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ORIGINAL INVESTIGATION

Acute effects of Naltrexone and GBR 12909 on ethanol drinking-in-the-dark in C57BL/6J mice

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Abstract
Rationale Recently, a simple procedure was described, drinking in the dark (DID), in which C57BL/6J mice self-administer ethanol to the point of intoxication. The test consists of replacing the water with 20% ethanol in the home cage for 2 or 4 h early during the dark phase of the light/dark cycle.
Objectives To determine whether the model displays predictive validity with naltrexone, and whether opioid or dopaminergic mechanisms mediate excessive drinking in the model.
Materials and methods Naltrexone or GBR 12909 were administered via intraperitoneal injections immediately before offering ethanol solutions, plain tap water, or 10% sugar water to male C57BL/6J mice, and consumption was monitored over a 2- or 4-h period using the DID procedure.
Results Naltrexone (0.5, 1, or 2 mg/kg) dose dependently decreased ethanol drinking but these same doses had no significant effect on the consumption of plain water or 10% sugar water. GBR 12909 (5, 10, and 20 mg/kg) dose dependently reduced the consumption of ethanol and sugar water but had no effect on plain water drinking.
Conclusions The DID model demonstrates predictive validity. Both opioid and dopamine signaling are involved in ethanol drinking to intoxication. Different physiological pathways mediate high ethanol drinking as compared to water or sugar water drinking in DID. DID may be a useful screening tool to find new alcoholism medications and to discover genetic and neurobiological mechanisms relevant to the human disorder.

Keywords Alcoholism · Naltrexone · GBR 12909 · Ethanol · C57BL/6J · Drinking · Natural reward · Water · Opioid · Dopamine

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Introduction

Alcoholism is a devastating disorder with enormous economic and medical costs to society. Very few *pharmaceutical* treatments are available. Animal models are useful to screen new compounds for potential efficacy and to explore etiology *of excessive ethanol drinking*. No animal model can completely reproduce all characteristics of alcoholism, but partial models have been developed to study features of alcoholism such as excessive ethanol-drinking behavior (Egli 2005; Finn et al. 2005; Rhodes et al. 2007).

Many procedures have been developed to study ethanol drinking in rodents, but it has been difficult to find a procedure in which mice or rats drink to the point of intoxication (Dole and Gentry 1984). Recently, a simple procedure was described, which we call drinking in the dark (DID), in which a majority of individuals within the genetically predisposed mouse strain C57BL/6J drink to the point of behavioral intoxication as indicated by motor impairment on the balance beam and rotarod (Rhodes et al. 2007). In brief, the water bottle is replaced with a bottle containing 20% ethanol for 2 or 4 h in the home cage starting 3 h after lights shut off. A comparison of 12 inbred strains showed that the quantity of ethanol intake (g/kg) in

this period is similar between single-bottle and 2-bottle choice, although the single bottle produces higher blood-ethanol levels. We have argued that the DID model may be useful to study genetic and neurobiological mechanisms underlying excessive ethanol drinking, possibly analogous to binge drinking (Rhodes et al. 2007).

One way of evaluating DID as a model to explore features relevant to alcoholism is to determine whether the model displays predictive validity (e.g., whether medications currently used to treat alcoholism reduce ethanol drinking in the model; Egli 2005). There is no gold standard for treating alcoholism, but one medication that has proven useful is naltrexone, a nonspecific, competitive antagonist of opioid receptors (Kiefer et al. 2003; Volpicelli et al. 1992). It has been argued that naltrexone is more effective than some other pharmaceutical agents such as disulfiram (Fuller et al. 1986) or fluoxetine (Egli 2005; Kranzler et al. 1995), although alternate views have been expressed (Brewer 1995; De Sousa 2004). Naltrexone has proven effective at reducing ethanol consumption in many different animal models including nonhuman primates (Boyle et al. 1998; Kornet et al. 1991), rats (Coonfield et al. 2002, 2004; Davidson and Amit 1997; Goodwin et al. 2001; Parkes and Sinclair 2000; Sharpe and Samson 2001), and mice (Fachin-Scheit et al. 2006), including several models using the C57BL/6 mouse genotype (Le et al. 1993; Middaugh and Bandy 2000; Middaugh et al. 2000, 2003; Phillips et al. 1997).

One pharmacological action of ethanol is to increase opioid signaling (Froehlich and Li 1994). It has been hypothesized that this activates the dopamine reward pathway by inhibiting GABAergic cells in the ventral midbrain that normally hold the dopamine system under inhibitory control (i.e., disinhibition of the dopamine reward system; Froehlich 1996). Consistent with this idea is that ethanol increases extracellular levels of dopamine in the nucleus accumbens of rats (Melendez et al. 2002) and C57BL/6J mice (Middaugh et al. 2003). It has been suggested that naltrexone may reduce high ethanol drinking by blocking ethanol-induced increases in dopamine signaling thereby diminishing ethanol reward (Froehlich 1996).

It is interesting that rats that are selectively bred for high ethanol preference display lower levels of dopamine D2 receptors in limbic areas of the brain as compared to unselected rats (McBride et al. 1993; but see McBride et al. 1997; Stefanini et al. 1992). The C57BL/6J strain displays fewer dopamine D2 receptors in the hippocampus and hypothalamus as compared to DBA/2J, a strain that is known to avoid ethanol (Ng et al. 1994). Moreover, dopamine receptor agonists decrease ethanol consumption in C57BL/6J (Ng and George 1994). Taken together, these results suggest that reduced dopamine function may contribute to the high ethanol drinking in C57BL/6J, and that drugs that result in increased dopamine signaling can reduce drinking in this strain.

