

**Effect of Duration and Pattern of Chronic Ethanol Exposure on Tolerance to the
Discriminative Stimulus Effects of Ethanol in C57BL/6J Mice**

Howard C. Becker and Alicia M. Baros

Research Service, Department of Veterans Affairs, Ralph H. Johnson Medical Center (HCB)

and

Charleston Alcohol Research Center, Center for Drug and Alcohol Programs,

Medical University of South Carolina

Charleston, SC 29425, USA (HCB and AMB)

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Address for Correspondence: Howard C. Becker, Ph.D., Charleston Alcohol Research Center, Center for Drug and Alcohol Programs, Medical University of South Carolina, 67 President Street, PO Box 250861, Charleston, SC, 29425

Telephone: 843-792-5207

Fax: 843-792-1724

Email: beckerh@musc.edu

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Abbreviations: BEC: blood ethanol concentration; FR: fixed ratio

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ABSTRACT

This study was conducted to examine whether amount and/or pattern (intermittent or continuous) of chronic ethanol exposure subsequently alters sensitivity to the discriminative stimulus effects of ethanol. Adult male C57BL/6J mice were trained to discriminate between 1.5 g/kg ethanol and saline in a two-lever food-reinforced operant procedure. Once ethanol discrimination was successfully acquired, generalization testing was conducted using a cumulative dosing procedure to generate a baseline dose-response function (0 - 2.5 g/kg ethanol). Discrimination training was then suspended while mice received chronic ethanol vapor or air exposure in inhalation chambers. The total amount of ethanol exposure was systematically increased, but delivered in an intermittent or continuous fashion. At 24 hr or 16 hr following inhalation treatment, ethanol discriminability was re-assessed using the same generalization testing procedures. Results indicated that discrimination performance in control (air-exposed) mice was similar to baseline. However, sensitivity to ethanol's discriminative cue following chronic ethanol treatment was reduced (as evidenced by rightward shifts in the dose-response functions and increased ED₅₀ values). The magnitude of this tolerance effect increased as a function of the number of chronic ethanol exposures as well as the total duration of ethanol exposure. Additionally, tolerance was more robust when generalization testing was conducted earlier (16 hr vs. 24 hr) after chronic ethanol treatment was halted (2-3 fold increase in ED₅₀ values). These results may have important clinical implications, as blunted sensitivity to ethanol's discriminative cue may contribute to enhanced ethanol self-administration behavior observed in these mice following similar chronic ethanol treatment.

Many biological and environmental factors are known to influence motivation to initiate ethanol drinking, as well as define parameters that lead to termination of intake. The perception of internal cues produced by ethanol consumption most likely plays an important role in regulating intake, as well as formulating expectations about the consequences of ethanol that shape future motivation to engage in drinking behavior. Use of drug discrimination procedures affords the opportunity to examine the ability of a subject to perceive the subjective interoceptive cues associated with exposure to a variety of psychotropic drugs, including ethanol (Preston and Bigelow, 1991). Studies in humans have demonstrated that ethanol produces a complex discriminative cue and, as is the case for many other drugs, subjective effects of ethanol were shown to correlate with discriminative responding (Duka et al., 1998; Jackson et al., 2001).

In preclinical studies, the discriminative stimulus effects of ethanol have been well established in several animal species and under a variety of experimental conditions (Grant, 1999; Hodge et al., 2006; Shelton and Grant, 2002). The interoceptive cue associated with ethanol is complex, dependent on both dose and timing (Grant and Colombo, 1992; Grant et al., 1997; Quertemont et al., 2003; Schechter, 1989), and mediated by central mechanisms involving several neurochemical systems (Besheer et al., 2003; Hodge and Cox, 1998; Hodge et al., 2001). While ethanol discriminability has been noted to be rather stable over time (i.e., stable stimulus control of responding over many discrimination training and testing sessions), a few studies in rats (Emmett-Oglesby, 1990; Lytle et al., 1994) and mice (Middaugh et al., 2003) have demonstrated reduced sensitivity to the discriminative cue of ethanol following chronic ethanol consumption. We recently demonstrated reduced sensitivity (tolerance) to the discriminative stimulus effects of ethanol following chronic exposure to the drug in C57BL/6J mice (Crissman et al., 2004). A key procedural feature of these studies is that chronic ethanol exposure was administered outside the context of discrimination training and testing. Similarly, tolerance to the discriminative stimulus effects of other drugs, such as opiates, stimulants, and

depressants, has been demonstrated when chronic exposure to the drugs was administered while discrimination training was suspended (Young, 1991).

Although the relationship between the discriminative stimulus effects of ethanol and its reinforcing effects is not entirely clear (Duka et al., 1999), this issue is of clinical relevance since an alteration in ethanol discriminability resulting from chronic ethanol exposure may have a significant influence on propensity to drink. That is, reduced ability to detect or perceive ethanol's subjective (intoxicating) effects may lead (contribute) to motivation for continued ethanol consumption. The present study was conducted to further explore the apparent tolerance effect observed in our initial findings using C57BL/6J mice (Crissman et al., 2004), by examining whether the amount (duration) and/or pattern (intermittent or continuous) of chronic ethanol exposure influences subsequent sensitivity to ethanol's discriminative cue.

