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ScienceDirect
Pharmacology, Biochemistry and Behavior 89 (2008) 556–562
PHARMACOLOGY BIOCHEMISTRY AND BEHAVIOR
www.elsevier.com/locate/pharmbiochembeh

Effects of disulfiram and dopamine beta-hydroxylase knockout on cocaine-induced seizures

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Received 4 December 2007; received in revised form 30 January 2008; accepted 6 February 2008
Available online 12 February 2008

Abstract

The antialcoholism drug disulfiram has shown recent promise as a pharmacotherapy for treating cocaine dependence, probably via inhibition of dopamine β-hydroxylase (DBH), the enzyme that catalyzes the conversion of dopamine (DA) to norepinephrine (NE). We previously showed that DBH knockout (*Dbh* −/−) mice, which lack NE, are susceptible to seizures and are hypersensitive to the psychomotor, rewarding, and aversive effects of cocaine, suggesting that disulfiram might exacerbate cocaine-induced seizures (CIS) by inhibiting DBH. To test this, we examined CIS in wild-type and *Dbh* −/− mice following administration of disulfiram or the selective DBH inhibitor nepicastat. We found that *Dbh* genotype had no effect on CIS probability or frequency, whereas disulfiram, but not nepicastat, increased the probability of having CIS in both wild-type and *Dbh* −/− mice. Both disulfiram and nepicastat increased CIS frequency in wild-type but not *Dbh* −/− mice. There were no genotype or treatment effects on serum cocaine levels, except for an increase in disulfiram-treated *Dbh* −/− mice at the highest dose of cocaine. These results suggest that disulfiram enhances CIS via two distinct mechanisms: it both increases CIS frequency by inhibiting DBH and increases CIS frequency in a DBH-independent manner.

Keywords: Norepinephrine; Dopamine beta-hydroxylase; Disulfiram; Nepicastat; Seizure; Cocaine

1. Introduction

Cocaine is considered the most potent stimulant of natural origin, yet despite a host of negative social, psychological, and medical consequences of the drug's abuse, there are currently no widely used pharmacotherapies for cocaine addiction. Recently, the compound disulfiram, also known as Antabuse has shown promise as a candidate pharmacotherapy. Disulfiram has been FDA approved for the treatment of alcohol dependence for over 50 years. Its efficacy is due to the inhibition of the enzyme aldehyde dehydrogenase, which induces an aversive reaction following alcohol consumption by elevating toxic aldehydes in the liver. This "Antabuse reaction" manifests as flushing, headache, nausea, weakness, dizziness, anxiety, vertigo, and ataxia. Interestingly, disulfiram has been shown to decrease cocaine intake regardless of concurrent alcohol consumption (Carroll et al., 1998, 2000; George et al., 2000; Petrakis et al., 2000), although the mechanisms by which it does so have not been fully elucidated. Nevertheless, because the combination of disulfiram and cocaine does not result in aldehyde accumulation, aldehyde dehydrogenase inhibition probably cannot account for the efficacy of disulfiram here. Because its major metabolite (diethyldithiocarbamate) is a copper chelator (Johansson, 1989), disulfiram affects enzymatic reactions that require copper as a cofactor. One hypothesis to account for disulfiram's efficacy in reducing cocaine intake is its inhibition of dopamine β-hydroxylase (DBH). DBH is a copper-containing monooxygenase enzyme that converts dopamine (DA) to norepinephrine (NE), thus controlling NE production and consequently the NE/DA ratio in noradrenergic

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0091-3057/$ - see front matter © 2008 Elsevier Inc. All rights reserved.
doi:10.1016/j.pbb.2008.02.009

neurons. Alteration of this ratio has been found to alter behavioral responsivity to cocaine in rodents and humans. For example, DBH knockout (*Dbh* −/−) mice are hypersensitive to the locomotor, rewarding, and aversive effects of cocaine (Schank et al., 2006). Pharmacological inhibition of DBH with disulfiram, which decreases the NE/DA ratio in the rodent brain (Goldstein et al., 1964; Musacchio et al., 1966; Karamanakos et al., 2001; Bourdelát-Parks et al., 2005), facilitates the development of behavioral sensitization to cocaine (Haile et al., 2003). Furthermore, a common polymorphism in the *Dbh* gene influences both DBH enzymatic activity and cocaine-induced paranoia (Zabetian et al., 2001; Kalayasiri et al., 2007).