Dopamine signaling can be increased pharmacologically in a variety of ways (e.g., dopamine agonists, monoamine oxidase inhibitors, reuptake blockers, reversal of the reuptake transporter). Each of these methods has advantages and disadvantages related to specificity and efficacy. One drug that has received relatively little attention with regard to reducing ethanol consumption is the dopamine reuptake blocker GBR 12909. The advantage of GBR 12909 is that it blocks the dopamine transporter protein with extremely high affinity and specificity, and hence, can specifically elevate dopamine signaling with diminished direct effects on other signaling systems such as serotonin or norepinephrine (Andersen 1989). It was recently tested for safety in treatment of cocaine addiction in a phase 1 clinical trial but the trial was aborted because the 75 mg dose showed some evidence of adverse cardiovascular effects (John Grabowski, University of Texas, Houston, and Roberta Kahn, National Institute on Drug Abuse, personal communication). Very few studies have examined whether GBR 12909 is efficacious in reducing ethanol drinking in any animal or human model.

The aims of this study were to (1) determine whether naltrexone reduces ethanol drinking in the DID model to evaluate the model for predictive validity, (2) test the hypothesis that opioid function is necessary for the drinking behavior in the DID model, (3) determine the acute effect of GBR 12909 on ethanol intake in DID to test the hypothesis that the behavior is mediated, in part, by dopamine signaling, and (4) determine the specificity of the effect of naltrexone and GBR 12909 on ethanol intake as compared to intake of plain tap water or sugar water.

Based on a large body of evidence suggesting that naltrexone reduces excessive drinking in animal models, we hypothesized that naltrexone would reduce ethanol drinking in the DID model. Because of the paucity of data on effects of GBR 12909 on ethanol drinking, we were less certain about the outcome of this experiment, but based on the hypothesis that reduced dopamine signaling contributes to excessive drinking in C57BL/6J, we predicted that GBR 12909 would reduce ethanol drinking (by increasing dopamine signaling). We hypothesized that these effects would be specific to ethanol (i.e., that naltrexone or GBR 12909 would not affect intake of plain tap water or sugar water) because we hypothesized that naltrexone and GBR 12909, at the doses administered, would reverse mechanisms associated with excessive drug-taking behavior but not interfere with mechanisms regulating "natural" motivated behaviors. It is well established that the pathways involved in drug motivation overlap extensively with those mediating natural drives (Kelley and Berridge 2002; Wise 2002), but key differences probably underlie pathological,

excessive drug-taking behavior, and these differences are the likely targets of useful medications (Baunez et al. 2005; Carelli et al. 2000; Carelli and Wondolowski 2003).

Materials and methods

Animals

Different animals were used for each experiment except where indicated. Male C57BL/6J mice were purchased from the Jackson Laboratory (Bar Harbor, ME). This inbred strain was chosen for their known high levels of ethanol consumption (Belknap et al. 1993; McClearn and Rodgers 1959). Animals arrived at the Beckman Institute Animal facility at 5 weeks of age and were acclimated for 18 days before testing. During the acclimation period, mice were housed four per cage for the first 11 days and then were transferred to individual cages for the remaining 7 days. Animals were housed in standard polycarbonate shoebox cages with Bed-o-Cob™ bedding. Rooms were controlled for temperature (21±1°C) and photoperiod (12:12 L:D). A reverse light/dark cycle was used in which lights turned on at 22:00 hours and off at 10:00 hours 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 h as described below. The Beckman Institute Animal Facility is Association for Assessment and Accreditation of Laboratory Animal Care 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 ethanol-drinking solutions were prepared from 200 proof absolute anhydrous ethanol (Pharmco-Aaper brand, Brookfield, CT) diluted to 10, 20, or 30% (v/v) using tap water. The 10% sugar-water drinking solution was prepared from food-grade plain white sugar (Meijer brand, Champaign, IL) dissolved in tap water at 10% (w/v) concentration. Food-grade sugar was used instead of sucrose or saccharin because the goal was to offer a palatable natural reward, not to separate taste from caloric value or isolate the contribution of purified sucrose. Naltrexone hydrochloride and GBR 12909 dihydrochloride (Sigma-Aldrich, St. Louis, MO) were dissolved in 0.9% saline and were administered via intraperitoneal (i.p.) injections in a volume of 0.005 ml/g. Naltrexone was administered at doses 0.5, 1, 2, 4, 8, and 16 mg/kg, and GBR 12909 at 5, 10, and 20 mg/kg. Doses of naltrexone were chosen based on the rodent ethanol drinking literature (e.g., Middaugh and Bandy 2000; Phillips et al. 1997) and on studies conducted by Rhodes on effects of naltrexone and GBR 12909 on wheel-running behavior (Li et al. 2004; Rhodes et al. 2001). They were prepared based on the salt, not the base form. GBR 12909 was heated slightly to dissolve into saline and then was filtered through a 0.22 μm Millipore syringe filter (Billerica, MA) to remove any debris that accumulated during the dissolving process following Rhodes et al. (2001).

DID procedure

A 2-day version of the procedure was used following Rhodes et al. (2007). In brief, starting 3 h 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 10, 20, or 30% 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 h. Intakes were recorded every 15 min for experiments 1–3 and after the 2-h period for experiments 4–10. After the 2-h period, the cylinders were replaced with water bottles. This procedure was repeated on day 2 except that animals were given an i.p. injection of saline, naltrexone, or GBR 12909 (see below for a description of the experimental design) immediately before their water bottles were replaced with the cylinders.

Experimental design

A within subjects design was used. In each experiment (described below), each individual mouse received saline and three different doses of naltrexone or GBR 12909 before receiving ethanol, plain tap water, or sugar water (except for experiment 7 where a single dose of naltrexone versus saline was used, see description under experiment 7 below). This was implemented by repeating the 2-day 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. The rationale for allowing the mice to experience the drinking solutions without injections on alternate days was to reduce the chance that a taste aversion might develop from always pairing a drinking solution with an injection. Each experiment began with 24 animals, and the order in which the four 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.