We previously have demonstrated that an intermittent pattern of chronic ethanol exposure, which involves repeated episodes of withdrawal, results in progressive intensification, of numerous withdrawal symptoms including several measures of seizure activity, anxiety, and stress (Becker, 1999; Veatch and Becker, 2002). This sensitization or "kindling" of ethanol withdrawal has been suggested to contribute to the problem of relapse and perpetuation of excessive drinking in dependent subjects. Indeed, our ethanol dependence model has been shown to subsequently enhance voluntary ethanol self-administration behavior (Becker and Lopez, 2004), and the development of this effect was accelerated when the chronic ethanol exposure was delivered in an intermittent, as opposed to a continuous, fashion (Lopez and Becker, 2005). Based on these findings, it was hypothesized that tolerance to the discriminative stimulus effects of ethanol would not only increase as a function of the duration of chronic ethanol exposure, but the magnitude of the tolerance effect would be greater when the chronic ethanol exposure is delivered in an intermittent rather than continuous pattern. The present study was designed to systematically vary the duration and pattern of chronic ethanol exposure to address this issue. Additionally, tolerance to ethanol's discriminative cue was assessed at

two time points (16 and 24 hours) following chronic ethanol exposure to examine temporal parameters of the phenomenon.

METHODS

Subjects. Experimentally naïve adult male C57BL/6 mice (The Jackson Laboratories, Bar Harbor, ME), weighing 23-29 g at the beginning of the experiment (n= 29), were housed in polycarbonate cages with wood shavings and stainless steel wire lids. The animals were maintained in a temperature and humidity-controlled AAALAC-approved facility under a 12-hr light/dark cycle (lights on at 0600 hr). After a two-week period of acclimation, the mice were individually housed and maintained at 85% of their free-feeding body weight by daily restricted feeding with standard rodent chow. Water was continuously available throughout the experiments, except during the experimental sessions. All procedures were approved by the Institutional Animal Care and Use Committee and followed the NIH Guide for the Care and Use of Laboratory Animals (1996).

Apparatus. Mice were trained and tested in six standard operant chambers (16 x 15 x 12.5 cm), each enclosed in ventilated and sound-attenuating cubicles (MED Associates, St. Albans, VT; modular mouse chamber model ENV-307). The operant chambers were equipped with two retractable levers, and a centrally located food-trough (equidistant from both levers) was programmed to dispense one 20 mg food pellet (P.J. Noyes, Lancaster, NH) when schedule requirements were met. Stimulus lights were located above each lever and a house light was located on the opposite wall. A microcomputer-based MED Associates interface and operating system was used to record responses and control schedule contingencies.

Study Design and General Procedure. The general study design entailed first establishing accurate ethanol discrimination performance, followed by generation of a baseline ethanol dose-response function through generalization testing (see below). Mice were then assigned to chronic ethanol and control groups, and treated as described below. Discrimination

training sessions were suspended during chronic ethanol (and control) treatments. After various chronic ethanol treatment regimens, ethanol discriminability was re-assessed in ethanol-exposed and control groups using the same generalization testing procedures employed to establish the baseline ethanol dose-response function.

The ethanol-exposed animals were further separated into two groups based on the pattern of chronic exposure. One group (MW group) received chronic intermittent ethanol exposure (with multiple intervening periods of withdrawal), while the remaining mice (CE group) received the same total amount of ethanol exposure, but in a continuous fashion. The number of cycles (intermittent exposure) and duration (continuous exposure) was systematically increased, but designed to equate total amount of ethanol exposure for MW and CE conditions. That is, MW mice received one, two, three, or four cycles of 16 hr exposure to EtOH vapor in inhalation chambers (described below), with each cycle separated by an 8-hr period of withdrawal (MWx1, MWx2, MWx3, and MWx4 conditions, respectively). In contrast, CE mice were exposed to 16, 32, 48, or 64 hr ethanol vapor, with no interruption (CE/16, CE/32, CE/48, and CE/64 conditions, respectively). A separate group of animals served as controls (CTL group) and received similar handling as MW and CE groups, but did not receive chronic ethanol vapor exposure.

At a time corresponding to 24 hr following final withdrawal from each of the treatment conditions, MW, CE, and CTL groups were re-tested to determine a new ethanol dose-response function. To examine whether testing at an earlier time point might influence the tolerance effect, a select number of CTL and ethanol-exposed groups (MWx2 versus CE/32 and MWx4 versus CE/64) also were tested 16 hr after withdrawal from the inhalation chambers. Following each of the generalization testing sessions, all mice received discrimination training sessions to ensure that criterion-level discrimination performance was maintained prior to the next chronic ethanol (or air) exposure treatment condition. Thus, a minimum of eight training sessions (saline versus 1.5 g/kg ethanol) separated generalization testing and the next chronic ethanol

(or air) treatment condition. Generalization tests following chronic ethanol (or control) treatment were conducted in ascending order with respect to number of cycles/duration of exposure (i.e., CTL, MWx1, CE/16; CTL, MWx2, CE/32; CTL, MWx3, CE/48; and CTL, MWx4, CE/64). Generalization tests at the 24 hr time point were conducted prior to testing at the 16 hr time point.

