during the final three pretraining trials on the 10 RPM fixed speed rotarod on session 0 (data not shown). Results after scheduled access to alcohol or water are shown in Fig. 7. There was a group X session interaction across test sessions 1 through 4 for the 10 RPM condition [$F(3,72) = 3.78$, $p < 0.02$)]. Although rotarod performance was not different between alcohol-experienced and naive groups after the first two sessions, performance was significantly decreased in the alcohol group after alcohol sessions 3 and 4. For the 15 RPM tests, there was only a main effect of session [$F(3,72) = 4.54$, $p < 0.01$)]. Post hoc analyses revealed that latencies to fall were significantly shorter on session 3 than on session 1.

3.3.4. Fixed speed rotarod following challenge injections

At both 10 and 15 RPM, the alcohol-experienced groups had longer latencies to fall from the rotarod than the alcohol naïve groups [$t(1,24) = 4.46$, $p < 0.05$] and [$t(1,24) = 8.90$, $p < 0.001$], respectively, demonstrating that the alcohol-experienced groups were tolerant to the intoxicating effects of alcohol (Fig. 8).

4. Discussion

Several results from these experiments suggest the efficacy of the scheduled fluid access procedure as a model of high and repeated alcohol consumption. The consistency of alcohol intakes and resulting BECs across multiple exposures are consistent with previously reported findings (Finn et al., 2005), and these BECs were sufficient to impair motor performance on three of the four tests employed. While there was some evidence for the development of tolerance to alcohol's impairing effects with repeated self-

administration, this was only seen using some tests of tolerance.

Signs of ethanol motor impairment in mice can be seen on many tasks of motor coordination at BECs greater than 1 mg/ml (Crabbe et al., 2003a). However, different assays of motor performance are influenced by different genetic factors in mice, indicating that this behavioral domain is complex (Crabbe et al., 2005). In the current series of studies, the average self-administered blood levels always exceeded 1 mg/ml after 30 min access to a 5% ethanol solution. Performance on the balance beam and screen test indicated that the mice had consumed alcohol doses that produced motor impairment in Experiment 1. Mice tested on the fixed speed rotarod at 10 RPM were clearly impaired in Experiments 2 and 3. They tended to perform worse after a 6.5 RPM test in Experiment 1, and were significantly impaired at this speed in Experiment 2. There was no evidence of motor impairment at 15 RPM in Experiment 3. We have found that fixed speed rotarod rates greater than 10 RPM do not further decrease latencies to fall in mice given intraperitoneal doses of ethanol (Rustay et al., 2003b). For the alcohol-exposed group in Experiment 3, the latencies to fall at 15 RPM were similar to the latencies at 10 RPM. However, the control group showed worse performance at 15 RPM than at 10 RPM (Fig. 7). This suggests that the highest RPM condition was more difficult for the control group, and the reduced latencies to fall at 15 RPM in the control group may have obscured any possible ethanol effect at 15 RPM. Overall, it was possible to detect motor impairment on the fixed speed rotarod under appropriate test conditions.

No significant motor impairment was seen after alcohol self-administration using the accelerating rotarod. This may be a characteristic of the task's sensitivity to detect dose-related ethanol-induced impairment. In a comparison of the sensitivity to ethanol of 21 inbred mouse strains on the accelerating rotarod, we found that ethanol doses between 1.0 and 1.5 g/kg can *enhance* performance in this test depending on the genotype and test parameters, while higher doses impair performance in all genotypes. C57BL/6J mice given 1.0 g/kg ip showed modestly enhanced performance in this experiment (Rustay et al., 2003a). In C57BL/6J mice following ip injection, doses between 1 and 1.5 g/kg have been shown to produce BECs averaging 1.28 mg/ml 30 min later (Crabbe et al., 2003a), BECs similar to those achieved following drinking in Experiments 1 and 2 (1.5 and 1.3 mg/ml, respectively). Thus, it is possible that performance on the accelerating rotarod was not decreased by alcohol consumption due to the specific dose effect characteristics of this task and mouse strain.

The development of tolerance was assessed both within and between groups in Experiments 2 and 3, at two RPM in each experiment. While not entirely consistent, results indicated that tolerance could be detected under appropriate conditions. Within groups, we predicted that mice chronically ingesting alcohol would show significant improvement in performance on the rotarod across test days with intoxicated practice, indicating that tolerance had developed. However, this did not occur in Experiment 2, where maximum performance had been reached by the 2nd or 3rd ethanol session. In rats, and using different measures of motor impairment, intoxicated practice does not necessarily increase the magnitude of tolerance, but rather increases its rate of acquisition and its longevity after cessation of alcohol exposure (Lê and Shaham, 2002; Lê et al., 1994). Therefore, the rapid development of modest within group tolerance may have resulted in our inability to detect it.

There was also no evidence for within-group tolerance at either RPM in Experiment 3 (Fig. 7). One major difference between Experiments 2 and 3 was the initial training to a criterion of performance (Table 1). In Experiment 2, mice of both the alcohol and control groups were learning the task during the consumption paradigm. Therefore, any performance impairments seen due to alcohol were also confounded with the incomplete state of learning the task. Throughout the course of the experiment, the control group learned the task to criterion (i.e., no animals fell from the fixed-speed rotarod by the end of testing), but the alcohol group did not. Thus, it is possible that alcohol-induced impairments in learning the task interfered with tolerance development and/or the effect of intoxicated practice in our alcohol group. The difference between the two rotarod rates could explain why there was a trend toward tolerance across days at 10 RPM, where alcohol administering animals were more impaired. That is, a ceiling effect may have been at work in Experiment 2.

