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Disulfiram Attenuates Drug-Primed Reinstatement of Cocaine Seeking via Inhibition of Dopamine β-Hydroxylase

Jason P Schroeder[1,6], Debra A Cooper[1,6], Jesse R Schank[1], Megan A Lyle[1], Meriem Gaval-Cruz[1], Yvonne E Ogbonmwan[1], Nikita Pozdeyev[2], Kimberly G Freeman[3], P Michael Iuvone[2,4], Gaylen L Edwards[3], Philip V Holmes[5] and David Weinshenker*[,1]

[1]Department of Human Genetics, Emory University School of Medicine, Atlanta, GA, USA; [2]Department of Pharmacology, Emory University School of Medicine, Atlanta, GA, USA; [3]Department of Physiology and Pharmacology, University of Georgia, Athens, GA, USA; [4]Department of Ophthalmology, Emory University School of Medicine, Atlanta, GA, USA; [5]Department of Psychology, University of Georgia, Athens, GA, USA

The antialcoholism medication disulfiram (Antabuse) inhibits aldehyde dehydrogenase (ALDH), which results in the accumulation of acetaldehyde upon ethanol ingestion and produces the aversive 'Antabuse reaction' that deters alcohol consumption. Disulfiram has also been shown to deter cocaine use, even in the absence of an interaction with alcohol, indicating the existence of an ALDH-independent therapeutic mechanism. We hypothesized that disulfiram's inhibition of dopamine β-hydroxylase (DBH), the catecholamine biosynthetic enzyme that converts dopamine (DA) to norepinephrine (NE) in noradrenergic neurons, underlies the drug's ability to treat cocaine dependence. We tested the effects of disulfiram on cocaine and food self-administration behavior and drug-primed reinstatement of cocaine seeking in rats. We then compared the effects of disulfiram with those of the selective DBH inhibitor, nepicastat. Disulfiram, at a dose (100 mg/kg, i.p.) that reduced brain NE by ~40%, did not alter the response for food or cocaine on a fixed ratio 1 schedule, whereas it completely blocked cocaine-primed (10 mg/kg, i.p.) reinstatement of drug seeking following extinction. A lower dose of disulfiram (10 mg/kg) that did not reduce NE had no effect on cocaine-primed reinstatement. Nepicastat recapitulated the behavioral effects of disulfiram (100 mg/kg) at a dose (50 mg/kg, i.p.) that produced a similar reduction in brain NE. Food-primed reinstatement of food seeking was not impaired by DBH inhibition. Our results suggest that disulfiram's efficacy in the treatment of cocaine addiction is associated with the inhibition of DBH and interference with the ability of environmental stimuli to trigger relapse.
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INTRODUCTION

Disulfiram (Antabuse) has been used for more than 50 years in the treatment of alcoholism (Fuller *et al*, 1986). Disulfiram inhibits aldehyde dehydrogenase (ALDH), which results in the accumulation of acetaldehyde on ethanol ingestion. This toxic metabolite produces aversive symptoms, such as flushing, nausea, and vomiting, and a desire to avoid this reaction encourages abstinence. Because 50–90% of patients who abuse cocaine also abuse alcohol (Weiss *et al*, 1988; Grant and Harford, 1990; Closser and Kosten, 1992; Khalsa *et al*, 1992), the belief was that discouraging alcohol consumption in cocaine- and alcohol-dependent individuals might lower cocaine use. Indeed, disulfiram was found to reduce alcohol and cocaine intake in this patient population (Carroll *et al*, 1993, 1998, 2000). Surprisingly, further studies revealed that disulfiram is at least as effective at treating cocaine addicts who do not consume alcohol, and may even be more effective (George *et al*, 2000; Petrakis *et al*, 2000; Carroll *et al*, 2004). Therefore, an ALDH-independent mechanism must be responsible for the ability of disulfiram to promote cocaine abstinence (Weinshenker and Schroeder, 2007; Gaval-Cruz and Weinshenker, 2009).

Cocaine increases extracellular levels of dopamine (DA), norepinephrine (NE), and serotonin in the brain by blocking plasma membrane monoamine transporters. Thus, pathways critical for the production or transmission of these neurotransmitters are a reasonable place to look for targets underlying the efficacy of disulfiram in the treatment of cocaine dependence. Because the primary metabolite of disulfiram, diethyldithiocarbamate, is a copper chelator (Hald and Jacobsen, 1948; Johnston, 1953), disulfiram impairs the function of many copper-containing enzymes, including ALDH, carboxylesterase, cholinesterase, and

*Correspondence: Dr D Weinshenker, Department of Human Genetics, Emory University School of Medicine, Whitehead 301, 615 Michael Street, Atlanta, GA 30322, USA, Tel: +1 404 727 3106, Fax: +1 404 727 3949, E-mail: dweinshenker@genetics.emory.edu
[6]These authors contributed equally to the work.
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dopamine β-hydroxylase (DBH). It is of particular interest that the inhibition of DBH by disulfiram reduces production of NE, with a concomitant increase in tissue levels of DA in rodents (Goldstein, 1966; Musacchio *et al*, 1966; Bourdélat-Parks *et al*, 2005). Disulfiram also decreases NE and its metabolites in the urine, blood, and CSF of humans (Takahashi and Gjessing, 1972; Major *et al*, 1979; Rogers *et al*, 1979; Hoeldtke and Stetson, 1980; Rosen and Lobo, 1987; Paradisi *et al*, 1991). We have shown that disulfiram has no effect on catecholamine levels in DBH knockout (*Dbh*−/−) mice, which lack NE, indicating that disulfiram's effects on NE and DA are mediated solely by DBH inhibition (Bourdélat-Parks *et al*, 2005). Disulfiram also inhibits cocaine-metabolizing enzymes and increases peak plasma cocaine levels under some conditions in humans (McCance-Katz *et al*, 1998a, b; Baker *et al*, 2007), but not in rodents (Gaval-Cruz and Weinshenker, 2008, 2009).