Discrimination Training. Mice were initially trained to alternate daily between the two available response levers under a fixed ratio-1 schedule of reinforcement (FR-1) during 15-min sessions. The reinforcement contingency was increased incrementally to an FR-20 schedule; i.e., every twentieth response was reinforced. Once stable (unbiased) lever responding under this schedule was established, mice were trained to discriminate ethanol (1.5 g/kg) from 0.9% saline. Ethanol or saline injections were administered intraperitoneally (i.p.) and at 5 min prior to the 15-min operant sessions. For each mouse, one lever was designated the vehicle lever and the other designated the drug lever. The mice were trained five days a week, receiving the training drug or vehicle according to a pre-determined schedule (which stipulated that animals did not receive the same treatment more than two days in a row). A performance criterion of 85% treatment-appropriate responding for 8 out of 10 consecutive sessions, with no more than 4 incorrect responses before 20 responses were made on the treatment-appropriate lever, was used to indicate successful discrimination training.

Generalization Testing Procedure. A cumulative dosing procedure was employed for generalization testing, similar to that previously described (Crissman et al., 2004). This allowed for the generation of a full ethanol dose-response curve within a single session both prior to (baseline) and following the various chronic ethanol exposure treatments (tolerance testing). The cumulative dosing procedure involved sequential administration of saline, 0.5, 0.5, 0.5, 0.5, 0.5 g/kg ethanol (i.p.), which corresponded to cumulative doses of 0, 0.5, 1.0, 1.5, 2.0, 2.5 g/kg

ethanol. The 2-min test sessions were initiated 5 min after each injection, during which responding was reinforced (FR-20) on either lever.

Chronic Ethanol Administration. Ethanol was administered by the inhalation route, as previously described (Becker and Lopez, 2004). Briefly, mice were placed in Plexiglas inhalation chambers (60 X 36 X 60 cm), with the housing conditions identical to that in the colony room. Ethanol (95%) was volatilized by passing air through an air stone (gas diffuser) submerged in the ethanol. The ethanol vapor was mixed with fresh air and delivered to the chambers at a rate of 5 l/min, which maintained the ethanol concentration in the chamber in the range of 19-22 mg/l ethanol and yielded blood ethanol concentrations (BEC) in the range of 150-200 mg/dl.

At the beginning of each 16-hr exposure period (1700 hr), intoxication was initiated in the MW group by administration of ethanol (1.6 g/kg; 8% w/v) along with the alcohol dehydrogenase inhibitor pyrazole (1.0 mmol/kg) to stabilize blood ethanol levels. Both drugs were injected i.p. in a volume of 0.02 ml/g body weight. The CE group received an injection of ethanol (1.6 g/kg) upon entry into the inhalation chamber, and pyrazole injections at the same time as the corresponding MW condition (every 24 hr) in order to maintain stable BEC. The CTL group was treated similarly as the CE group, but given an injection of pyrazole along with saline (rather than ethanol) and maintained in control (air) chambers. In this way, all mice received similar handling, with the number and timing of pyrazole injections equated across groups (but dependent on the number of cycles (MW group) and duration (CE and CTL groups) of inhalation exposure). Immediately following final removal from the inhalation chambers, blood samples were collected from all mice and subsequently analyzed for blood ethanol content.

Ethanol Samples and Measurement. Chamber ethanol concentration was monitored daily (at 0900 hr). Air samples (2 ml) were collected with a syringe through a port in the

inhalation chamber wall. The samples were then transferred to Venoject™ tubes for later analysis using an enzymatic spectrophotometric assay procedure previously described (Becker and Lopez, 2004). Ethanol concentration in the chambers is expressed as mg/l air.

Blood samples were collected from the retro-orbital sinus with heparinized capillary tubes. The samples were centrifuged for phase separation and 5 µl of plasma were injected into an Analox Instrument analyzer (Lunenburg, MA). Blood ethanol concentration (expressed as mg/dl) was recorded by measuring oxygen uptake generated by the oxidation of ethanol to acetaldehyde and hydrogen peroxide by ethanol oxidase.

Data Analysis. Results from generalization tests are expressed as mean percentage of total responses on the ethanol-appropriate lever (ethanol-appropriate responses/total session responses) and response rates (total session responses/min). Discrimination data collected at the 24 and 16 hr time points were analyzed separately. Data from dose-response curves were subjected to non-linear regression analysis (SPSS 12.0, NLR & CNLR) fitting a two parameter (ED_{50} and slope) logistic function. Differences between curves were assessed by residual F-tests. Potency ratio analyses were conducted to compare determined ED_{50} values for the control conditions with corresponding treatment groups (MW, and CE conditions). Initial analysis showed no evidence for differences in slopes and that parameter was shared across curves in all analyses. Since analysis of curves (and ED_{50} values) for the CTL group revealed no significant difference across the different generalization test sessions, these data were combined to determine an overall ED_{50} value for the CTL condition. This was used in separate analyses to evaluate the effects of increasing the number of chronic ethanol cycles in the MW condition and the effects of increasing the duration of exposure in the CE condition (with Bonferroni-corrected tests for multiple comparisons). Comparison of ED_{50} values generated from similar treatment groups determined at 24 hr vs. 16 hr time points was analyzed by residual F-tests. Response rate data were analyzed by 2-way ANOVAs, with treatment group

as a between-subjects factor and ethanol dose as a repeated measure. Post hoc comparisons were performed using Tukeys HSD test, when appropriate.

