Acute Effects of Acamprosate and MPEP on Ethanol Drinking-in-the-Dark in Male C57BL/6J Mice

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Background: Recently, a simple procedure in mice, Drinking-in-the-Dark (DID), was hypothesized to have value for medication development for human alcoholism. In DID, mice are offered intermittent, limited access to ethanol over a series of days during the dark phase that results in rapid drinking to intoxication in predisposed genotypes.

Methods: We measured the effects of acamprosate or MPEP, metabotropic glutamate 5 receptor (mGluR5) antagonist, on intake of 20% ethanol, plain tap water or 10% sugar water using the DID procedure in male C57BL/6J mice.

Results: Acamprosate (100, 200, 300, or 400 mg/kg) dose dependently decreased ethanol drinking with 300 mg/kg reducing ethanol intake by approximately 20% without affecting intake of plain water or 10% sugar water. MPEP (1, 3, 5, 10, 20, or 40 mg/kg) was more potent than acamprosate with 20 mg/kg reducing ethanol intake by approximately 20% and for longer duration without affecting intake of plain water or 10% sugar water.

Conclusions: These results support the hypothesis that mGluR5 signaling plays a role in excessive ethanol intake in DID and suggest DID may have value for screening novel compounds that reduce overactive glutamate signaling for potential pharmaceutical treatment of excessive ethanol drinking behavior.

Key Words: Alcoholism, Acamprosate, MPEP, Ethanol, C57BL/6J, Drinking, Glutamate, Metabotropic glutamate receptor 5, mGluR5.

ALCOHOLISM IS A complex disorder (e.g., heterogeneous among individuals, polygenic, gene by gene, gene by environment, and environment by environment interactions) with devastating costs to society (Hines et al., 2005). Although several medications are prescribed including acamprosate, naltrexone, and disulfiram, these are only marginally effective in some individuals (Egli, 2005; Heilig and Egli, 2006). Hence, there is a strong need for better medications and also more medications tailored for individual differences. Animal models are useful to screen potential new medications and to identify etiology. Many animal models are available. No single model can capture all features of alcoholism. However, certain features of the human disorder can be represented in a model.

One characteristic feature of human alcoholism is repeated excessive ethanol consumption to the point of intoxication. Unfortunately, this has been difficult to demonstrate in any animal model. For example, although it is long known that C57BL/6J mouse genotype drinks 10 to 15 g/kg ethanol per day in the 24-hour 2-bottle test (McClearn and Rodgers, 1959), the ethanol is taken sporadically over the 24 hour period, and animals rarely reach a high enough level of alcohol in their blood at any given time to become intoxicated as measured by motor impairment (Dole and Gentry, 1984). Recently, an alternate version was described where ethanol is only offered for a short time during the early phase of the dark period (Rhodes et al., 2005). This method has the advantage that mice of C57BL/6J genotype reliably drink to behavioral intoxication (i.e., motor impairment) and reach blood-ethanol levels above 1 mg/ml at predictable time periods when effects of medications can be measured (Kamdar et al., 2007; Rhodes et al., 2007). The procedure has since been dubbed drinking-in-the-dark (DID), and is now being used by a growing number of investigators (Kamdar et al., 2007; Moore et al., 2007; Ryabinin et al., 2008; Sparta et al., 2008).

Note that as a model for only some aspects of human alcoholism, DID has features that are clearly different from the human condition. For example, in DID, mice are given limited access to ethanol, whereas humans control the availability of their alcohol. On the other hand, as shown in Rhodes et al. (2007), DID behavior is genetically correlated with many other ethanol-related behavioral traits. Moreover, recent studies suggest that the model may be useful for medication development. For example, naltrexone reduces excessive ethanol drinking in DID at doses that have no effect on intake of alternative fluids, including those that are naturally rewarding such as plain water or sugar water (Kamdar et al.,

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2007). Other investigations using the DID method have established that signaling at corticotrophin releasing factor receptor 1 (CRF1) (Sparta et al., 2008) and GABA receptors (Moore et al., 2007) also play a role in excessive ethanol intake in DID using the C57BL/6J genotype. We propose DID is not "better" than any other model but rather that it may be useful for medication development because it shows the predicted responses to medications moderately effective for ameliorating human alcoholism (i.e., predictive validity) and it is simple, hence conducive for screening new compounds for potential efficacy (Kamdar et al., 2007).