Noradrenergic transmission has been implicated in the modulation of seizure activity (reviewed by Weinshenker and Szot, 2002). Enhancement of noradrenergic transmission suppresses seizure activity (Lindvall et al., 1988; Weinshenker et al., 2001; Kaminski et al., 2005), whereas norepinephrine depletion with 6-hydroxydopamine or disulfiram exacerbates seizures and facilitates seizure kindling (Corcoran et al., 1974; Callaghan and Schwark, 1979; McIntyre, 1980; Abed, 1994; Amabeouku and Syce, 1997), and *Dbh* −/− mice have increased susceptibility to seizure induced by flurothyl, pentylenetetrazole, kainic acid, and sound (Szot et al., 1999).

Approximately 31% of all drug-related emergency room episodes are related to cocaine abuse (SAMHSA, 2005). Cocaine-induced seizures are a manifestation of the toxicity associated with the drug, and estimates are that 8–12% of patients admitted to emergency departments with cocaine intoxication have seizures (Derlet and Albertson, 1989; Dhuna et al., 1991; Koppel et al., 1996). These seizures can be resistant to common anticonvulsant drugs, such as benzodiazepines and barbiturates, and constitute a major fraction of cocaine-related deaths (Dhuna et al., 1991; Benowitz, 1993). In addition, there have been several reports of individuals without a history of epilepsy developing seizures following treatment with therapeutic doses of disulfiram (Liddon and Satran, 1967; Price and Silberfarb, 1976a,b; McConchie et al., 1983; Daniel et al., 1987).

Concurrent use of cocaine and disulfiram is now on the rise, as disulfiram is under evaluation as a pharmacotherapy for cocaine dependence. Because pharmacological or genetic inhibition of DBH increases the sensitivity to seizures and the behavioral effects of cocaine, we sought to examine the effects of DBH and disulfiram on susceptibility to cocaine-induced seizures (CIS). We measured the probability of having a seizure and the frequency of CIS following a high dose of cocaine (60 mg/kg) in both wild-type (*Dbh* +/+) and *Dbh* −/− mice. We hypothesized that (1) *Dbh* −/− mice would be hypersensitive to cocaine-induced seizures (CIS) and (2) disulfiram would exacerbate CIS in a *Dbh* genotype-dependent manner. To further examine whether disulfiram affects cocaine responses via a DBH-dependent mechanism, we also tested the selective DBH inhibitor nepicastat (Stanley et al., 1997). To determine whether the effects of these drugs could be attributed to changes in cocaine metabolism, we also measured peak serum cocaine levels.

2. Methods

2.1. Animals and housing

Adult *Dbh* +/+ and −/− mice maintained on a mixed 129/SvEv and C57BL6/J background were developed and generated as previously described (Thomas et al., 1995; Thomas and Palmiter, 1998). Genotypes were confirmed by PCR. All mice were reared in a specific pathogen-free facility with a 12-h light/dark cycle (lights on at 0700 h, lights off at 1900 h); food and water were available ad libitum. Naïve mice between 3 and 6 months of age were used for all experiments, as were both male and female mice. No sex differences were observed, and results were combined. Experimental protocols were approved by the Emory University IACUC and met the guidelines of the Association for Assessment and Accreditation of Laboratory Animal Care.