RESULTS

Discrimination Acquisition and Baseline Generalization Testing. All mice acquired the discrimination and met the training criterion after an average of 17.2 ± 0.6 sessions. Baseline generalization testing revealed ethanol dose-dependently increased selection of the ethanol-appropriate lever. Analysis of the dose-response function indicated a mean ED_{50} value of 0.72 ± 0.04 g/kg ethanol. Mice registered 40-60 responses per min during each 2 min test period of the cumulative dosing procedure. Analysis of response rates indicated a significant decrease only at the highest (2.5 g/kg) ethanol dose ($F[5,168] = 7.74$, $p < 0.0001$). Subsequent generalization testing conducted in animals following varying durations of air exposure in control inhalation chambers (CTL group) produced similar ethanol dose-response curves (mean ED_{50} value = 0.67 ± 0.03 g/kg) that did not significantly differ from baseline.

Generalization Testing at 24 hr Following Chronic Ethanol Treatment. In all cases, chronic ethanol exposure, whether delivered in a continuous (CE groups) or intermittent (MW groups) fashion, resulted in a significant shift to the right in the ethanol dose-response function 24 hr after removal from the inhalation chambers (**Fig. 1**). Potency ratio analyses indicated significant 1.5-2 fold increases in the calculated ED_{50} values for all MW and CE groups compared to the CTL groups (all F 's > 35.0 , all p 's < 0.001). Nonlinear regression analyses also revealed significantly greater shifts to the right in the ethanol dose-response curves for CE/32 and CE/48 groups compared to the corresponding MWx2 and MWx3 groups (p 's < 0.05) (**Fig. 1B and 1C**).

Analysis of response rate data for each exposure condition indicated significant main effects of Dose (all F 's > 6.20 , p 's < 0.001). In each case (**Fig. 1A-D**), all groups (MW, CE, and CTL) exhibited significantly lower rates of responding at the 2.5 g/kg ethanol dose in comparison to saline (p 's < 0.05). ANOVA also revealed a significant Group x Dose interaction

for response rates in mice tested after 64 hr of chronic ethanol exposure (CE/64 and MWx4 groups) and air (CTL group) in inhalation chambers ($F[10,130] = 2.55, p < 0.008$) (**Fig. 1D**). Post-hoc analysis indicated that the CE/64 group registered significantly lower rates of responding at all test doses, while the MWx4 group exhibited lower response rates for saline and 0.5 g/kg ethanol doses in comparison to CTL mice (p 's < 0.05). Additionally, response rates were significantly lower in the CE/64 group compared to the MWx4 group for all ethanol doses (p 's < 0.05).

Generalization Testing at 16 hr Following Chronic Ethanol Treatment. Exposure to two or four cycles of 16 hr ethanol treatment (MWx2 and MWx4 groups, respectively) as well as 32 hr or 64 hr continuous ethanol exposure (CE/32 and CE/64 groups, respectively) produced robust shifts to the right in the ethanol dose-response function compared to controls when testing was conducted 16 hr after the chronic treatment regimen (**Fig. 2**). Potency ratio analyses indicated significant 2.5-3 fold increases in the calculated ED_{50} values for both MW and CE conditions compared to the CTL groups (all F 's > 45.0 , all p 's < 0.001). Nonlinear regression analyses also revealed significantly greater shifts to the right in the ethanol dose-response curves for MWx2 and MWx4 groups compared to the corresponding CE/32 and CE/64 groups (p 's < 0.05) (**Fig. 2A and 2B**).

Analysis of response rates revealed a significant Group x Dose interaction ($F[10,130] = 3.92, p < 0.0001$), with significantly lower rates of responding in MWx2 mice compared to CE/32 and CTL mice at all doses except 2.5 g/kg, and the CE/32 group responding at a lower rate than CTL at the 0.5, 1.0, and 1.5 g/kg test doses (p 's < 0.05) (**Fig. 2A**). Likewise, a significant Group x Dose interaction ($F[2,26] = 25.26, p < 0.001$) indicated both MWx4 and CE/64 groups exhibited significantly lower rates of responding in comparison to the CTL group across all doses and the MWx4 group registered lower response rates compared to CE/64 mice at the saline and 0.5 g/kg test doses (p 's < 0.05) (**Fig. 2B**).