Another common method to detect tolerance is to compare an alcohol experienced versus an alcohol naïve group following a challenge injection at the end of the ethanol exposure period (Crabbe et al., 1979; Darbra et al., 2002; Erwin et al., 1992; Gatto et al., 1987). This method is usually used not to assess the rate of tolerance development, but rather its extent when complete. Thus, after multiple opportunities to consume ethanol, both alcohol and water drinking mice were administered a challenge injection of alcohol and tested on the rotarod. Despite 15 total alcohol exposures in Experiment 2, rotarod performance at 6.5 RPM was comparable in the alcohol and water groups in response to a challenge injection; however, there was a non-significant tendency for alcohol-experienced mice to perform better when tested at 10 RPM (see Fig. 5). However, the between-groups tests for tolerance were significant at both RPM in Experiment 3 (Fig. 8). In summary, the results of the between groups tests in Experiments 2 and 3 suggest that tolerance developed under conditions where the task was not being acquired during testing, and where the task was more challenging.

Although there was some variation in daily intakes of ethanol in all experiments, animals generally self-administered between 2.00 and 2.50 g/kg in 30 min, and reasonably stable alcohol intakes were seen throughout testing. These

intakes resulted in BECs that consistently exceeded 1 mg/ml on average in all experiments. Table 3B and Fig. 3 both hint at a tendency for animals chronically self-administering alcohol to show slightly higher intake levels later in the experiment. Thus, no strong tendency was seen for the tolerance that did develop in these experiments to be accompanied by a pattern of increasing intakes.

Other methods can also achieve relatively high intakes. For instance, rat and mouse lines that have been selectively bred for alcohol preference in either free choice or limited access drinking paradigms may consume 5–12 g/kg in a 24 h period (for reviews, see Grahame et al., 1999; McBride and Li, 1998). However, there is a great deal of variability in the amount of alcohol consumed and the resulting BECs in both rats and mice. Significant and sustained elevations of BECs from self-administration can be achieved following long-term or intermittent consumption to ethanol sufficient to support a state of physical dependence, or may be seen following periods of access separated by periods of withdrawn access following presumptive physical dependence (O'Dell et al., 2004; Roberts et al., 1996; Rodd-Henricks et al., 2001; Waller et al., 1982) Some studies have shown that the BECs of C57BL/6J mice self-administering ethanol in an unlimited access two-bottle preference test may exceed 1 mg/ml, but these levels are only transiently maintained (Dole and Gentry, 1984). Sustained BECs in C57BL/6J mice have also been reported following more complex schedules of access (Becker and Lopez, 2004; Middaugh et al., 2003; Mittleman et al., 2003). Our scheduled access procedure may provide an easy way to reduce variability and precisely investigate consumption that leads to alcohol-induced motor impairment and tolerance.

In Experiments 2 and 3, we modified the restricted fluid access paradigm to increase the frequency and contiguity of the alcohol exposures and, therefore, the likelihood that tolerance would develop during consumption (Cox and Tiffany, 1997). Access to alcohol was provided every other day as opposed to every 3rd day as in Experiment 1. Ultimately, this modification might facilitate investigations of chronic excessive alcohol consumption. Offering alcohol daily might enhance tolerance development even more, as has been shown in some other paradigms (Crabbe et al., 1979). Experiment 2 demonstrated that alcohol intakes can remain stable for nearly a month.

In these studies, it is possible that the scheduled fluid access may have adversely affected our mice physiologically and/or behaviorally (Toth and Gardiner, 2000). However, the daily performance of the control mice on the rotarod was either similar to or better than their baseline performance during ad libitum access. Despite the modest fluid restriction, body weights also remained stable for the duration of the experiment suggesting that this level of restriction is tolerated well. In other studies, we have gradually increased the period of water availability that follows alcohol to as long as 10 h without any reductions in the amount of alcohol consumed (Finn et al., 2005). Other studies have shown no abnormalities in rats chronically maintained on a 3-h access schedule as evidenced by daily clinical examinations and comprehensive postmortem evaluations (Hughes et al., 1994). Collectively, this evidence suggests that the limited availability of fluid in the present studies did not induce detrimental physiological or behavioral effects in the mice. In another method we are exploring, ethanol is substituted for water for a limited period during the circadian dark cycle, a period where mice are known to perform most of their drinking (Freund, 1970). C57BL/6J mice will also self-administer ethanol to blood levels similar to those we report here when drinking in the dark (Rhodes et al., 2005).

In essence, we have demonstrated that a procedure that schedules access to alcohol can produce high and stable levels of alcohol consumption leading to motor impairment. We have modest evidence under some conditions that repeated self-administration resulted in functional tolerance as well. While we cannot speak directly to the nature of the reinforcing aspects of the alcohol consumed in this procedure, the present findings validate the method as a potential model for studying one important component of alcoholism, alcohol self-administration that leads to repeated motor impairment and the development of tolerance.

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