2.2. Cocaine-induced seizures

Mice were given 3 injections of saline, disulfiram (100 mg/kg, i.p.), or the selective DBH inhibitor nepicastat (100 mg/kg, i.p.), with 2 h between each injection. Two hours following the last injection, all mice were injected with a high dose of cocaine (60 mg/kg, i.p.). This dose was found to induce seizures in ~50% of mice of the same strain during a pilot study. Mice were observed for 30 min following cocaine administration, and the latency to first seizure and seizure frequency were recorded. The first seizure and/or ataxia typically occurred within 2–4 min postinjection. Seizures were defined as repetitive, rapid periods of jumping, wild-running, tonic–clonic activity, or a loss of the righting reflex. N=9–16 for each treatment group.

2.3. Cocaine metabolism

Mice were given 3 injections of either saline or disulfiram (100 mg/kg, i.p.) each 2 h apart. Two hours following their last injection, they were injected with cocaine (20 or 60 mg/kg, i.p.). These two doses were chosen because the 20-mg/kg dose supports a conditioned place preference in control mice, but elicits a conditioned place aversion in *Dbh* −/− mice, while the 60-mg/kg dose was the one used to produce seizures in this study. Mice were decapitated 5 min later. We chose this time point because cocaine levels peak in mice ~5 min after i.p. cocaine administration (Benuck et al., 1987). Trunk blood was collected in microcentrifuge tubes containing 5 µl each of NaF (132 mg/ml) and $(COOK)_2$ (106.7 mg/ml). Blood was centrifuged for 5 min at 10000 rpm and serum was isolated and frozen until analysis. Serum cocaine levels were measured by HPLC. N=3–8 for each genotype and treatment group.

2.4. Measurement of serum cocaine levels by HPLC

Cocaine was quantified in mouse serum by extraction with preparatory columns and isocratic HPLC with UV detection. Varian Bond Elut Certify C18 (130 mg) preparatory columns were placed on a vacuum manifold and pretreated with 2 ml of methanol and then 2 ml of 100 mM phosphate buffer (pH 6) at

constant pressure (5 psi). Two hundred µl of calibrators, controls, and samples were mixed by vortexing with 20 µl of a 10 µg/ml nalorphine solution (internal standard), 1.8 ml of saline, and 1 ml of 100 mM phosphate buffer (pH 6). Columns were washed with 6 ml of Milli-Q water, then 3 ml of 1 M acetic acid solution, and finally vacuum dried. Next, the columns were washed with 6 ml of methanol, and then cocaine was eluted with 2 ml of dichloromethane:isopropanol (80:20) containing 2% ammonium hydroxide. Final sample eluates were dried to residue with streaming nitrogen; residues were redissolved in 250 µl of mobile phase, then 100 µl of each sample was injected into the HPLC system [Waters model 510 pump, Waters 717 sample injector, Waters 2587 UV detector; a Phenomenex C18 column (5 micron, 4.5 mm ID × 150 mm L)]. The flow rate of the mobile phase was 1.5 ml/min. Cocaine, BE, and nalorphine were detected at a fixed wavelength of 214 nm. The mobile phase contained 8% acetonitrile, 12% methanol, and 80% of a solution of 12 mM KH_2PO_4 (pH 2.5). Calibrators were spiked mouse serum at concentrations of 100, 500, 1000, 5000, and 10,000 ng/ml. Cocaine and benzoylecgonine concentrations were expressed in ng/ml.

2.5. Drugs

All drugs were freshly prepared before being used and injected i.p. in a volume of 10 ml/kg. Cocaine HCl (Sigma Aldrich, St Louis, MO) and nepicastat (Roche Biosciences, Palo Alto, CA) were dissolved in 0.9% NaCl. Disulfiram (Sigma Aldrich, ST Louis, MO) was injected as a suspension in 0.9% NaCl following sonication.

2.6. Statistical analyses

The effect of disulfiram and nepicastat on the probability of an animal exhibiting a cocaine-induced seizure was analyzed using the χ^2 distribution. The effect of the DBH inhibitors on the number of cocaine-induced seizures exhibited in a 30-minute period and serum cocaine levels were analyzed using a 2-way completely randomized design ANOVA with post-hoc Bonferroni comparisons. A p-value of <0.05 was considered significant. Outliers were detected using the Grubb's test. One subject (disulfiram-treated *Dbh*−/−) met this criteria and was removed from all statistical analysis.