Effect of Increasing Cycles and Duration of Chronic Ethanol Treatment. A

summary of mean ED₅₀ values derived from ethanol dose-response functions generated from generalization testing at 24 or 16 hr following exposure to air (CTL group), or chronic ethanol exposure delivered in an intermittent (MW group) or continuous (CE group) fashion is presented in Table 1. Analysis of MW groups revealed a significant main effect of Cycles ($F[4,46] = 143.78, p < 0.001$), indicating significantly greater ED₅₀ values generated 24 hr following exposure to one, two, three, or four cycles of 16 hr ethanol vapor in comparison to controls (p 's < 0.01). Post-hoc tests also indicated that experience with four cycles of chronic ethanol exposure (MWx4 group) yielded a significantly greater ED₅₀ value compared to all other MW groups (p 's < 0.01). Analysis of CE groups indicated that the rightward shift in dose-response functions determined 24 hr after varying durations of chronic ethanol exposure produced significantly higher ED₅₀ values in comparison to the CTL group ($F[4,46] = 136.94, p < 0.001$). Post-hoc tests indicated that exposure to 32, 48, and 64 hr ethanol exposure yielded significantly higher ED₅₀ values in comparison to the CE/16 condition (p 's < 0.01), while CE/32, CE48, and CE64 groups did not differ from one another (Table 1).

A similar pattern of results was obtained from analysis of generalization testing data determined 16 hr following MW treatment ($F[2,22] = 113.33, p < 0.001$) and CE treatment ($F[2,22] = 58.57, p < 0.001$) (Table1). Post-hoc analyses revealed that ED₅₀ values were significantly higher for the MWx4 group compared to the MWx2 group, with both groups producing significantly higher ED₅₀ values than the CTL condition (p 's < 0.01). Similarly, both CE/32 and CE/64 groups produced significantly greater ED₅₀ values in comparison to the CTL group (p 's $< .01$), but there was no significant difference between CE/32 and CE/64 groups.

For each of the experimental conditions tested at both 16 hr and 24 hr following chronic ethanol treatment, ED₅₀ values generated at 16 hr post-withdrawal were significantly greater than those determined from generalization testing that was conducted 24 hr following chronic

ethanol exposure (Table 1). This impression was supported by analysis of MW groups ($F[1,10] = 135.57, p < 0.001$ and $F[1,10] = 42.84, p < 0.001$ for MWx2 and MWx4 groups, respectively), and CE groups ($F[1,10] = 31.70, p < 0.05$ and $F[1,10] = 5.53, p < 0.05$ for CE/32 and CE/64 groups, respectively).

Blood Ethanol Concentrations Following Chronic Ethanol Vapor Exposure. Blood ethanol levels determined immediately upon removal from the inhalation chambers did not significantly differ among MW and CE conditions that were tested at the 24 hr time point ($F[1,7] = 1.62, p > 0.14$) or the 16 hr time point ($F[1,3] = 2.11, p > 0.11$) (Table 2).

DISCUSSION

Results from this study agree with findings reported by others in mice (Middaugh et al., 2003) and rats (Emmett-Oglesby, 1990; Lytle et al., 1994) that demonstrated reduced sensitivity to the discriminative stimulus effects of ethanol after a period of chronic ethanol consumption. In these previous studies, animals were given the opportunity to consume ethanol on a voluntary basis while discrimination training was suspended. In the present study, inhalation chambers delivering ethanol vapor were used to better control the amount and timing of chronic ethanol exposure in relation to generalization testing. Total amount (duration) of ethanol exposure was systematically increased, with the pattern of chronic exposure differing (continuous vs. intermittent) prior to generalization testing. Results indicated that the magnitude of tolerance to ethanol's discriminative cue increased with increasing amount of chronic ethanol exposure (as evidenced by rightward shifts in the dose-response curves generated from generalization testing). This was true for both continuous (CE condition) and intermittent (MW condition) patterns of chronic ethanol exposure, and the tolerance effect was even more robust when generalization testing occurred earlier (16 hr vs. 24 hr) after chronic ethanol treatment. These results also corroborate our previous work indicating reduced sensitivity to ethanol's discriminative cue, with the tolerance effect dissipating when testing was conducted 48 hr following chronic (64 hr) ethanol treatment in inhalation chambers (Crissman et al., 2004).

While increasing the total amount of chronic ethanol exposure generally increased the magnitude of tolerance to ethanol's discriminative cue, the effect was influenced by the pattern in which the chronic ethanol was delivered (continuous vs. intermittent). When generalization testing was conducted 24 hr following inhalation treatment, the rightward shift in ethanol dose-response curves was greater after an intermediate amount of chronic ethanol vapor exposure (32 and 48 hr) was delivered in a continuous rather than intermittent fashion (see Figure 1 and Table 1). However, when generalization testing was conducted at 16 hr following inhalation

treatment, the rightward shift in ethanol dose-response curves was significantly greater in mice that were exposed to chronic ethanol vapor in an intermittent compared to a continuous fashion (see Figure 2 and Table 1). Thus, both total amount (duration) and the pattern of chronic ethanol exposure appear to influence subsequent sensitivity to ethanol's discriminative cue, with the relative magnitude of the tolerance effect varying as a function of when generalization testing is conducted.