Statistical analysis

Data were analyzed in two ways. First, 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. The data were also analyzed considering dose and (the logarithm, base 10, of dose+1 with saline=1) as an ordered, continuous variable (rather than a factor), using a mixed effects linear model with animal entered as the random effect (to account for repeated measures; i.e., block diagonal variance–covariance matrix with animals as the blocks). For these models, the linear, quadratic, and cubic terms were evaluated to estimate the dose–response curve. In experiment 7, dose, concentration of ethanol in the drinking water, and the interaction between dose and concentration of ethanol were entered as factors in a mixed model with the animal entered as a random effect (i.e., for repeated measures). A p value less than 0.05 was considered significant.

Experiments 1–3: effect of low doses of naltrexone on the intake of 20% ethanol, plain tap water, or 10% sugar water ($n = 24$/experiment)

Experiment 1 Mice received either saline or 0.5, 1, or 2 mg/kg of naltrexone immediately before their water bottles were replaced with cylinders containing 20% ethanol according to the experimental design described above.

Experiment 2 Mice received either saline or 0.5, 1, or 2 mg/kg of naltrexone immediately before their water bottles were replaced with cylinders containing plain tap water.

Experiment 3 Mice received either saline or 0.5, 1, or 2 mg/kg of naltrexone immediately before their water bottles were replaced with cylinders containing 10% sugar water.

Experiments 4–6: effect of high doses of naltrexone on plain tap water, 20% ethanol, or 10% sugar water ($n = 24$/experiment)

Experiment 4 One mouse died during the acclimation period before any injections were given, thus the sample size dropped from 24 to 23. Mice received 4, 8, and 16 mg/kg naltrexone immediately before their water bottles were replaced with cylinders containing plain tap water.

Experiment 5 The same animals used in Experiment 4 were retested to examine the effect of high doses of naltrexone on 20% ethanol drinking. Mice received 4, 8, and 16 mg/kg naltrexone immediately before their water bottles were replaced with cylinders containing 20% ethanol. One animal appeared sick on the second day of the experiment and was humanely euthanized, thus the sample size dropped from 23 to 22.

Experiment 6 The same animals used in Experiment 4 and 5 were tested a third time to determine the effects of naltrexone on consumption of 10% sugar water. Mice received 4, 8, and 16 mg/kg naltrexone immediately before their water bottles were replaced with cylinders containing 10% sugar water. One animal died during injections, and thus the sample size dropped from 22 to 21.

Experiment 7: effect of an 8-mg/kg naltrexone on the intake of 10, 20, and 30% ethanol ($n = 24$)

The purpose of this experiment was to determine whether the effect of naltrexone on ethanol intake depends on the concentration of ethanol in the drinking water. Mice received either saline or 8 mg/kg naltrexone immediately before their water bottles were replaced with cylinders containing either 10, 20, or 30% ethanol. The experimental design was similar to the previous experiments in that a 2-day DID procedure was repeated in the same animals twice a week (Monday–Tuesday, and Thursday–Friday, with Wednesday off) but this time for 3 weeks (with weekends off). Each animal received all combinations of treatments in a permuted order with the following constraints: animals alternated between receiving saline and naltrexone on injection days and animals always received the same type of fluid within a 2-day trial.

Experiments 8–10: effect of GBR 12909 on the consumption of 20% ethanol, plain tap water, and 10% sugar water ($n = 24$/experiment)

Experiment 8 Mice received 5, 10, and 20 mg/kg of GBR 12909 immediately before their water bottles were replaced with cylinders containing 20% ethanol.

Experiment 9 Mice received 5, 10, and 20 mg/kg of GBR 12909 immediately before their water bottles were replaced with cylinders containing plain tap water according to the experimental design described above.

Experiment 10 Mice received 5, 10, and 20 mg/kg of GBR 12909 immediately before their water bottles were replaced

with cylinders containing 10% sugar water according to the experimental design described above.

Results

Experiments 1–3: effect of low doses of naltrexone on the consumption of 20% ethanol, plain tap water, and 10% sugar water

Experiment 1 Figure 1 shows the effect of naltrexone on 20% ethanol consumption. It shows data from experiments 1 (low doses) and 5 (high doses) to allow for the combined visualization of all doses. Naltrexone administered at 0.5, 1, or 2 mg/kg decreased ethanol consumption in a dose-dependent manner resulting in levels of consumption over a 2-h period that were 86, 72, and 75% levels of saline-treated mice, respectively (Fig. 1, low doses) [$F(3, 69)=4.56$, $P=0.006$]. The 2-h ethanol intake after a saline injection was significantly greater than the intake after 1 or 2 mg/kg of naltrexone (both $P<0.05$); no other post hoc pair-wise comparisons were significant.

The difference in the cumulative intake of ethanol in response to the 2 mg/kg naltrexone as compared to saline was significant from 45 min through 2 h. The effect size increased from 45 to 90 min, after which the cumulative differential remained consistent (Fig. 1b).

Experiment 2 Naltrexone administered at 0.5, 1, or 2 mg/kg had no significant effect on plain tap water consumption (Fig. 2; low doses) [$F(3, 69)=0.95$, $P=0.42$].