One active area in medication development (besides opioid, CRF1, and GABA mentioned above) is pharmacological blockade of glutamate signaling. Specifically, overactive glutamate signaling is hypothesized to contribute to ethanol withdrawal and relapse, and relapse is considered one of the major obstacles in treatment of alcohol addiction (Koob, 2003). Acamprosate, which was recently approved by the United States Food and Drug Administration, is a medication that is hypothesized to act, in part, by blocking NMDA and/or metabotropic glutamate receptors (Harris et al., 2003; Rammes et al., 2001). Current consensus is that acamprosate has moderate therapeutic benefit in reducing relapse in some individuals and medications are now being developed based on these hypothesized mechanisms (De Witte et al., 2005; Littleton and Zieglgansberger, 2003).

Previous studies using rats have established efficacy for acamprosate and MPEP (metabotropic glutamate receptor 5 antagonist) in animal models of alcohol withdrawal (Schroeder et al., 2005; Spanagel et al., 1996), relapse (Bachteler et al., 2005; Backstrom et al., 2004; Cole et al., 2000; Quertemont et al., 2002) and reinforcement (Besheer et al., 2008), and new data in mice support the hypothesis that part of the behavioral effects of acamprosate is via blockade of mGluR5 (Blednov and Adron Harris, 2008).

Data on effects of acamprosate or MPEP on simpler models of ethanol drinking behavior under free choice or limited access conditions are less available. The predictions for these models are also less clear. On the one hand, because relatively low levels of ethanol are experienced during free choice drinking even in predisposed genotypes, it is not clear whether ethanol ever achieves a high enough concentration in the brain to cause neuroadaptations related to overactive glutamate signaling, withdrawal or relapse. On the other hand, it has been demonstrated that in these models animals can experience blood ethanol levels approaching 1 mg/ml for brief periods (Dole and Gentry, 1984), and when that is repeated it might be sufficient. This is supported by data suggesting that MPEP reduces ethanol consumption in a 4 bottle choice situation in male C57BL/6J mice (with the 4 choices being plain water, 3%, 6% or 12% ethanol, Lominac et al., 2006) and in a 2-bottle choice paradigm in mice that were backcrossed onto C57BL/6J (Olive et al., 2005). The goals of this study were to test the value of DID for screening potential novel medications based on hypothesized mechanisms of action of acamprosate, and to determine whether signaling at metabotropic glutamate 5 receptors (mGluR5) is required for excessive ethanol intake in DID.

METHODS

Animals

Male C57BL/6J mice (n = 192 total) were purchased from the Jackson Laboratory (Bar Harbor, ME). This inbred strain was chosen for their known high levels of ethanol consumption. Animals arrived at the Beckman Institute Animal facility at 5 weeks of age and were acclimated (remained undisturbed) for 18 days prior to testing. During the acclimation period, mice were housed 4 per cage for the first 11 days and then were transferred to individual cages, where they remained for the duration of the study. Animals were housed in standard polycarbonate shoebox cages with Bed-o-Cob™ bedding. Rooms were controlled for temperature (21 ± 1°C) and photo-period (12:12 L:D). A reverse light/dark cycle was used in which lights turned on at 2200 hour and off at 1000 hour Central Standard Time. Red incandescent lamps were kept on continuously so that investigators could handle mice during the dark phase. Food (Harlan Teklad 7012) and water were provided ad libitum, except when ethanol was substituted for water for 2 or 4 hours as described below. The Beckman Institute Animal Facility is AAALAC approved. All procedures were approved by the University of Illinois Institutional Animal Care and Use Committee and adhered to National Institutes of Health guidelines.