The efficacy of disulfiram in treating cocaine dependence has been attributed to several different mechanisms, including a decrease in cocaine reward, an increase in cocaine aversion, and as a 'DA replacement therapy' that elevates DA levels and restores normal reward function in hypodopaminergic addicts (Weinshenker and Schroeder, 2007; Sofuoglu *et al*, 2008; Gaval-Cruz and Weinshenker, 2009); however, the data have been ambiguous. Different human laboratory studies report that genetic or pharmacological DBH inhibition increases cocaine-induced paranoia and decreases, increases, or has no effect on psychostimulant-induced euphoria (Hameedi *et al*, 1995; McCance-Katz *et al*, 1998a, b; Cubells *et al*, 2000; Petrakis *et al*, 2000; Baker *et al*, 2007; Kalayasiri *et al*, 2007; Sofuoglu *et al*, 2008). In rodents, disulfiram decreases the locomotor-activating effects of acute cocaine administration, but facilitates cocaine sensitization (Maj *et al*, 1968; Haile *et al*, 2003).

The available human and animal data provide us a hazy picture of how disulfiram discourages cocaine use. The influence of disulfiram on the reinforcing properties of cocaine is yet to be investigated in an animal model, and whereas DBH inhibition has been suggested to underlie disulfiram's efficacy, this hypothesis has not been tested directly. In an effort to resolve these issues, we assessed the effects of disulfiram in operant rat paradigms of drug taking (cocaine self-administration) and relapse (cocaine-primed reinstatement) at doses that inhibit DBH in the brain. To determine whether the effects of disulfiram were mediated by inhibition of DBH, we used the selective DBH inhibitor, nepicastat. Nepicastat is a direct, competitive inhibitor of DBH with greater potency than disulfiram ($IC_{50} = 9$ nM for nepicastat *vs* $IC_{50} \cong 1\,\mu$M for disulfiram; Green, 1964; Goldstein, 1966; Stanley *et al*, 1997), as well as better selectivity (does not chelate copper, no significant interaction with a panel of other enzymes and receptors tested, including ALDH and tyrosine hydroxylase, the rate-limiting enzyme in catecholamine biosynthesis) (Stanley *et al*, 1997; K Walker, Roche Biosciences, personal communication).

MATERIALS AND METHODS

Subjects

Male Sprague–Dawley rats (175–200 g) were purchased from Charles River (Wilmington, MA). All subjects were maintained in a temperature-controlled environment on a 12-h reverse light/dark cycle with the lights on from 1900 to 0700 hours with *ad libitum* access to food and water. Rats were acclimated to the vivarium for 1 week before catheter-implantation surgery. All self-administration sessions occurred during the dark cycle and were performed using standard methods with minor modifications (McFarland and Kalivas, 2001; Fuchs *et al*, 2006). All animals were treated in accordance with NIH policy, and experiments were approved by the Emory IACUC committee.

Drug Doses

In initial pilot experiments, we tested the effects of disulfiram (10, 25, 50, 75, 100, or 200 mg/kg, i.p.) and nepicastat (50 or 100 mg/kg, i.p.) on brain catecholamine levels and operant responding for food. Disulfiram was obtained from Sigma-Aldrich (St Louis, MO), sonicated in sterile saline, and injected as a suspension. Nepicastat was obtained from Synosia Therapeutics (South San Francisco, CA), sonicated in sterile saline containing 1.5% DMSO and 1.5% Cremaphor EL (Sigma), and injected as a suspension. We chose the 100 mg/kg dose of disulfiram based on four criteria. First, 100 mg/kg was the maximum dose that significantly inhibited DBH, but did not impair the ability of rats to perform operant responses. Second, the 100 mg/kg dose has been shown by others to alter other behavioral effects of cocaine in rats, such as locomotor activity and sensitization (Haile *et al*, 2003). Third, the 100 mg/kg dose inhibits ALDH in rats and is in the range typically used for alcohol studies (Deitrich and Erwin, 1971; Yourick and Faiman, 1991; Karamanakos *et al*, 2001). Fourth, the 100 mg/kg dose is therapeutically relevant. The typical therapeutic dose for the cocaine studies performed in humans is 250–500 mg per day (Carroll *et al*, 1998; McCance-Katz *et al*, 1998a, b), which translates to $\sim$3–7 mg/kg for a 70 kg human, or $\sim$10-fold lower than we used in our study. Because of their higher metabolic rate, rodents require much larger doses of psychoactive drugs to produce behavioral and neurochemical effects compared with humans, and the 3–7 mg/kg dose has been shown to inhibit DBH in humans with a magnitude similar to the 100 mg/kg dose in rats (compare Vesell *et al*, 1971; Major *et al*, 1979; Rogers *et al*, 1979; Paradisi *et al*, 1991 human studies to our current rat study). Thus, use of the 100 mg/kg dose in rats is a close functional match to therapeutic doses in humans. We chose the 10 mg/kg dose of disulfiram for an additional experiment because it was the maximum dose in our pilot studies that did not significantly reduce brain NE levels. The 50 mg/kg dose of nepicastat was chosen to match the level of DBH inhibition observed with the 100 mg/kg dose of disulfiram.