Experiment 3 Naltrexone administered at 0.5, 1, or 2 mg/kg had no significant effect on the intake of 10% sugar water [$F(3, 69)=0.38$, $P=0.77$]. It is notable that the animals consumed much more sugar water than plain tap water or ethanol at all doses (1–2 ml versus 0.2 to 0.5 ml), and a

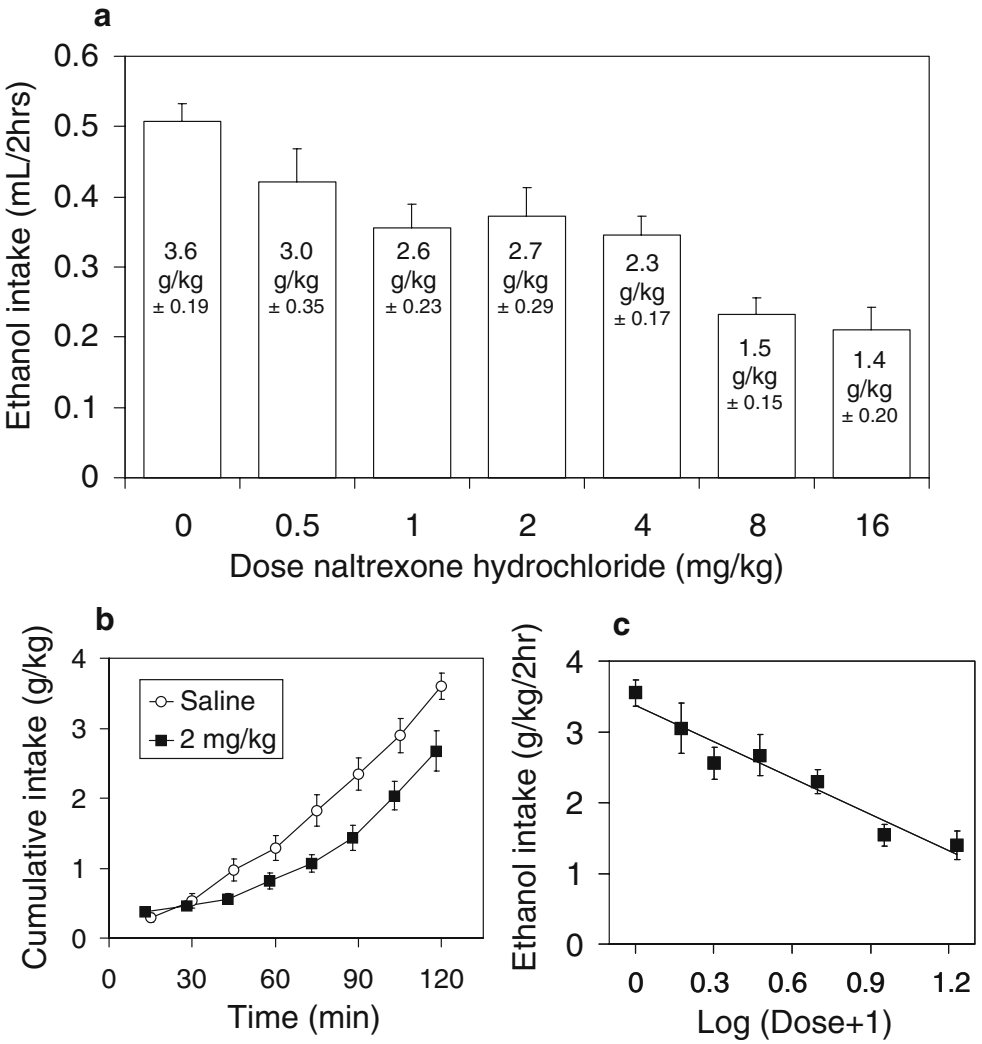

Fig. 1 Naltrexone dose dependently reduced ethanol DID. **a** Mean±SEM cumulative consumption of 20% ethanol in milliliters (g/kg is shown within the *bars*) 2 h after an injection of 0 (saline), 0.5, 1, 2, 4, 8, or 16 mg/kg naltrexone ($n=22$–24 per group except saline where $n=46$). These data were collected in two separate experiments (1 and 5). No mean difference was observed for the saline groups between experiments so the saline groups were combined. The animals used for the high doses (4, 8, and 16 mg/kg) were used previously in experiment 4 to test the effect of naltrexone on plain tap water consumption. The animals for the low doses were naïve. **b** Cumulative consumption of ethanol in grams per kilogram in 15 min intervals in response to saline or 2 mg/kg naltrexone ($n=24$ per group). Similar curves for 0.5 and 1 mg/kg were observed (data not shown). **c** Mean±SEM cumulative consumption of 20% ethanol (g/kg) plotted against the logarithm of dose+1 in milligrams per kilogram. The best fit linear regression through these data weighted by the standard errors is shown

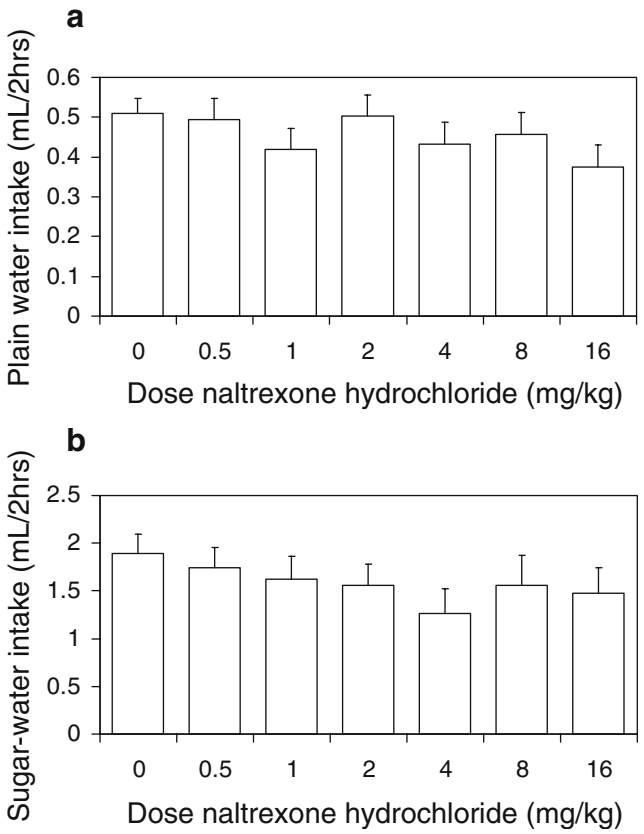

Fig. 2 Naltrexone had no significant effect on the consumption of plain tap water or 10% sugar water. Mean±SEM consumption (ml) of **a** plain tap water or **b** 10% sugar water in 2 h after i.p. injections of saline or six different doses of naltrexone hydrochloride. These data were collected in four separate experiments (2 and 4 for plain water and 3 and 6 for 10% sugar water). Within each graph, the saline groups were combined. The animals used for the high doses (4, 8 and 16 mg/kg) in the sugar water graph (**b**) were used previously in experiment 4 to test the effect of high doses of naltrexone on plain tap water consumption. The rest of the animals were naïve