Drugs and Drinking Solutions

The 20% ethanol drinking solution was prepared from 200 proof absolute anhydrous ethanol (Pharmco-Aaper brand, Brookfield, CT) diluted to 20% (v/v) using tap water. The 10% sugar water drinking solution was prepared from sucrose (Sigma Aldrich, St. Louis, MO) dissolved in tap water at 10% (w/v) concentration. Acamprosate (generously provided by Dr. Robert Messing, Ernest Gallo Clinic and Research Center, University of California, San Francisco), and 6-Methyl-2-(phenylethynyl) pyridine (MPEP) (Sigma Aldrich, St. Louis, MO) were dissolved in 0.9% saline and were administered via intraperitoneal (IP) injections in a volume of 10 ml/kg. Acamprosate was administered at doses 100, 200, 300, or 400 mg/kg, and MPEP at 1, 3, 5, 10, 20, or 40 mg/kg based on the literature (Busse et al., 2004; Chester et al., 2001; Cole et al., 2000; Escher and Mittleman, 2006; Hodge et al., 2006; Kim et al., 2004; Lominac et al., 2006; McGeehan and Olive, 2003; Olive et al., 2005; Spooren et al., 2002).

Drinking-in-the-Dark Procedure

The original description of the Drinking in the Dark procedure used 4 consecutive days of alcohol presentations (Rhodes et al., 2005). This was modified for examination of drug effects in order to implement a within subjects design where a number of doses of a drug can be examined within the same individual within a short amount of time (Kamdar et al., 2007). Following Kamdar et al. (2007), starting 3 hours after lights shut off, the water bottles were replaced with 10 ml graduated cylinders fitted with double ball bearing sipper tubes (to prevent leakage) containing either 20% ethanol, plain tap water, or 10% sugar water (see above). This was done in the home cages where animals were singly housed. The cylinders remained in place for 2 hours for experiments 1 to 4, and 4 hours for experiments 5 to 8 (see Table 1). Duration of ethanol exposure was lengthened for MPEP experiments 5 to 8 because preliminary data (not shown) demonstrated MPEP reduced ethanol intake up to 4 hours after administration whereas effects of acamprosate waned after 2 hours. Intakes were recorded every 15 minutes for experiments 1 to 4 and every 30 minutes for experiments 5 to 8. After the 2- or 4-hour periods the cylinders were replaced with water bottles. This procedure was repeated on day 2 except that animals were given

Table 1. List of Experiments

Experiment	*n*	Drug	Doses (mg/kg)	Drinking solution
1	24	Acamprosate	Saline, 100, 200, 400	20% ethanol
2_a	24	Acamprosate	Saline, 100, 200, 400	Water
2_b		Acamprosate	Saline, 100, 200, 300	Water
3_a	24	Acamprosate	Saline, 100, 200, 300	10% sucrose
3_b		Acamprosate	Saline, 100, 200, 300	Water
3_c		Acamprosate	Saline, 100, 200, 300	20% ethanol
4_a	24	MPEP	Saline, 1, 3, 10	20% ethanol
4_b		MPEP	Saline, 1, 3, 10	Water
5_a	24	MPEP	Saline, 10, 20, 40	20% ethanol
5_b		MPEP	Saline, 1, 3, 5	20% ethanol
6_a	24	MPEP	Saline, 1, 3, 5	20% ethanol
6_b		MPEP	Saline, 10, 20, 40	20% ethanol
7_a	24	MPEP	Saline, 1, 3, 5	Water
7_b		MPEP	Saline, 1, 3, 5	10% sucrose
8_a	24	MPEP	Saline, 10, 20, 40	Water
8_b		MPEP	Saline, 10, 20, 40	10% sucrose

an intraperitoneal (ip) injection of saline, acamprosate or MPEP (see below) immediately before their water bottles were replaced with the cylinders. Similar to Hodge et al. (2006) and Olive et al. (2005) but unlike other previous studies with MPEP (Lominac et al., 2006; Schroeder et al., 2005), no pretreatment interval was used between ip injection and presentation of the drinking solutions.