It is unclear why the tolerance effect was generally greater in MW compared to CE groups at 16 hr, but the opposite relationship was sometimes observed when testing was conducted 24 hr following inhalation treatment. There is some evidence indicating that the pattern of chronic ethanol treatment can influence the development, expression, and retention of tolerance to various physiological and behavioral effects of ethanol. For example, in some cases discontinuous (intermittent or spaced) ethanol treatment resulted in more accelerated tolerance development and faster loss of tolerance in comparison to when ethanol was delivered in a more continuous (or massed) fashion (Holloway et al., 1992; Maier and Pohorecky, 1987; Pohorecky and Roberts, 1991). In a similar vein, results from this study suggest that chronic intermittent ethanol exposure in comparison to continuous exposure may produce greater tolerance to ethanol's discriminative cue when the effect is assessed at earlier time points following the chronic treatment, with the effect dissipating at a faster rate. Future studies will need to examine additional time points to more fully characterize both the time course and manner in which the pattern of chronic ethanol treatment influences tolerance to ethanol's discriminative effects.

It is possible that some degree of tolerance developed even before chronic exposure to ethanol vapor in inhalation chambers as a function of discrimination training, which involved repeated administration of the 1.5 g/kg ethanol training dose. Although we did not directly test this (comparing repeated generalization tests during the course of training), repeated generalization testing in CTL mice consistently yielded similar ethanol dose-response curves.

Further, the corresponding calculated ED₅₀ values did not significantly differ from that generated at baseline. Additionally, these ED₅₀ values are very similar to that determined in our previous work with C57BL/6J mice and a 1.5 g/kg ethanol training dose (Becker et al., 2004). It should be noted that in the present study all shifts in the ethanol dose-response functions for ethanol-exposed mice (MW and CE groups) were analyzed relative to the CTL condition, which did not differ from baseline generalization testing.

While sensitivity to the ethanol cue was blunted in both MW and CE conditions following varying amounts of ethanol vapor exposure in comparison to controls, this apparent tolerance to ethanol's discriminative cue was overcome by higher test doses of ethanol. That is, although the rightward shift in ethanol dose-response curves indicated higher doses of ethanol were required for MW and CE mice to detect the ethanol cue, accurate discrimination performance was achieved with the highest (2.5 g/kg) dose of ethanol for all groups. Analysis of response rate data indicated a general decrease as the test dose of ethanol increased and as the amount of chronic ethanol exposure increased. However, since reduced responding did not systematically vary between MW and CE conditions and all groups exhibited accurate discrimination at the highest ethanol test dose, the tolerance effect does not appear to be attributable to changes in general operant performance.

In the present study, discrimination training was suspended during the period of chronic ethanol treatment in inhalation chambers. Thus, decrements in discrimination performance exhibited by MW and CE groups might be related to this delay in testing. However, as indicated above, all groups demonstrated the ability to accurately perform the discrimination task when challenged with a sufficiently high enough dose of ethanol. This was true for the longest interval of time between testing (24 hr after 64 hr of continuous or intermittent chronic ethanol exposure). Further, generalization testing in CTL mice revealed discrimination performance that did not significantly differ from that established during baseline, suggesting that reduced sensitivity to the ethanol cue in ethanol-exposed groups could not be due simply to the

experimentally imposed hiatus in discrimination testing. Additionally, saline administration (as part of the cumulative dosing procedure) did not generalize to the ethanol cue whether testing was conducted 16 hr or 24 hr following inhalation treatment, indicating that there was no residual ethanol from chronic treatment that may have influenced (contaminated) discrimination performance. Finally, blood ethanol levels resulting from chronic ethanol treatment were similar for all groups prior to testing (Table 2). Thus, it is highly unlikely that changes in discrimination performance in MW and CE groups could be attributed to differences in level of intoxication even across increasing amounts (cycles/duration) of exposure.

There has been some controversy as to whether tolerance truly develops to the discriminative stimulus properties of drugs (Colpaert, 1999; Young, 1991). On the one hand, discrimination performance for a wide range of drugs has been noted to be very stable even over prolonged periods of training and testing. This is certainly supported by results from this study where repeated generalization testing yielded similar ethanol dose-response curves in CTL mice over a period of about six months. Further, little or no tolerance has been reported to drug discriminative cues when chronic treatment with the drug was administered concurrent with discrimination training sessions (Sannerud and Griffiths, 1993; Young et al., 1996). However, when discrimination training is suspended during chronic drug treatment, tolerance has been demonstrated for a variety of drugs, including benzodiazepines (Sannerud and Griffiths, 1993), opiates (Bespaloz et al., 1999; Walker et al., 1997; Young et al., 1996), psychomotor stimulants (Woods and Emmett-Oglesby, 1986; Young et al., 1992), and cannabinoids (Wiley et al., 1993). Results from the present study and other previous reports (Crissman et al., 2004; Emmett-Oglesby, 1990; Lytle et al., 1994; Middaugh et al., 2003) indicate that similar effects are observed for ethanol. Shifts to the right in dose-response curves have been interpreted as reflecting reduced capacity to detect the drugs' discriminative cue following chronic treatment.