different scale was used on the y-axis of Fig. 2b versus Fig. 2a or Fig. 1.

Experiments 4–6: effect of high doses of naltrexone on the intake of plain tap water, 20% ethanol, and 10% sugar water

Experiment 4 Naltrexone administered at 4, 8, or 16 mg/kg had no significant effect on plain tap water consumption (Fig. 2, high doses) [$F(3, 64)=1.1$, $P=0.35$]. A combined analysis of data from experiment 2 and 4 (Fig. 2), considering dose or the logarithm of dose+1 as a continuous variable, showed no significant linear, quadratic, or cubic terms, indicating no significant dose response, although the linear term was marginally nonsignificant for dose [$F(1, 138)=3.8$, $P=0.06$] and log(dose+1) [$F(1, 138)=3.8$, $P=0.06$].

Experiment 5 The same animals in which we observed no significant effect of 4, 8, or 16 mg/kg of naltrexone on the consumption of plain tap water in Experiment 4 showed a strong dose-dependent decrease in the consumption of 20% ethanol (Fig. 1, high doses) [$F(3, 63)=23.1$, $P<0.0001$]. Consumption levels were reduced to 65, 43, and 39% of saline levels, respectively. Ethanol intake (in 2 h) after a saline injection was significantly greater than the intake after all doses of naltrexone (all $P<0.05$), and the intake after 4 mg/kg was greater than 8 and 16 mg/kg (both $P<0.05$), but 8 and 16 mg/kg did not differ from each other.

A combined analysis of data from experiment 1 and 5 (Fig. 1), considering dose as a continuous variable, showed significant linear [$F(1, 135)=55.9$, $P<0.0001$] and quadratic [$F(1, 135)=14.0$, $P= 0.0003$] coefficients but no significant cubic term indicating a convex shape to the dose-response curve. The best polynomial equation predicting the intake of ethanol (g/kg 2 h^{-1}) in response to naltrexone was $3.34-0.346(\text{dose})+0.014(\text{dose}^2)$ where dose equals the quantity of naltrexone hydrochloride in mg/kg. The logarithm of (dose+1) showed a significant linear coefficient [$F(1, 137)=70.4$, $P<0.0001$] and nonsignificant quadratic and cubic terms. The equation was $3.4-1.762\log(\text{dose}+1)$ (Fig. 1c).

Experiment 6 The same animals in which we observed a strong dose-dependent decrease in ethanol consumption after 4, 8, and 16 mg/kg of naltrexone in Experiment 2 showed no significant decrease in the consumption of 10% sugar water at the high doses (Fig. 2c) [$F(3, 58)=1.2$, $P=0.30$]. A combined analysis of all doses from experiment 3 and 6, considering dose or the logarithm of dose+1 as a continuous variable, showed no significant linear, quadratic, or cubic terms, indicating no significant dose response, although the linear term was marginally nonsignificant for log(dose+1) [$F(1, 153)=3.2$, $P=0.08$].

Experiment 7: effect of 8 mg/kg of naltrexone on the intake of 10, 20, and 30% ethanol

Naltrexone (8 mg/kg) reduced the intake of ethanol (g/kg) by approximately 50% relative to saline regardless of whether the ethanol was offered as a 10, 20, or 30% solution [$F(1, 110)=72.0$, $P<0.0001$] (Fig. 3). Mice drank slightly less ethanol in g/kg when it was offered as a 10% solution as compared to a 20 or 30% solution [$F(2, 110)=5.7$, $P=0.005$] but there was no difference between 20 or 30%. The animals appeared to adjust the dose by altering the volume of the fluid consumed (Fig. 3). The interaction between naltrexone and ethanol concentration was not significant [$F(2,110)=0.18$, $P=0.83$].

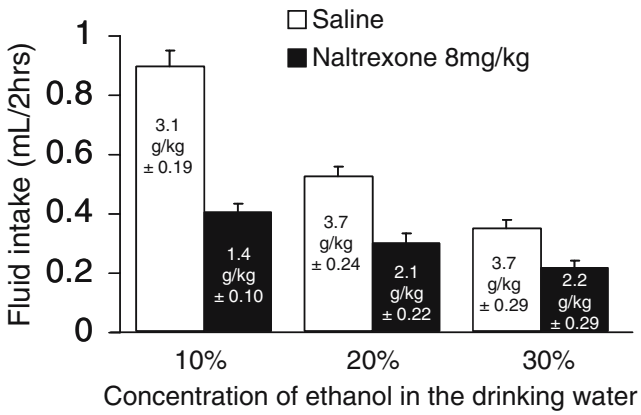

Fig. 3 Naltrexone (8 mg/kg) reduced ethanol consumption in grams per kilogram by approximately 50% regardless of whether ethanol was offered as a 10, 20, or 30% solution. Mean±SEM consumption of 10, 20, or 30% in milliliters (g/kg is shown within the *bars*) after saline (*open bars*) or 8 mg/kg naltrexone (*closed bars*)