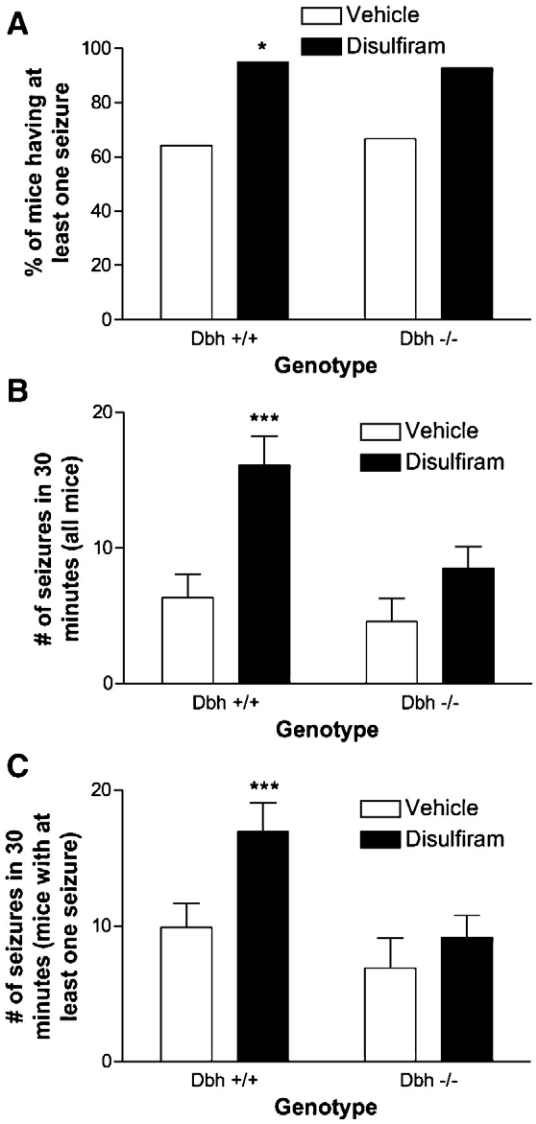

Fig. 1. The effects of disulfiram and *Dbh* genotype on cocaine-induced seizures. *Dbh* +/+ and *Dbh* −/− mice were pretreated with vehicle or disulfiram (3 doses of 100 mg/kg, i.p., each dose spaced 2 h apart). Two hours after the last pretreatment, mice were injected with cocaine (60 mg/kg, i.p.), and behavior was observed for 30 min. Shown is (A) the percent of all mice tested having at least one seizure, (B) the mean ± SEM seizures observed in 30 min in all mice tested, and (C) the mean ± SEM seizures only in mice that had at least one seizure. $N = 14$–20 per genotype and treatment group. *$p<0.05$, ***$p<0.001$ compared to vehicle control for that genotype.

3. Results

3.1. Disulfiram and cocaine-induced seizures

Dbh genotype alone had no effect on CIS probability; 64.2% of *Dbh* +/+ and 66.6% of *Dbh* −/− mice had seizures following cocaine administration (Fig. 1A). Disulfiram pretreatment increased the probability of having at least one cocaine-induced seizure in both *Dbh* +/+ and *Dbh* −/− mice with similar magnitude (*Dbh* +/+, 64.2% saline vs. 95% disulfiram; *Dbh* −/−, 66.6% saline vs. 92.9% disulfiram) (Fig. 1A). This effect was statistically significant in *Dbh* +/+ mice, but did not quite reach significance in *Dbh* −/− mice (*Dbh* +/+, $\chi^2 = 5.346$, $p = 0.02$; *Dbh* −/−, $\chi^2 = 3.027$, $p = 0.08$).