Experimental Design

In each experiment listed in Table 1, each individual mouse received saline and 3 different doses of acamprosate or MPEP before receiving ethanol, plain tap water or sugar water as described above. This was implemented by repeating a 2-day version of the DID procedure in the same animals twice a week (Monday-Tuesday, and Thursday-Friday, with Wednesday off) for 2 weeks (with the weekend off). Thus, before an animal received an injection they always had 1 day of access to a drinking solution without injections, and 1 or 2 days where they were left undisturbed (See Fig. 1). The rationale for leaving the animals undisturbed was to separate episodes of the 2-day cycle and to reduce the chance for carry over effects of the injection for following presentations of drinking solutions. Each experiment consisted of 24 animals and the order in which the 4 injections were administered was permuted such that each of the 24 animals received the injections in a different order. This was done so that order of injections would not need to be considered in the statistical analysis.

In experiments 2 to 8, the entire 2 week procedure described above (and shown in Fig. 1) was repeated in the same animals using a different drinking solution. These repeated tests are indicated by subscripts b and c in Table 1, representing second and third tests, respectively, in the same 24 animals.

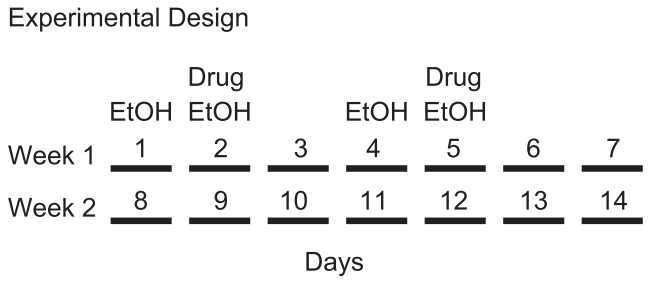

Fig. 1. A schematic diagram of the experimental design. "EtOH" indicates days when water bottles were replaced with a drinking solution for 2 or 4 hours (either 20% ethanol, plain water or 10% sugar water). "Drug" indicate days when injections of acamprosate or MPEP were given. Each animal received 3 doses plus saline in counterbalanced order over the 2 weeks, on days 2, 5, 9, and 12. These injections occurred immediately before replacing the water bottles with the drinking solutions. Each of these injection days was preceded with a day when animals were offered the drinking solutions without any injections, days 1, 4, 8 and 11. All other days, animals remained undisturbed without a drinking solution besides their normal water bottle.

Statistical Analysis

Data were analyzed using a repeated measures analysis of variance with dose as the within-subjects factor using SAS (Release 8.01) Proc Mixed. Tukey post hoc tests were used to determine which doses yielded significantly different responses from each other. A *p*-value less than 0.05 was considered significant.

RESULTS

Experiments 1 to 3: Acamprosate

Under baseline conditions (average of the first days of the 2-day cycles; see methods), in a 2 hour period during the onset of the dark phase of the light dark cycle, animals drank an average of 0.44 ml (±0.02 SE) of 20% ethanol, 0.53 ml (±0.04) plain tap water and 1.46 ml (±0.10) 10% sugar water (see Fig. 2). Under the intermittent conditions when it was available, C57BL/6J showed a significant increase in ethanol consumed as the days progressed (Fig. 2*A*; $F_{3,68} = 17.4$, $p < 0.0001$; all Tukey posthoc pairwise comparisons significant, $p < 0.05$, except day 1 vs. 4 and 4 vs. 8). Changes over days for plain water were not significant (Fig. 2*A*). Sugar water intake increased after the first day, and thereafter was maintained at a plateau (Fig. 2*A*; $F_{3,68} = 3.8$, $p = 0.01$; only Tukey comparisons with day 1 were significant).

Acamprosate reduced ethanol intake in a dose-dependent manner (Fig. 2*B*; $F_{3,69} = 12.5$, $p < 0.0001$; all Tukey post hoc comparisons significant, $p < 0.05$, except saline versus 100 mg/kg and 100 vs. 200 mg/kg) with 300 mg/kg producing approximately 20% reduction in intake. Acamprosate did not reduce intake of plain tap water except at highest dose,