A possible explanation for the demonstrated tolerance to ethanol's discriminative cue following chronic treatment with the drug may relate to the emergence of distinct interoceptive

cues associated with rebound or withdrawal from chronic ethanol exposure. These rebound or withdrawal-related cues may serve to counteract or reduce sensitivity to the discriminative stimulus effects of the drug itself. Drug rebound stimulus effects have been demonstrated for several substances, including chlordiazepoxide, amphetamine, and nicotine (Barrett et al., 2004; Barrett et al., 2001; Barrett and Smith, 2005). Likewise, distinct rebound-like cues associated with ethanol withdrawal have been demonstrated under a number of experimental conditions (Gauvin et al., 1993; Gauvin et al., 1992; Lal et al., 1988). This may be especially relevant in the present study since we have shown that withdrawal effects not only increase with amount of chronic ethanol exposure, but also become sensitized over repeated withdrawal episodes (Becker, 1999). The noted decreased response rates during generalization testing in the present study, even after saline administration, might reflect withdrawal-related disruption in operant performance. In fact, decreased responding was most evident in the MW group when generalization testing was conducted at 16 hr following chronic treatment. This corresponds to the time when tolerance to ethanol's discriminative stimulus effects was most robust. Thus, the emergence of interoceptive cues associated with ethanol withdrawal may have played a contributing role in the observed reduced sensitivity to ethanol's cue following chronic treatment with the drug.

Regardless of the mechanism underlying the observed tolerance effect, altered sensitivity to the subjective (discriminative) cues associated with ethanol following chronic treatment with the drug may have significant clinical implications. While the relationship between the discriminative stimulus properties of ethanol and its reinforcing effects are not entirely clear (Duka et al., 1999), altered perception of ethanol's subjective effects may influence motivation to continue drinking in individuals with a history of heavy consumption. In this vein, it is interesting that using the same chronic ethanol exposure model, we have reported enhanced voluntary ethanol self-administration behavior in mice following chronic intermittent ethanol exposure (Becker and Lopez, 2004; Lopez and Becker, 2005). Thus, reduced ability to detect

or perceive ethanol's intoxicating effects may lead (contribute) to increased ethanol consumption, with excessive intake ultimately resulting in full-blown relapse to uncontrollable drinking. In support of this notion, there are some clinical studies that indicate reduced capacity to detect (discriminate) internal cues associated with ethanol intoxication in individuals that are heavy drinkers (Hiltunen, 1997; Jackson et al., 2001; Schuckit and Klein, 1991). Future studies will need to further explore the potential relationship between blunted sensitivity to ethanol cues and enhanced propensity to self-administer the drug that result from a history of chronic ethanol exposure/drinking.

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FOOTNOTES

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FIGURE LEGENDS

Fig. 1: Generalization testing 24 hr after a total of 16 hr (**Panel A**), 32 hr (**Panel B**), 48 hr (**Panel C**), or 64 hr (**Panel D**) ethanol vapor exposure delivered in an intermittent (MW groups) or continuous (CE groups) fashion in comparison to air controls (CTL). Data are expressed as mean \pm s.e.m. percent ethanol-appropriate responding (top) and response rates (bottom) as a function of ethanol dose.

Fig. 2: Generalization testing 16 hr after a total of 32 hr (**Panel A**) or 64 hr (**Panel B**) ethanol vapor exposure delivered in an intermittent (MW groups) or continuous (CE groups) fashion in comparison to air controls (CTL). Data are expressed as mean \pm s.e.m. percent ethanol-appropriate responding (top) and response rates (bottom) as a function of ethanol dose.

Table 1. Mean \pm s.e.m. ED₅₀ values calculated from ethanol dose-response functions generated during generalization testing at 24 hr or 16 hr following an increasing amount of chronic ethanol exposure delivered in an intermittent (MW condition) or continuous (CE condition) manner. Repeated ED₅₀ determinations for the CTL group were collapsed for analysis. Discrimination training was suspended during chronic inhalation treatment.

Group	ED ₅₀ Values at 24 hr (g/kg)	ED ₅₀ Values at 16 hr (g/kg)
Control	0.67 \pm 0.03	0.67 \pm 0.03
MWx1	1.03 \pm 0.05 *	
MWx2	0.94 \pm 0.04 *	1.62 \pm 0.07 * #
MWx3	0.98 \pm 0.05 *	
MWx4	1.35 \pm 0.07 *	2.15 \pm 0.14 * #
CE/16	0.96 \pm 0.04 *	
CE/32	1.23 \pm 0.04 * †	1.44 \pm 0.07 * † #
CE/48	1.28 \pm 0.05 * †	
CE/64	1.36 \pm 0.07 *	1.77 \pm 0.12 * † #

Significantly differs from controls (* p < 0.01)

Significantly differs from corresponding MW group († p < 0.05)

Significantly differs from corresponding group tested at 24 hr (# p < 0.001)

Table 2. Mean \pm s.e.m. blood ethanol concentrations determined immediately upon removal from inhalation chambers for groups that received chronic ethanol vapor exposure in an intermittent (MW groups) or continuous (CE groups) manner and then tested 24 hr or 16 hr later (generalization testing). Numbers in parentheses represent group sizes. There were no significant differences in the degree of intoxication (BEC) among MW and CE groups prior to generalization testing.