Dbh genotype alone also had no effect on CIS frequency (Fig. 1B); however, disulfiram increased CIS frequency in *Dbh* +/+ but not *Dbh* −/− mice (Fig. 1B). ANOVA revealed a significant effect of pretreatment, with disulfiram significantly increasing the number of seizures during the 30-minute test period following cocaine administration in wild-type (WT) but not knockout (KO) mice ($F = 12.58$, $df = 1$; $p < 0.001$ for WT). An additional ANOVA was performed only on data from subjects exhibiting at least one cocaine-induced seizure. Analysis of seizure frequency in these animals showed the

same effect of disulfiram, as pretreatment with the drug significantly increased seizure frequency only in wild-type mice ($F=14.13$, $df=1$; $p<0.001$) (Fig. 1C).

3.2. Nepicastat and cocaine-induced seizures

To further determine whether the effects on CIS from disulfiram were mediated by DBH inhibition, we tested the selective DBH inhibitor nepicastat. In contrast to disulfiram, nepicastat pretreatment did not affect the probability of having a cocaine-induced seizure in wild-type mice, and in fact significantly reduced this probability in *Dbh* −/− mice ($\chi^2=5.35$; $p<0.05$) (Fig. 2A).

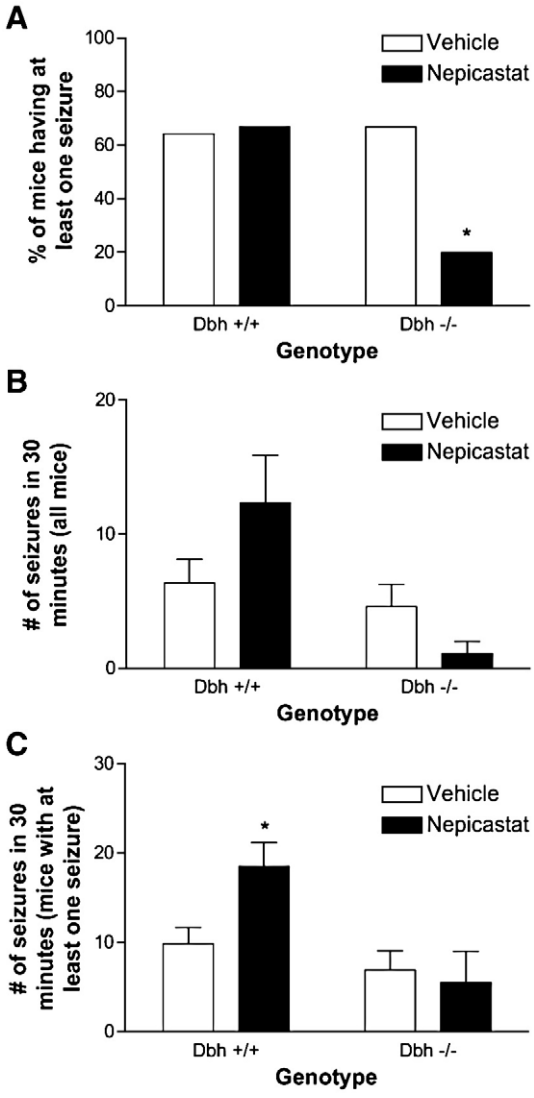

Fig. 2. The effects of nepicastat and *Dbh* genotype on cocaine-induced seizures. *Dbh* +/+ and *Dbh* −/− mice were pretreated with vehicle or nepicastat (3 doses of 100 mg/kg, i.p., each dose spaced 2 h apart). Two hours after the last pretreatment, mice were injected with cocaine (60 mg/kg, i.p.), and behavior was observed for 30 min. Shown is (A) the percent of all mice tested having at least one seizure, (B) the mean±SEM seizures observed in 30 min in all mice tested, and (C) the mean±SEM seizures only in mice that had at least one seizure. $N=9$–15 per genotype and treatment group. *$p<0.05$ compared to vehicle control for that genotype.