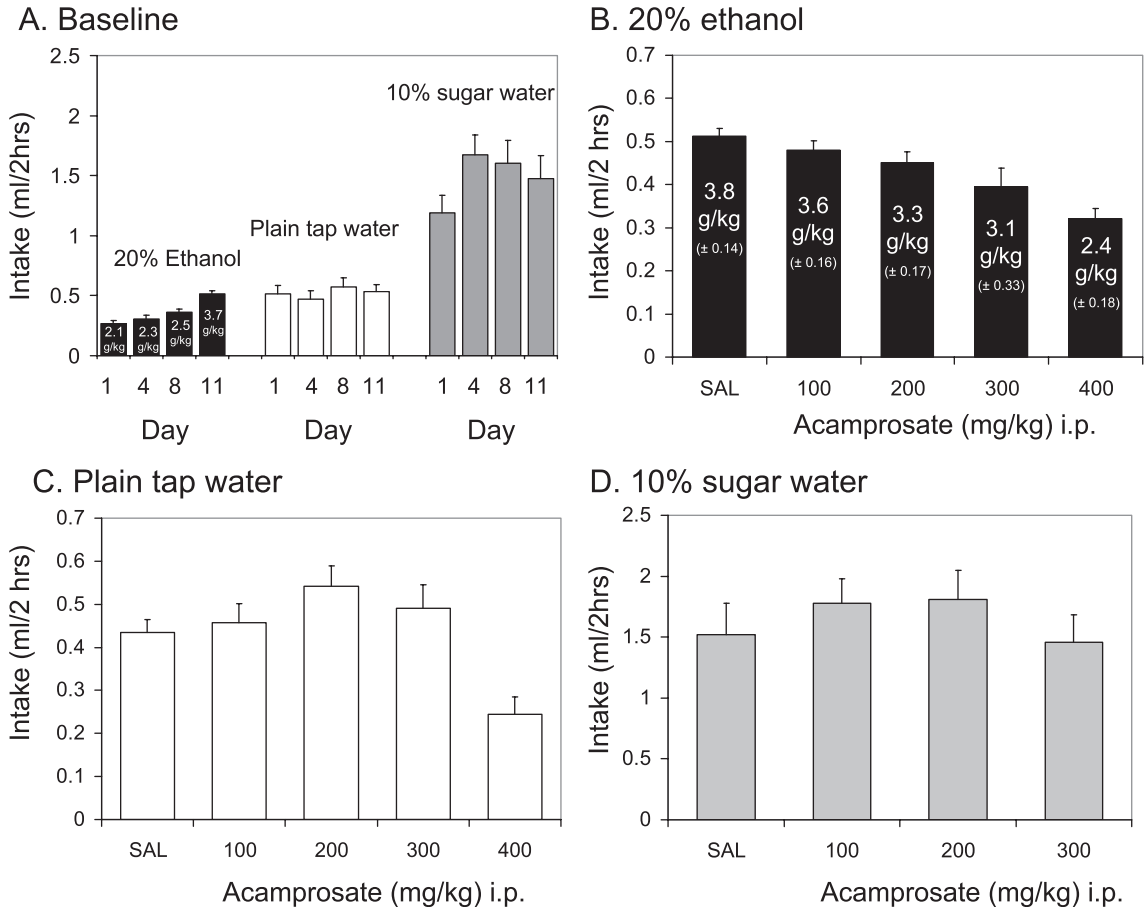

Fig. 2. Effects of acamprosate on intake of 20% ethanol, plain tap water or 10% sugar water in C57BL/6J mice using the 2-hour DID procedure. (**A**) Baseline intake of the 3 fluids on days when animals did not receive injections. (**B**) Acamprosate dose-dependently reduced ethanol intake with 300 mg/kg (i.p.) reducing intake by approximately 20%. (**C**) Acamprosate had no effect on plain water intake except at the highest dose (400 mg/kg) and (**D**) no effect on sugar water intake up to 300 mg/kg. Standard errors shown.

400 mg/kg (Fig. 2*C*; $F_{3,183} = 4.6$, $p = 0.001$; only Tukey comparisons with 400 mg/kg were significant). Doses lower than 400 mg/kg showed trends for increased intake of water. Acamprosate did not affect intake of 10% sugar water up to 300 mg/kg (Fig. 2*D*). Doses less than 200 mg/kg showed trends for increasing sugar water intake.