Experiments 8–10: effect of GBR 12909 on the intake of 20% ethanol, plain tap water, and 10% sugar water

Experiment 8 GBR 12909 decreased ethanol consumption in a dose-dependent manner (Fig. 4). [$F(3, 68)=16.8$, $P<0.0001$]. Consumption levels were reduced to 78, 56, and 34% saline levels for doses 5, 10, and 20 mg/kg, respectively. All post hoc pair-wise comparisons between doses were significant (all $P<0.05$). Considering dose as a continuous variable, the linear coefficient was significant [$F(1, 68)=48.4$, $P<0.0001$] but not the quadratic or cubic terms. The best linear equation predicting intake of ethanol (g/kg 2 hr^{-1}) in response to GBR 12909 was $3.40-0.116$(dose). The logarithm of (dose+1) showed a significant linear coefficient [$F(1, 70)=45.2$, $P<0.0001$] and nonsignificant quadratic and cubic terms. The equation was $3.7-1.716\log(\text{dose}+1)$.

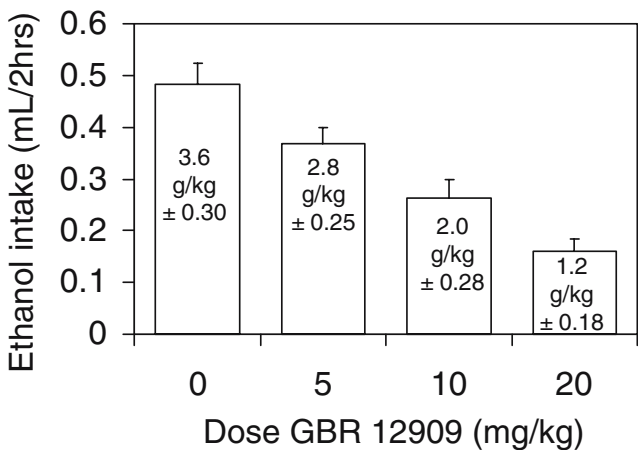

Fig. 4 GBR 12909 dose dependently reduced intake of 20% ethanol using the DID procedure. Mean±SEM intake of 20% ethanol in milliliters (g/kg shown within *bars*) in 2 h after i.p. injections of saline or GBR 12909 dihydrochloride ($n=24$ per group)

Experiment 9 GBR 12909 had no significant effect on the consumption of plain tap water (Fig. 5a) [$F(3, 69)=1.7$, $P=0.19$]. Considering dose or the logarithm of dose+1 as a continuous variable, neither the linear, quadratic, or cubic terms were significant, indicating no significant dose response.

Experiment 10 GBR 12909 decreased the intake of 10% sugar water in a dose-dependent manner (Fig. 5b) [$F(3, 69)=18.9$, $P<0.0001$]. Consumption levels were reduced to 73, 55, and 51% saline levels, respectively. The post hoc analysis indicated that all pair-wise comparisons were significant except 10 versus 20 mg/kg. Considering dose as a continuous

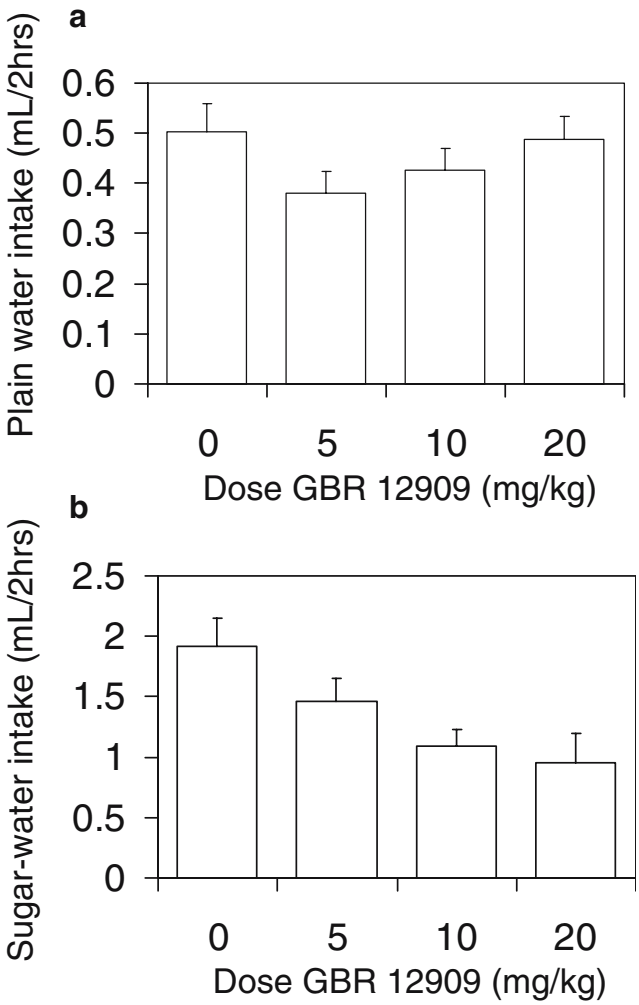

Fig. 5 GBR 12909 had no significant effect on the consumption of plain tap water but reduced the intake of 10% sugar water in a dose-dependent manner. Mean±SEM intake (ml) of **a** plain tap water or **b** 10% sugar water in 2 h after i.p. injections of saline or GBR 12909 dihydrochloride ($n=24$ per group)

variable, the linear [$F(1, 69)=44.6$, $P<0.0001$] and quadratic terms [$F(1, 69)=12.2$, $P=0.0008$] were significant but not the cubic term. The best polynomial equation predicting the intake of 10% sugar water (ml) in response to GBR 12909 was $1.91-0.123(\text{dose})+ 0.0038(\text{dose}^2)$. The logarithm of (dose+1) showed a significant linear coefficient [$F(1, 70)=45.2$, $P<0.0001$] and nonsignificant quadratic and cubic terms. The equation was $1.92-0.736\log(\text{dose}+1)$.