Group	BEC (mg/dl) tested at 24 hr	BEC (mg/dl) tested at 16 hr
MWx1	183.3 \pm 3.4 (10)	
MWx2	179.9 \pm 6.2 (9)	194.2 \pm 6.2 (9)
MWx3	181.4 \pm 9.5 (8)	
MWx4	161.9 \pm 9.7 (9)	202.7 \pm 2.1 (10)
CE/16	174.1 \pm 8.2 (10)	
CE/32	198.4 \pm 7.4 (9)	208.3 \pm 11.6 (10)
CE/48	164.2 \pm 5.0 (10)	
CE/64	176.0 \pm 8.5 (10)	178.5 \pm 6.4 (9)

Figure 1

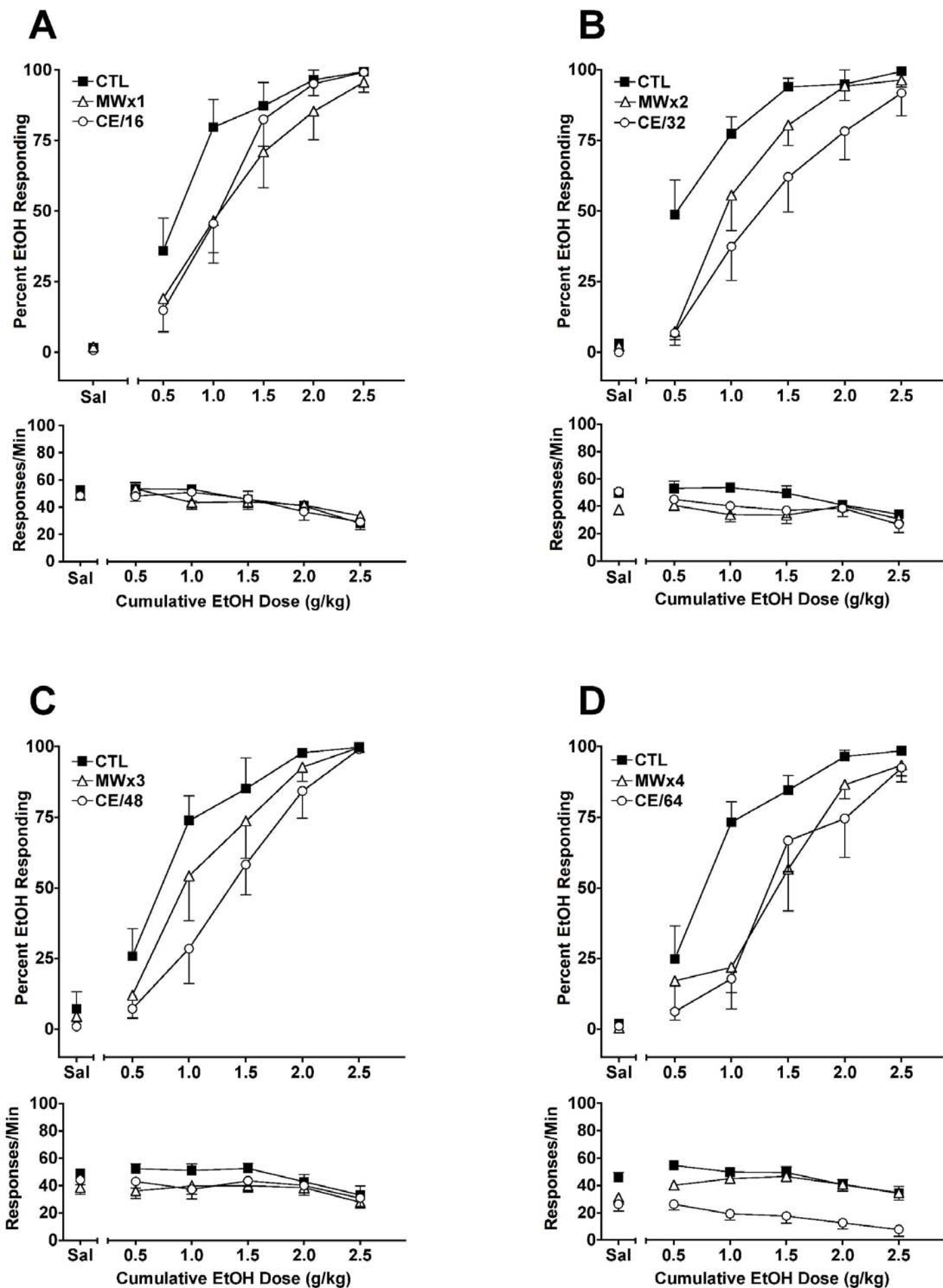


Figure 2