Experiments 4 to 8: MPEP

Under baseline conditions (the first days of the 2-day exposures), over a 4 hour period, animals drank an average of 0.99 ml (±0.04 SE) of 20% ethanol, 1.1 ml (±0.07) plain tap water and 2.8 ml (±0.20) 10% sugar water (see Fig. 3). Ethanol consumption increased on the last day of intermittent exposure as compared to previous days (Fig. 3*A*; $F_{3,69} = 5.2$, $p = 0.003$; only Tukey comparisons with day 11 were significant). Changes over days for plain water were not significant (Fig. 3*A*). Sugar water intake increased after the first day, and thereafter maintained at a plateau (Fig. 3*A*; $F_{3,63} = 5.0$ $p = 0.004$; only Tukey comparisons with day 1 were significant).

MPEP (1, 3, 5, 10, 20, or 40 mg/kg) reduced ethanol intake in a dose-dependent manner (Fig. 3*B*). This was observed in both the 2 hour period following injection ($F_{6,295} = 8.3$, $p < 0.0001$) and the 4 hour period ($F_{6,276} = 8.3$, $p < 0.0001$). Tukey posthoc analysis indicated that ethanol intake within the 4-hour period was lower after 20 mg/kg or 40 mg/kg as compared to saline, and that 40 mg/kg was different from all other doses ($p < 0.05$). Within the 2-hour period, ethanol intake was significantly reduced as compared to saline at doses 10 mg/kg or higher. MPEP had no effect on intake of plain water or sugar water except for slightly reducing intake of plain water at the highest dose (40 mg/kg) but only for the 2 hour time-point ($F_{6,137} = 5.0$, $p < 0.0001$; posthoc tests indicated 40 mg/kg differed from all others) not after 4 hours ($F_{6,137} = 0.8$, $p = 0.57$) (Fig. 3*C* and *D*).

DISCUSSION

Acamprosate and MPEP are hypothesized to reduce probability for relapse by ameliorating overactive gluatamate signaling associated with ethanol withdrawal (Littleton and Zieglgansberger, 2003; Lominac et al., 2006). The main finding of this study is that a simple procedure, Drinking-in-the-Dark, which previously was established to involve opioid reward (Kamdar et al., 2007) and CRF1 signaling