Quantification of Catecholamine Levels

Rats were injected with disulfiram (10 or 100 mg/kg, i.p.), nepicastat (50 mg/kg, i.p.), or vehicle (saline for disulfiram, 1.5% DMSO + 1.5% Cremaphor EL in saline for nepicastat; 1 ml/kg, i.p.). After 2 h, rats were killed by administrating CO_2, brains were removed, and the frontal cortex was dissected on ice and was frozen. The frontal cortex was chosen because it contains comparable amounts of NE and

DA, and thus can be used to assess the DBH inhibition accurately. NE and DA levels were determined using HPLC followed by the coulometric detection. DA and NE concentrations were normalized to wet tissue weight for each sample.

Analytical samples from saline- and disulfiram-treated rats were prepared by adding 10 volumes of ice-cold mobile phase (0.1 mM $NaHSO_4$, monohydrate 0.1 mM EDTA, 0.2 mM octane sulfonic acid, 6.5% acetonitrile (pH 3.1)), and sonicated until completely homogenized. Samples were centrifuged at 13.2 r.p.m. $\times$ 1000 for 30 min at 4°C, and the supernatant was removed from the tubes. The supernatant was centrifuged again at 13.2 r.p.m. $\times$ 1000 for 30 min at 4°C using a 22-μm filter column. The resulting eluant was injected using an ESA 542 Autosampler (ESA Biosciences, Chelmsford, MA) onto a Synergi Max-RP 4 μm (150 $\times$ 4.6 mm) with Security Guard precolumn filter with Max-RP cartridges (Phenomenex, Torrance, CA) at a constant rate of 1 ml/min maintained by ESA 584 pumps. An ESA CoulArray 5600A detector with a potential set at −150, 200 mV was used to visualize the peaks. The retention time and height of NE and DA peaks were compared with reference standard solutions (Sigma). Peak heights were quantified by CoulArray software (ESA Biosciences).

Analytical samples of vehicle and nepicastat-treated rats were prepared by adding 70 μl of ice-cold 0.1 N perchloric acid and 0.04% sodium metabisulfite to the tissue, and then sonicating until homogenized. Samples were centrifuged at 15 r.p.m. $\times$ 1000 for 10 min at 4°C. This supernatant was injected at a constant flow rate of 1 ml/min onto an Ultrasphere ODS 250 $\times$ 4.6 mm column, 5 μm (Beckman Coulter, Fullerton, CA) with mobile phase (0.1 mM EDTA; 0.35 mM sodium octyl sulfate; 0.6% phosphoric acid; 5% acetonitrile (pH 2.7)). A coulometric electrochemical array detector (Agilent Technologies; guard cell set at 600 mV and analytical cell at 300 mV) was used to visualize the peaks. The retention time, height, and area of NE and DA peaks were compared with reference standard solutions (Sigma) and quantified by ChemStation chromatography software (Agilent Technologies).

Food Training

Rats were trained to lever-press for food in standard rat operant chambers (Med Associates, St Albans, VT) before the drug exposure to facilitate acquisition of drug self-administration, as described (Fuchs *et al*, 2006). Each chamber was equipped with a houselight, two levers (active and inactive), and stimulus lights above both the levers. Fan motors provided ventilation and masked noise in each chamber. A microcomputer with Logic '1' interface and MED-PC software (MED Associates) controlled schedule contingencies and recorded data. Animals had access to a water bottle and received 45-mg food pellets following active lever presses on a fixed ratio 1 (FR1) schedule, that is, the rat received a reinforcer following each active lever press. The food training sessions lasted for 8 h, or until the animal met criteria, defined as at least 70% selection of the active lever and at least 100 food pellets obtained. Most rats met criteria on the first day of food training, but a few required 2–3 days.

Surgery

Following food training, rats were anesthetized with isoflurane and implanted with indwelling jugular catheters using standard methods. Briefly, catheters were inserted into the jugular vein and anchored with suture material and tissue adhesive. The catheter was then threaded subcutaneously through the skin between the shoulder blades, and the catheter was anchored. Catheters were flushed daily with 0.05 ml gentamicin (4 mg/ml) and 0.1 ml heparin solution (30 U/ml in sterile saline). Catheter patency was verified periodically by infusing 0.08–0.12 ml of methohexital sodium (10 mg/ml, IV; Eli Lilly, Indianapolis, IN), which produces a rapid loss of muscle tone only when administered intravenously.

Cocaine Self-Administration

Daily self-administration sessions were run for 2 h on a FR1 schedule. At the start of each session, both active and inactive levers were extended, and rats received a noncontingent infusion of cocaine (0.5 mg/kg). During the training, each press of the active lever resulted in a cocaine infusion (0.5 mg/kg in a volume of 167 μl/kg) accompanied by a discrete flashing light above the lever. Following a 20-s timeout period (during which time active lever presses did not result in drug infusion), the stimulus light was extinguished, and responses were again reinforced. Responses on the inactive lever had no programmed consequences. To prevent overdose, we terminated the session early if the number of cocaine infusions exceeded 40.

Once rats reached a stable level of responding (number of drug infusions varied by $<20\%$ of the mean, and preference for the active lever was at least 75% for 3 consecutive days, with a minimum of 5 total days of cocaine self-administration), the effects of disulfiram were assessed. Rats received an injection of saline (2 ml/kg, i.p.) or disulfiram (100 mg/kg, i.p.) 2 h before the self-administration session. The rats were then allowed 1–2 days of self-administration sessions with no pretreatment. The following day, rats received the opposite pretreatment (saline or disulfiram) 2 h before the self-administration session in a counterbalanced manner.

Extinction

Following the completion of the maintenance phase of cocaine self-administration, lever pressing was extinguished in daily 2-h sessions during which presses on the previously active lever no longer resulted in delivery of cocaine or presentation of cocaine-paired cues. Behavior was considered extinguished when active lever presses over 3 consecutive days were $<25\%$ of the average number of active lever presses during the last 3 days of maintenance.