Discussion

Many different animal models of excessive ethanol drinking have been described (for a review see Crabbe et al. 1994; Cunningham et al. 2000). At best, each of these models captures only a subset of features relevant for understanding alcoholism. Advantages of the DID model are that it is very simple to implement, short in duration (2 days), and reliably produces behavioral intoxication (Rhodes et al. 2005). Previously, we argued that the DID model demonstrates face validity by showing that the predisposed genotype C57BL/6J self-administers ethanol to the point at which they display motor impairment (Rhodes et al. 2007). Now we extend this result by showing that the model displays predictive validity with opioid and dopaminergic mechanisms. In this study, we show that the opioid antagonist, naltrexone, which has proven to reduce ethanol drinking in human alcoholics (Volpicelli et al. 1992) and many animal models including nonhuman primates (Boyle et al. 1998; Kornet et al. 1991), rats (Coonfield et al. 2002, 2004; Davidson and Amit 1997; Goodwin et al. 2001; Parkes and Sinclair 2000; Sharpe and Samson 2001), and mice (for a review see Egli 2005; Fachin-Scheit et al. 2006; Le et al. 1993; Middaugh and Bandy 2000; Middaugh et al. 2000, 2003; Phillips et al. 1997), also reduces ethanol drinking in DID. Moreover, the effect seems to be relatively specific to consumption of ethanol because naltrexone, given at the same doses, did not significantly reduce the consumption of plain tap water or sugar water. Note that the acute effect of naltrexone in reducing ethanol intake may disappear (Middaugh and Bandy 2000) or even reverse (i.e., increase ethanol intake) with repeated administration in C57BL/6J mice (Phillips et al. 1997).

In contrast, GBR 12909 reduced the consumption of both ethanol and sugar water (but not plain tap water). These data support the idea that dopaminergic mechanisms are involved in high ethanol drinking in DID, but suggest that the mechanism targeted by GBR 12909 is relatively nonspecific, affecting both normal reinforcement of consumption of sweet fluids and excessive ethanol drinking, but not plain tap water drinking. Taken together, these results suggest that intake of ethanol, plain water, and sugar water are influenced by different motivational pathways. Results suggest that ethanol's effect on opioid signaling is necessary for excessive ethanol intake in the DID model, whereas naltrexone-sensitive opioid signaling is not necessary for natural motivations for palatable sugar water or plain water under basal nonfluid-deprived conditions. Based on these results, we conclude that the DID model may be useful to explore mechanisms underlying high ethanol drinking and for screening new compounds for potential efficacy.

Opioid signaling is necessary for high ethanol drinking in DID

The observation that naltrexone significantly reduced ethanol intake but not the intake of the alternative fluids suggests that different pathways underlie motivation for ethanol drinking as compared to plain tap water or sugar water in the DID model. These results suggest that, in this model, opioid signaling plays a necessary role in high ethanol drinking but not in sugar-water drinking or plain-water drinking. The result adds to the vast literature suggesting that the opioid system is involved in excessive ethanol drinking (for a review see Froehlich and Li 1994). Moreover, it points to a specific role for the opioid system in initiating high ethanol intake as compared to the intake of a sugar reward or plain water. This is important because other animal models of high ethanol drinking, such as schedule-induced polydipsia, fail to show specificity for effects of naltrexone on ethanol drinking as compared to plain water drinking (Escher and Mittleman 2006). This result is also significant because one feature that has not yet been clearly elucidated is how motivation for ethanol is different from motivation for natural rewards (Kelley and Berridge 2002). Some have argued that the only difference is in the degree to which drugs stimulate or suppress the nervous system by altering chemical neurotransmission and signaling pathways (Di Chiara et al. 1993). These data point to opioid signaling in the differential motivation for ethanol. Future studies could further delineate the mechanism by centrally administering specific opioid antagonists (that target the different receptor subtypes) at specific sites in the brain before DID. This would enable a determination of which opioid signal is necessary and where in the brain it has to occur to elicit high ethanol drinking in DID.

One could argue that the specificity of naltrexone's effect on ethanol intake may have been related to differential motivation for ethanol versus the alternative fluids. For example, it is possible that the animals were more motivated to consume plain water or sugar water than ethanol, hence making consumption of the natural rewards harder to pharmacologically suppress, but that too would

indicate that different mechanisms underlie ethanol drinking versus intake of the natural rewards. Unfortunately, it is not possible to gage relative levels of motivation for the different solutions in this study. Volume of fluid consumed is not an appropriate indicator of motivation for ethanol because the animals may have adjusted the volume to self-administer a desired plane of intoxication. The data shown in Fig. 3 is consistent with this hypothesis by showing that the mice increased the volume of fluid to achieve a similar dose of ethanol (in g/kg) when the concentration of ethanol was decreased from 30 to 10%.

Dopamine signaling in DID

Dopamine signaling is widely known to play a role in reward and reinforcement and has been implicated in alcoholism and high ethanol drinking in many different animal models (for a review see Tupala and Tiihonen 2004). When ethanol is consumed, dopamine levels transiently increase in extracellular spaces, and this dopamine surge has been hypothesized to function in reinforcement and to contribute to addiction (Melendez et al. 2002). Because GBR 12909 binds to the dopamine transporter with high affinity and specificity, it increases dopamine in extracellular spaces for a longer period than the drugs of abuse (Andersen 1989) and does not produce the surge in dopamine that some have suggested is required for addiction, making it potentially valuable as a substitution therapy (Preti 2000). Our results are consistent with the idea that GBR 12909 can substitute for the drugs of abuse, but suggest that the effect is not specific to drugs as GBR 12909 appears to reduce motivation for natural pleasures as well. A transient increase in dopamine also occurs when rodents taste sweet fluids, which may underlie reinforcement of sugar water drinking in rodents (Avena et al. 2006). GBR 12909 may have reduced consumption of ethanol (Fig. 3) and sugar water (Fig. 4b) in the mice by an equivalent mechanism. The dopamine surge that normally accompanies consumption of these fluids may have been replaced with a longer lasting increase in dopamine, which thereby reduced reinforcement of the appetitive behaviors toward acquiring the sweet taste (or high calories) or the ethanol reward. Alternatively, GBR 12909 may have made the animals feel sick or had indirect effects on drinking the solutions because of the effects on locomotor activity (Rhodes et al. 2001), but the lack of effect on plain tap water drinking diminishes the likelihood of these hypotheses.