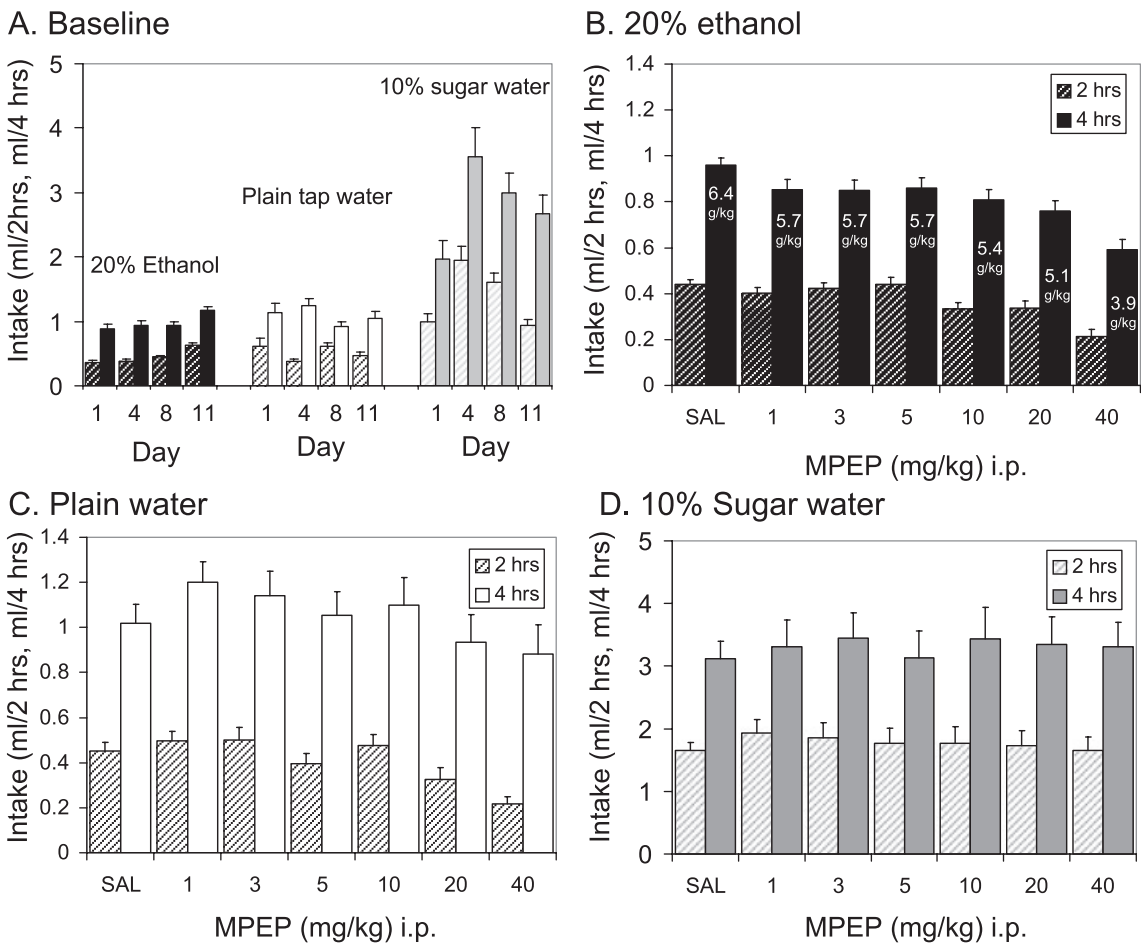

Fig. 3. Effects of MPEP on intake of the alternative fluids using the 4-hour DID procedure. Average intake over 2 and 4 hours is shown as adjacent bars throughout. (**A**) Baseline intake of the 3 fluids on days when animals did not receive injections. (**B**) MPEP reduced ethanol intake in a dose-dependent manner with 10 or 20 mg⁄kg reducing intake by approximately 20% over 2 or 4 hours, respectively. (**C**) MPEP had no effect on plain water intake except at the highest dose (40 mg⁄kg) and only for the 2 hour time-point. (**D**) No effect on sugar water intake was observed. Standard errors shown.

mechanisms (Sparta et al., 2008), may also be useful for screening novel medications based on hypothesized mechanisms of acamprosate (i.e., glutamate receptor antagonists) (Littleton and Zieglgansberger, 2003).

Drinking-in-the-Dark

Drinking-in-the-Dark represents a simple procedure for rapidly inducing high ethanol intake in genetically predisposed mice (Rhodes et al., 2007). Previous studies have established that naltrexone reduces ethanol drinking in DID without affecting intake of plain water or sugar water supporting the hypothesis that opioid signaling associated with ethanol reward contributes to excessive ethanol intake in this model (Kamdar et al., 2007). Current strategies for medication development are aimed at blocking relapse (Littleton and Zieglgansberger, 2003), and whereas some individuals may maintain complete abstinence with naltrexone (O'Brien et al., 1996; Rosner et al., 2008), possibly related to blunted opioid signaling elicited from drug-paired contextual cues (Bechtholt and Cunningham, 2005), the traditional hypothesized mechanism requires that ethanol is consumed otherwise there is nothing to antagonize (Littleton and Zieglgansberger, 2003). This is consistent with the notion that naltrexone may prevent a "slip" (a priming dose) into becoming a full blown relapse but is unlikely to prevent relapse from stress or cue-induced craving (Rosner et al., 2008).

Growing evidence suggests that ethanol reward plays a role in DID, but it is not known whether other features relevant for alcoholism such as stress, craving or withdrawal contribute to DID behavior. Ethanol is provided for brief episodes and animals repeatedly experience intoxicating levels of ethanol along with repeated periods when ethanol is withheld (Kamdar et al., 2007; Rhodes et al., 2007). Moreover, the escalating intakes shown in Figs 2*A* and 3*A* suggest tolerance or withdrawal may contribute, but alternatives such as acclimation or sensitization to the taste are also possible. It is notable that escalating intakes are not always observed such as when ethanol is offered daily (Rhodes et al., 2005) as opposed the procedure used here where ethanol is withheld on alternate days. This may be analogous to the alcohol deprivation effect established in rats (Heyser et al., 2003; Koob, 2000).