Cocaine-Primed Reinstatement

The day after extinction criteria were met, rats were pretreated with saline (2 ml/kg, i.p.) or disulfiram (10 or 100 mg/kg, i.p.). After 2 h, they were administered a noncontingent priming injection of cocaine (10 mg/kg, i.p.) and placed in the operant chambers under extinction

conditions (ie, presses on the 'active' lever had no programmed consequences) for 2 h. Rats then underwent a second round of extinction, as described above. When extinction criteria were met, rats were again tested for cocaine-primed reinstatement, but received the opposite pretreatment (saline or disulfiram) in a counterbalanced manner (order was randomized). Some of the rats used for the reinstatement tests were the same ones that received disulfiram at the end of the maintenance phase of cocaine self-administration, whereas others were from a separate group that did not receive any pretreatments during maintenance. We found no differences in reinstatement, and these groups were combined. To determine whether the effects of disulfiram on reinstatement were mediated by DBH inhibition, separate groups of rats went through cocaine self-administration and extinction, then were pretreated with vehicle (1.5% DMSO, 1.5% Cremaphor EL in saline, 1 ml/kg, i.p.) or nepicastat (50 mg/kg, i.p.) before counterbalanced reinstatement sessions, as described for disulfiram.

Food Self-Administration

Separate groups of rats were used for the food self-administration and reinstatement experiments. Rats were maintained on a restricted diet of 16 g of normal rat chow per day, given in the evening at least 1 h after self-administration sessions had ended. Parameters of food self-administration were identical to the cocaine self-administration experiments, except that rats received a food pellet instead of a cocaine infusion for each active lever press, and sessions lasted for 1 h and were terminated if the reinforcers obtained exceeded 60.

Food-Primed Reinstatement

Food-primed reinstatement of food seeking was performed using a modified version of published protocols (Sun and Rebec, 2005; Peters and Kalivas, 2006). Once the maintenance criteria for operant food self-administration were met (maintenance criteria and extinction criteria were identical to those used for cocaine-primed reinstatement), rats were pretreated with vehicle (1.5% DMSO, 1.5% Cremaphor EL in saline, 1 ml/kg, i.p.) or nepicistat (50 mg/kg, i.p.). After 2 h, they were placed in the operant chambers and the reinstatement session was started. Three food pellets were delivered non-contingently in the first 10 s of the session and the levers were presented to the subjects. As during extinction, responses on either of the levers had no programmed consequence. Throughout the 60-min food reinstatement session, a food pellet was delivered every 3 min noncontingently, and responses on the formerly active and inactive levers were recorded. Rats then underwent a second round of maintenance and extinction training for operant food self-administration, as described above, and were then tested for food-primed reinstatement following the opposite pretreatment (vehicle or nepicistat) in a counterbalanced manner (order was randomized).

Data Analyses

Catecholamine level data were analyzed by Student's *t*-test, and self-administration data were analyzed by ANOVA followed by Bonferroni *post hoc* tests using Prism 4.0 for Macintosh.

RESULTS

Disulfiram Inhibits DBH and Decreases Brain NE Levels

Dopamine β-hydroxylase is the enzyme in the catecholamine biosynthetic pathway that converts DA to NE in noradrenergic neurons. Thus, inhibition of DBH has the unique effect of simultaneously decreasing NE production and increasing DA (Figure 1). To confirm previous reports that systemic disulfiram administration inhibits DBH in the rat brain, we measured NE, DA, and the NE/DA ratio in the frontal cortex following administration of saline or disulfiram (100 mg/kg, i.p.). We chose the frontal cortex because it contains NE and DA in similar concentrations, thereby allowing the detection of both decreases and increases in these neurotransmitters. As expected, disulfiram was a *bona fide* DBH inhibitor, as it decreased NE, increased DA, and decreased the NE/DA ratio (Figure 2). Inhibition of other catecholamine biosynthetic enzymes would have had different patterns, such as decreases in both NE and DA following tyrosine hydroxylase inhibition.

Disulfiram has no Effect on Self-Administration of Food or Cocaine

To ensure that we were using a dose of disulfiram that did not impair the ability of rats to perform an operant task, we assessed responding for food pellets following saline or disulfiram (100 mg/kg, i.p.) administration. Disulfiram had no effect on food responding; all rats obtained the maximum number of reinforcers possible during the session (61), regardless of pretreatment ($n = 4$ per group). To determine whether disulfiram altered the reinforcing or aversive effects of cocaine, we assessed maintenance levels of responding for cocaine infusions (0.5 mg/kg per infusion) following saline or disulfiram (100 mg/kg, i.p.). Disulfiram had no effect on cocaine self-administration (Figure 3). Repeated-measures ANOVA revealed no significant effects for active lever presses ($F_{23,2} = 0.77$, $p = 0.48$) or reinforcers obtained ($F_{23,2} = 0.97$, $p = 0.4$). Inactive lever presses were negligible (0–2 presses per animal) and did not differ between groups.

Figure 1 Catecholamine biosynthetic pathway. Because DBH converts DA to NE in noradrenergic neurons, inhibition of DBH is unique in its ability to decrease NE while increasing DA.