The literature is generally consistent with the hypothesis that GBR 12909 reduces motivation for natural and drug rewards. In one published abstract from the Research Society on Alcoholism, i.p. injections of 20 or 40 mg/kg of GBR 12909 reduced ethanol intake in selectively bred, alcohol preferring P rats (Murphy et al. 1988). In this study, a limited access paradigm was used where animals had access to two-bottles (10% ethanol or water) for 4 h per day for several days (the phase of light/dark cycle when ethanol was offered was not given). The authors noted that the 20 mg/kg dose reduced the ethanol intake only during the first hour and that the 40 mg/kg dose, although it reduced ethanol intake from 2.8 to 0.2 g/kg in 4 h, also reduced food intake by 90%.

In another study with P rats, GBR 12909 directly infused into the nucleus accumbens over a 240 min period, at doses ranging from 10 to 200 μM per minute, had no significant effect on ethanol intake (Engleman et al. 2000). They used a 1-h-limited access, 2-bottle choice paradigm with 15% ethanol offered during the last 60 min of the GBR 12909 infusion. To the best of our knowledge, the only other studies that examined effects of GBR 12909 on ethanol intake were done in male Long-Evans rats that were REM (rapid-eye-movement)-sleep deprived (Aalto and Kiianmaa 1986, 1987). No effect was observed, but the rats only drank 2 g/kg over the entire 24-h period under baseline (saline) conditions. They used a 24-h 2-bottle choice paradigm with 10% ethanol. I.p. injections of 5 to 20 mg/kg were given twice a day, and intake over the entire 24 h was measured during and after REM sleep deprivation (which elevates drinking from approximately 1 g/kg day^{-1} to 2 g/kg day^{-1}).

Taken together, these data suggest that GBR 12909 might reduce ethanol intake in animals that are genetically predisposed for high ethanol intake, but only when GBR 12909 is administered i.p. (and hence can affect all regions receiving dopamine input) and not when it is administered only to the nucleus accumbens. Moreover, the data from Murphy et al. (1988) suggest that GBR 12909 might cause some negative side-effects on food consumption when administered at high doses. This is consistent with van der Hoek and Cooper (1994) who observed a decrease in duration of feeding a sweetened palatable diet in male hooded rats after i.p. administration of 15 or 20 (but not 5 or 10) mg/kg of GBR 12909. Taken together, these results suggest that a replacement therapy aimed at increasing dopamine signaling can reduce normal reinforcement for food and palatable drinks in addition to attenuating excessive ethanol drinking.

Limitations of the DID model

The DID model captures important features of alcoholism such as drinking to the point of intoxication (Rhodes et al. 2007) and rapid initiation to high levels of drinking (Rhodes et al. 2005). The model is useful because these features have been difficult to reproduce in other animal models but other important features of alcoholism such as dependence and tolerance have not yet been demonstrated

with DID (Cicero 1980). Ethanol drinking in DID does not always escalate over several days, which would be expected if the mice were becoming tolerant, although it is not known whether levels would escalate if the drinking episodes continued for more than 12 days (Rhodes et al. 2005). Moreover, it remains to be determined whether DID can lead to dependence as measured by a sign of withdrawal when alcohol is withheld (e.g., anxiety or seizure after ethanol is withheld after repeated DID exposure). Future studies will explore these questions. The issue of whether the single bottle is a limitation was examined in Rhodes et al. (2007). In that study, DID was measured using a single bottle or two-bottles (one with tap water) across 12 standard inbred strains including C57BL/6J. Results showed intake of ethanol (g/kg) was roughly equivalent when animals had a single bottle or 2-bottle choice. The main difference was that with a single bottle animals reach higher blood-ethanol levels than with two bottles (Rhodes et al. 2007).

Conclusions

Excessive ethanol drinking in C57BL/6J in the recently described DID model is reduced with the opioid antagonist naltrexone or the high-affinity dopamine reuptake blocker GBR 12909. The effect of naltrexone was specific (no effect was observed on intake of plain water or 10% sugar water at doses which significantly affected ethanol intake), whereas the effect of GBR 12909 was nonspecific (it reduced intake of ethanol or sugar water but *not* plain water). Future studies will further explore the specificity of opioid signaling in excessive ethanol intake relative to dopamine signaling in motivation for drugs and natural rewards. It is interesting that the effect of naltrexone and GBR 12909 is different for excessive wheel running as compared to excessive ethanol drinking. In mice selectively bred for high levels of running, naltrexone (at the doses used here) had no effect on running (Li et al. 2004), whereas GBR 12909 strongly reduced running (Rhodes et al. 2001). Future experiments will also explore a possible role for glutamate signaling with such drugs as acamprosate and the metabotropic glutamate receptor (mGluR5) antagonist, 2-methyl-6-(phenylethyl)-pyridine (Hodge et al. 2006). In conclusion, the DID model is a high-throughput, easily transportable tool that can be used to screen novel compounds for their potential efficacy in reducing high levels of ethanol drinking and to identify genetic and neurobiological mechanisms for excessive ethanol-drinking behavior.

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