Note that the escalating intakes were for the first day of the repeated 2-day cycles (when animals were not given injections; see Fig. 1). The medication effects were measured on day 2 when intake levels stabilized. This was indicated by the saline data which showed no differences among days (data not shown). Moreover, the saline intake levels were comparable to previous studies that established stable levels of drinking after the first day of DID (Kamdar et al., 2007; Rhodes et al., 2005, 2007).

Acamprosate

Although the mechanisms of action of acamprosate are not known, it has antagonist properties at mGluR5 and NMDA receptors and these are implicated in its antirelapse effects (Blednov and Adron Harris, 2008; Harris et al., 2002). The idea is that overactive glutamate signaling occurs as a neuroadaptation during ethanol exposure and persists for some time after ethanol is withheld contributing to withdrawal and/or craving (Littleton and Zieglgansberger, 2003). Novel medications, such as MPEP (mGluR5 antagonist) and iminoguanidine JR 220 (NMDA antagonist with novel site of action), are being developed based on these hypothesized mechanisms (personal communication with John Littleton, University of Kentucky). Hence, the results of this study are important because they suggest that the DID model, simple as it is, can be used to screen potential novel compounds designed based on hypothesized mechanisms of action of acamprosate.

mGluR5

The literature in rats shows that MPEP reduces operant self-administration of ethanol (Schroeder et al., 2005), reduces reinstatement of operant responding by drug-associated cues, and reduces the alcohol deprivation effect (Backstrom et al., 2004). Moreover, MPEP reduces the discriminative stimulus effects of ethanol in rats, providing direct evidence that it can substitute for ethanol (Besheer et al., 2006). In C57BL/6J mice, MPEP dose dependently reduced operant responding for ethanol but not plain water (Hodge et al., 2006; Lominac et al., 2006).

Fewer studies are available for simpler models of free choice drinking. A study in "wildtype" mice backcrossed onto C57BL/6J found that 10 mg/kg or 20 mg/kg reduced ethanol intake and preference in a 16 hour 2-bottle choice situation where animals were fluid restricted prior to alcohol presentation for 8 h/d (Olive et al., 2005). In another study, MPEP reduced intake and preference for ethanol in a 4 bottle free choice situation in male C57BL/6J mice without fluid restriction (Lominac et al., 2006). In this study 5 consecutive days of ip injections of a single dose of MPEP (10 mg/kg) reduced ethanol intake for up to 6 days. However, such long-lasting effects were not observed here. In our design, animals experienced repeated injections of MPEP, but these were separated by days when ethanol was offered without any injections (the first day of the 2-day cycles) and 1 or 2 days when animals remained undisturbed between cycles (without ethanol; see Fig. 1). The minimum duration between the previous MPEP injection and measure of baseline intake in the following cycle was 48 hours. By this time, ethanol intake levels either returned to baseline levels or increased (rather than decreased; see Figs 2*A* and 3*A*). Hence, it appears that MPEP effects on ethanol intake in this study waned within 48 hours.

Conclusions and Future Directions

We conclude that DID models features of human alcoholism that are relevant for medication development including alterations in the glutamate neurotransmitter system hypothesized to underlie withdrawal or relapse. Development of the DID model would benefit from exploration of possible roles of dependence, withdrawal, stress, anxiety, or conditioned cues. For example, one possibility is to test whether animals display conditioned place preference for contexts paired with DID (Zombeck et al., 2008). Another is to measure animals on a variety of behavioral tasks related to anxiety such as elevated plus maze, open field, zero maze, light/dark box on days when ethanol is withheld after DID (Kliethermes et al., 2004). To examine the possible role of stress, corticosterone levels could be measured in the blood across the circadian rhythm, throughout the entire DID procedure, including off days. Such data would help establish which features of the complex traits (e.g., alcoholism, excessive ethanol intake, stress, craving) are represented in the simple DID model. Furthermore, it would provide rationale for using DID to screen specific classes of compounds (e.g., those modulating glutamate, GABA, or CRH signaling systems).

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