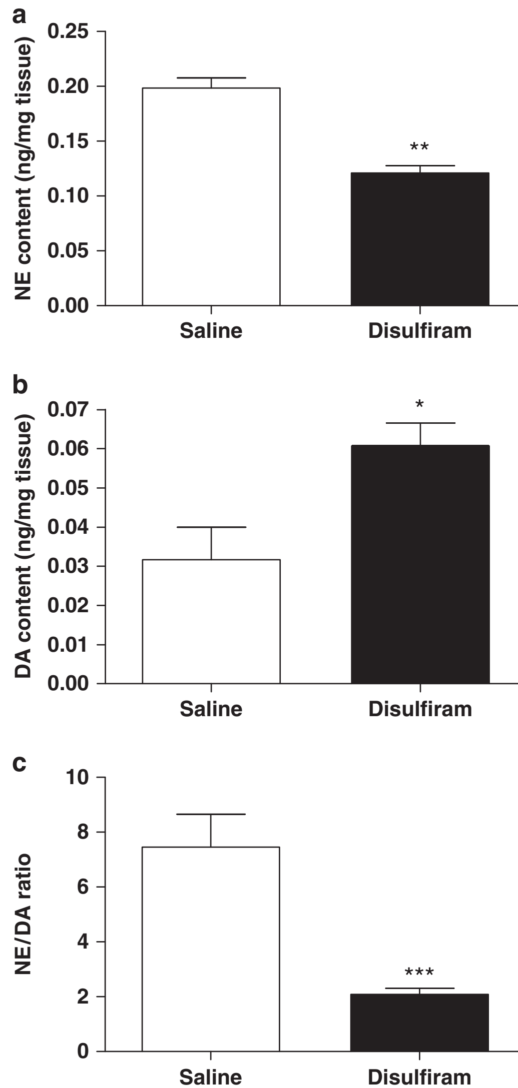

Figure 2 Effect of disulfiram on catecholamine levels in the rat frontal cortex. Shown is the mean ± SEM for (a) NE levels, (b) DA levels, and (c) the NE/DA ratio in the frontal cortex of rats after treatment with saline or disulfiram (single injection of 100 mg/kg, i.p., catecholamines measured 2 h after disulfiram administration by HPLC followed by electrochemical detection; $N = 6$ per group). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ compared with saline.

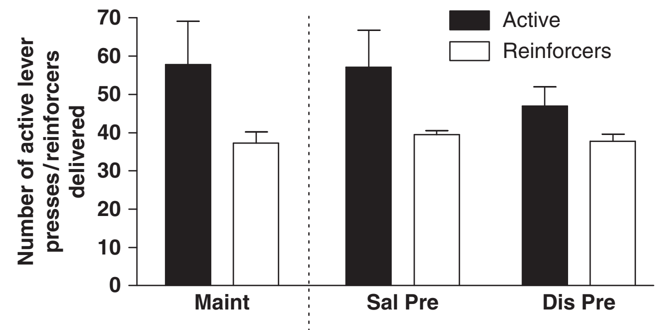

Figure 3 Disulfiram does not affect maintenance of cocaine self-administration. After reaching maintenance levels for operant cocaine self-administration (Maint), rats were pretreated with saline (Sal Pre) or disulfiram (100 mg/kg, i.p.; Dis Pre) 2 h before cocaine self-administration sessions. Shown are mean ± SEM active lever responses and number of reinforcers obtained over a 2-h session. Maintenance values reflect an average number of responses and reinforcers obtained over the last 3 days of maintenance. Occasional active lever pressing during the 20-s timeout periods result in more active lever presses than reinforcers received. $N = 8$ per group.

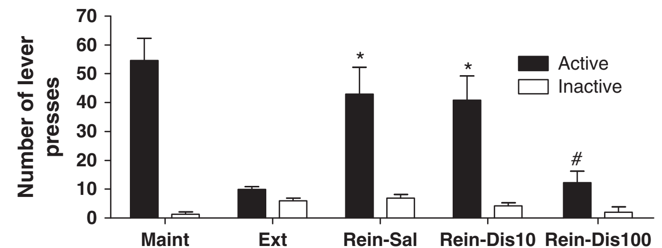

Figure 4 Disulfiram blocks cocaine-primed reinstatement. Once maintenance (Maint) and extinction (Ext) criteria for operant cocaine self-administration were met, rats were pretreated with saline (Rein-Sal, $N = 13$) or disulfiram (10 or 100 mg/kg, i.p.) (Rein-Dis10, $N = 6$ and Rein-Dis100, $N = 7$) 2 h before cocaine prime (10 mg/kg, i.p.) and placement into the self-administration chambers. Shown are active and inactive lever responses. Maintenance values reflect an average of the last 3 days of maintenance sessions, and extinction values reflect an average of the last 3 days of extinction. $^{*}p < 0.05$ compared with active lever responses during extinction, $^{\#}p < 0.05$ compared with active lever responses during cocaine-induced reinstatement tests with saline pretreatment ($N = 7$ per group).

Disulfiram Blocks Cocaine-Primed Reinstatement of Cocaine Seeking

We next tested the effects of disulfiram on drug-primed reinstatement of cocaine seeking. Following the attainment of stable self-administration and extinction, rats were treated with saline or disulfiram (100 mg/kg, i.p.) before a noncontingent priming injection of cocaine (10 mg/kg, i.p.). Rats that were pretreated with saline showed a robust reinstatement of responding on the previously active lever following cocaine prime. In contrast, disulfiram pretreatment completely blocked cocaine-primed reinstatement (Figure 4). ANOVA revealed a significant effect of treatment phase ($F_{4,51} = 8.17$, $p < 0.0001$), and Bonferroni *post hoc* analysis showed a significant difference between extinction responding and cocaine-primed reinstatement following saline pretreatment ($t = 3.62$, $p < 0.05$), but not between extinction responding and disulfiram pretreatment ($t = 0.22$, $p > 0.05$). In addition, there was a significant difference between reinstatement responding with saline pretreatment and disulfiram pretreatment ($t = 2.81$, $p < 0.05$). There was no effect of pretreatment on inactive lever responding.

We next tested the ability of a lower dose of disulfiram (10 mg/kg, i.p.) to attenuate cocaine-primed reinstatement. This dose of disulfiram, which we found in pilot studies to be the highest one that does not significantly reduce NE levels in the frontal cortex (vehicle = 0.32 ± 0.04 ng/mg tissue, disulfiram = 0.29 ± 0.08, $p > 0.05$, $n = 4$ per group), did not impair cocaine-primed reinstatement (Figure 4). Bonferroni *post hoc* analysis showed a significant difference between extinction responding and cocaine-primed reinstatement following saline ($t = 3.62$, $p < 0.05$) or low-dose

disulfiram pretreatment ($t = 2.69$, $p < 0.05$), but not between saline and low-dose disulfiram pretreatment ($t = 0.18$, $p > 0.05$).

Nepicastat Blocks Cocaine-Primed Reinstatement of Cocaine Seeking

The previous experiments indicated that a dose high enough to inhibit DBH is required for the efficacy of disulfiram in blocking cocaine-primed reinstatement. However, because DBH has many other targets, it was unclear whether DBH inhibition alone was sufficient to block reinstatement. Thus, we repeated the self-administration experiments with the selective DBH inhibitor, nepicastat, at a dose (50 mg/kg, i.p.) that inhibited DBH to a similar extent as the effective dose of disulfiram (100 mg/kg, i.p.) (Figure 5), and found that nepicastat pretreatment mimicked the effects of disulfiram in several ways. First, nepicastat had no affect on the maintenance phase of cocaine self-administration (Figure 6). Repeated-measures ANOVA revealed a nonsignificant trend for active lever presses ($F_{26,2} = 3.36$, $p = 0.06$) and no effect on reinforcers obtained ($F_{26,2} = 0.38$, $p = 0.69$). Inactive lever presses were negligible and did not differ between groups. Second, nepicastat blocked cocaine-primed reinstatement (Figure 7). Repeated-measures ANOVA revealed a significant effect of treatment phase ($F_{3,23} = 18.14$, $p < 0.0001$), and Bonferroni *post hoc* analysis showed a significant difference between extinction responding and cocaine-primed reinstatement following saline pretreatment ($t = 5.17$, $p < 0.01$) and between vehicle pretreatment and nepicastat

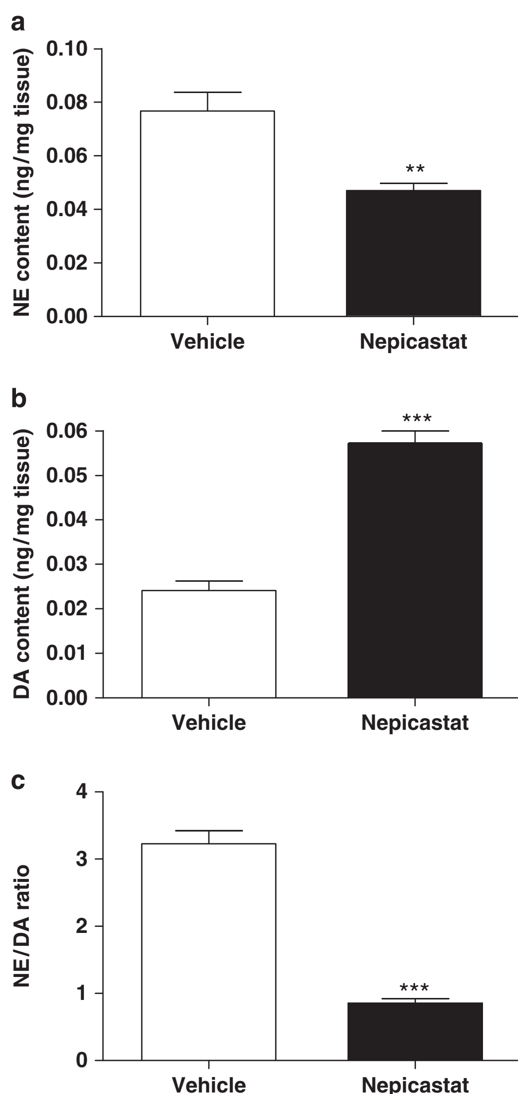

Figure 5 Effect of nepicastat on catecholamine levels in the rat frontal cortex. Shown is the mean ± SEM for (a) NE levels, (b) DA levels, and (c) the NE/DA ratio in the frontal cortex of rats after treatment with vehicle or nepicastat (single injection of 50 mg/kg, i.p., catecholamines measured 2 h after nepicastat administration by HPLC followed by electrochemical detection; $N = 8$ per group). $^{**}p < 0.01$, $^{***}p < 0.001$ compared with vehicle.

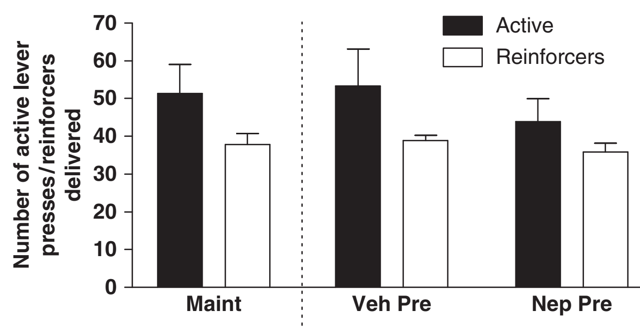

Figure 6 Nepicastat does not affect maintenance of cocaine self-administration. After reaching maintenance levels of operant cocaine self-administration (Maint), rats were pretreated with vehicle (Veh Pre) or nepicastat (50 mg/kg, i.p.; Nep Pre) 2 h before cocaine self-administration sessions. Shown are mean ± SEM active lever responses and number of reinforcers obtained over a 2-h session. Maintenance values reflect an average number of responses and reinforcers obtained over the last 3 days of maintenance. Occasional active lever pressing during the 20-s timeout periods result in more active lever presses than reinforcers received. ($N = 6$ per group).

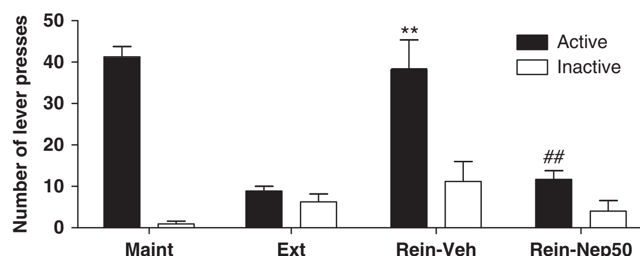

Figure 7 Nepicastat blocks cocaine-primed reinstatement. Once maintenance (Maint) and extinction (Ext) criteria for operant cocaine self-administration were met, rats were pretreated with vehicle (Rein-Veh) or nepicastat (50 mg/kg, i.p.; Rein-Nep50) 2 h before cocaine prime (10 mg/kg, i.p.) and placement into the self-administration chambers. Shown are mean ± SEM active and inactive lever responses. Maintenance values reflect an average of the last 3 days of maintenance sessions, and extinction values reflect an average of the last 3 days of extinction. $^{**}p < 0.01$ compared with active lever responses during extinction, $^{\#\#}p < 0.01$ compared with active lever responses during cocaine-induced reinstatement tests with vehicle pretreatment ($N = 6$ per group).

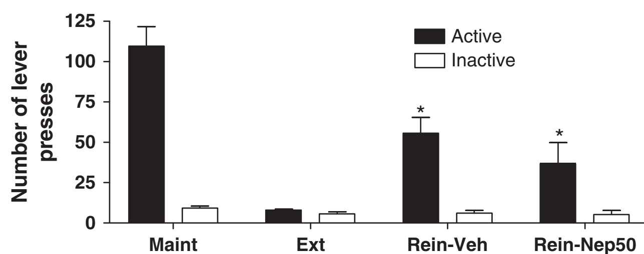

Figure 8 Nepicastat does not affect food-primed reinstatement of food seeking. Once maintenance (Maint) and extinction (Ext) criteria for operant food self-administration were met, rats were pretreated with vehicle (Rein-Veh) or nepicastat (50 mg/kg, i.p.; Rein-Nep50) 2 h before food prime (three pellets at beginning of the session, then one pellet every 3 min over the 60 min session) and placement into the self-administration chambers. Shown are mean ± SEM active and inactive lever responses. Maintenance values reflect an average of the last 3 days of maintenance sessions, and extinction values reflect an average of the last 3 days of extinction. *$p<0.05$ compared with active lever responses during extinction ($N=7$ per group).

pretreatment ($t=4.67$, $p<0.01$), but not between extinction responding and cocaine-primed reinstatement following nepicastat pretreatment ($t=0.5$, $p>0.05$). Pretreatment had no effect on inactive lever responding. Third, nepicastat (50 mg/kg, i.p.) had no effect on food responding; all rats obtained the maximum number of reinforcers possible during the session (61), regardless of pretreatment ($n=8$ per group).

Because the neural and molecular pathways underlying reinstatement of cocaine and food seeking are partially overlapping (Nair *et al*, 2009), we tested whether the attenuation of reinstatement by DBH inhibition was specific to cocaine, and found that nepicastat did not significantly reduce food-primed reinstatement of food seeking (Figure 8). Repeated-measures ANOVA revealed a significant effect of treatment phase ($F_{3,27}=29.49$, $p<0.0001$), and Bonferroni *post hoc* analysis showed a significant difference between extinction responding and cocaine-primed reinstatement following vehicle or nepicastat pretreatment (vehicle $t=4.27$, $p<0.05$; nepicastat $t=2.57$, $p<0.05$), but not between cocaine-primed reinstatement following vehicle and nepicastat pretreatment ($t=1.70$, $p>0.05$). These results indicate that the blockade of cocaine-primed reinstatement by nepicastat cannot be attributed to an inability to perform the operant task and that DBH inhibition does not impair reinstatement of responding for a natural reward.

DISCUSSION

Disulfiram has shown promise as a treatment for cocaine dependence in several clinical trials (Carroll *et al*, 1993, 1998, 2000, 2004; Petrakis *et al*, 2000; George *et al*, 2000; Grassi *et al*, 2007; Pettinati *et al*, 2008). Because concurrent alcohol use is not necessary for disulfiram to have beneficial effects on cocaine addiction, an ALDH-independent mechanism is likely. Furthermore, whatever the underlying molecular mechanism, why disulfiram treatment reduces cocaine use remains unclear; several human laboratory studies have reported conflicting results over how DBH inhibition influences the rewarding and aversive effects of cocaine. The purpose of our study was therefore twofold. First, to gain insight into which aspects of addiction were being altered in the clinic, we determined which 'phase' of cocaine self-administration (ie, maintenance *vs* reinstatement) was affected by disulfiram in rats. Second, to test the hypothesis that disulfiram was acting through DBH inhibition, we used a lower dose of disulfiram that does not inhibit DBH and the selective DBH inhibitor, nepicastat.

Treatments that alter the reinforcing effects of cocaine, such as dopaminergic manipulations, typically change cocaine self-administration behavior (Koob *et al*, 1994). Given the history of NE manipulations and cocaine self-administration, it is not surprising that disulfiram had no effect on maintenance responding for cocaine. NE transporter (NET) inhibitors themselves do not support self-administration, and neither NET inhibitors nor adrenergic receptor antagonists alter cocaine self-administration (Yokel and Wise, 1976; Roberts *et al*, 1977; Woolverton, 1987; Howell and Byrd, 1991; Skjoldager *et al*, 1993; Tella, 1995; Wee *et al*, 2006; Weinshenker and Schroeder, 2007; Gaval-Cruz and Weinshenker, 2009).

Drug addiction is a chronic relapsing disorder (Hunt *et al*, 1971; Leshner, 1997), as patients in treatment often slip back into drug taking after periods of sobriety. Several types of stimuli can trigger drug craving and lead to relapse, including reexposure to the drug, stress, and drug-associated cues; these stimuli also trigger reinstatement in the rat model. The reliability, species generality, as well as face and construct validity of the reinstatement model are high, because they recapitulate many of the features of human addiction (Panlilio and Goldberg, 2007). In contrast to the lack of data to support an influence on the maintenance phase of psychostimulant self-administration, the role of NE in the reinstatement of drug seeking is clear (Erb *et al*, 2000; Weinshenker and Schroeder, 2007; Gaval-Cruz and Weinshenker, 2009). Central infusion of NE itself, or the facilitation of NE transmission with reuptake inhibitors or inhibitory autoreceptor antagonists, induces reinstatement in rats and nonhuman primates (Lee *et al*, 2004; Platt *et al*, 2007; Brown *et al*, 2009). Conversely, blockade of β-adrenergic receptors prevents stress-induced reinstatement, whereas blockade of α1-adrenergic receptors prevents drug-primed reinstatement (Leri *et al*, 2002; Zhang and Kosten, 2005). Because we examined cocaine-primed reinstatement, it is likely that reinstatement was blunted following disulfiram or nepicastat pretreatment due to reduced NE production and a failure to engage α1-adrenergic receptors. The ability of DBH inhibition to block cocaine-primed reinstatement provides further support for the critical role of NE in this paradigm, and we propose that the clinical efficacy of disulfiram, through DBH inhibition and reduction of NE, reduces the risk for relapse. Most disulfiram clinical trials to date have not been designed to examine cocaine relapse specifically. It will be important to build measures into future trials that can distinguish between abstinence due to altered subjective drug effects *vs* healthier responses to environmental triggers.

The evidence available suggests that blockade of cocaine-primed reinstatement by disulfiram involves the impairment of neurotransmission in the nucleus accumbens

(NAc). Both DA release and glutamate release in the NAc are essential for cocaine-primed reinstatement (Schmidt *et al*, 2005; Kalivas, 2009). Noradrenergic neurons project to the mesocorticolimbic DA system, and NE promotes DA transmission, primarily through activation of α1-adrenergic receptors. For example, depletion of NE, or attenuation of α1-adrenergic receptor signaling through genetic, pharmacological, or neurotoxic ways, impairs psychostimulant-induced DA release in the NAc (Darracq *et al*, 1998; Drouin *et al*, 2002; Ventura *et al*, 2003). It is important to note that although DBH inhibition increases tissue levels of DA, it decreases DA release because NE-mediated excitation of DA neurons is reduced (Schank *et al*, 2006; Weinshenker and Schroeder, 2007; Weinshenker *et al*, 2008). Thus, the failure of a cocaine prime to provoke DA release in the NAc may underlie the efficacy of disulfiram in this paradigm. Although proof of a direct role for NE in regulating cocaine-induced glutamate release in the NAc is lacking, we have recently found that α1-adrenergic receptors are enriched in presumptive glutamatergic terminals throughout the mesocorticolimbic system (Rommelfanger *et al*, 2009), and we predict that a loss of noradrenergic tone may also attenuate the glutamate release essential for cocaine-primed reinstatement.

Although the blockade of cocaine-primed reinstatement by disulfiram could involve several targets, our results strongly suggest that it is mediated primarily by DBH inhibition, NE reduction, and a decrease in α1AR signaling, as the effects of disulfiram require a dose that significantly inhibits DBH and are mimicked by the selective DBH inhibitor, nepicastat (present study), and the α1AR antagonist, prazosin (Zhang and Kosten, 2005). What remains unclear is why a reduction of NE/α1AR signaling hampers drug-primed reinstatement, but not the maintenance phase of cocaine self-administration. Earlier findings revealed that blockade of α1ARs does not affect 'conventional' operant responding for cocaine, but does attenuate the escalation of cocaine self-administration elicited by long-access 'binge' paradigms or previous drug sensitization (Zhang and Kosten, 2007; Wee *et al*, 2008). Altogether, these results suggest that although NE does not have a critical role in the primary reinforcing effects of cocaine, as measured by standard operant self-administration, it does have significant effects under conditions that escalate or reinstate drug-seeking behavior. Furthermore, medications that impair NE production, such as disulfiram or nepicastat, may short circuit the ability of environmental triggers to promote relapse, and therefore make promising pharmacotherapies for the treatment of dependence on cocaine and other stimulants.

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DISCLOSURE

JPS, DAC, JRS, MAL, MG, YEO, NP, KGF, PMI, GLE, and PVH, except for income received from their primary employers, have received no financial support or compensation from any individual or company entity over the past 3 years for research or professional service, and there are no personal financial holdings that could be perceived as constituting a conflict of interest. DW, over the past 3 years, has received research funds from Cephalon Pharmaceuticals, the developer of therapeutics for central nervous system disorders, and is co-inventor on a patent concerning the use of selective DBH inhibitors for the treatment of cocaine dependence (US-2010-0105748-A1; 'Methods and Compositions for Treatment of Drug Addiction').

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