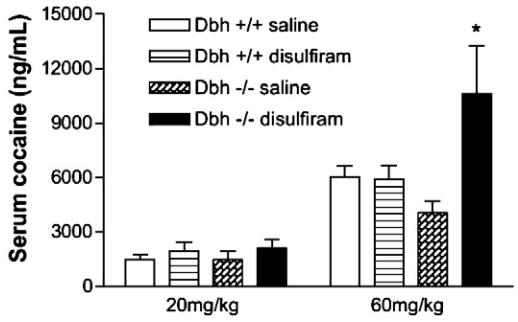

Fig. 3. The effects of disulfiram and *Dbh* genotype on cocaine metabolism. *Dbh* +/+ and *Dbh* −/− mice were pretreated with vehicle or disulfiram (3 doses of 100 mg/kg, i.p., each dose spaced 2 h apart). Two hours after the last pretreatment, mice were injected with cocaine (20 or 60 mg/kg, i.p.), and blood was collected 5 min later. Shown is the mean±SEM peak serum cocaine levels as measured by HPLC. $N=6$–8 per genotype and treatment group. *$p<0.05$ compared to vehicle control for that genotype.

The pattern of nepicastat effects on CIS frequency was similar to that of disulfiram. Nepicastat pretreatment increased CIS frequency in wild-type but not *Dbh* −/− mice. The effect on *Dbh* +/+ mice did not quite reach significance when all mice were included in the analysis ($F=0.3672$, $df=1$; $p>0.05$) (Fig. 2B), but did reach significance when data from animals exhibiting at least one seizure were analyzed ($F=1.518$, $df=1$; $p<0.05$) (Fig. 2C).

3.3. Cocaine metabolism

To determine whether the disulfiram-related rise in CIS probability and frequency could be attributed to drug effects on cocaine metabolism, we measured peak serum cocaine levels in *Dbh* +/+ and −/− mice after administration of 20 or 60 mg/kg of cocaine. We found that cocaine serum levels were unaffected either by *Dbh* genotype or by disulfiram in most cases. The exception was an increase in serum cocaine levels by disulfiram in *Dbh* −/− mice at the high dose of cocaine, and ANOVA revealed a genotype×treatment interaction ($F=5.312$, $df=1$; $p<0.05$) (Fig. 3). These results indicate that changes in cocaine metabolism do not underlie the effects of disulfiram on CIS.

4. Discussion

Cocaine-induced seizures account for approximately 10% of cocaine-related emergency room visits to hospitals and are a common manifestation of cocaine toxicity. While there are no widely accepted pharmacotherapies for cocaine addiction, disulfiram has shown recent promise as a treatment for cocaine dependence. Unfortunately, this treatment may be hazardous to some patients, due to its mechanism of action. Disulfiram inhibits the enzyme DBH, which could lead to increases in seizures and cocaine sensitivity. Therefore, we chose to examine how pharmacological DBH inhibition and DBH genotype affects CIS probability and frequency in an animal model. In order to assess this, we pretreated *Dbh* +/+ and −/− mice with disulfiram or nepicastat, a direct, selective DBH inhibitor, prior to administering a high dose of cocaine. We hypothesized that

Dbh −/− mice would be hypersensitive to CIS and that disulfiram would exacerbate CIS in a *Dbh* genotype-dependent manner. We also assessed changes in serum cocaine levels to determine whether the effects of these drugs could be attributed to changes in cocaine metabolism.

Disulfiram had two distinct effects on CIS; it increased the probability of having a seizure in *Dbh* +/+ and *Dbh* −/− mice and increased CIS frequency in *Dbh* +/+ mice only. Nepicastat did not increase seizure probability, but increased the frequency of CIS in *Dbh* +/+ mice only. These results indicate that pharmacological DBH inhibition is responsible for increasing the frequency of CIS, while disulfiram's ability to raise the probability of CIS is mediated by a DBH-independent mechanism. Given disulfiram's mechanism of action as a copper chelator, the inhibition of cocaine metabolic enzymes, such as cholinesterase and carboxylesterase, could underlie its effects on CIS probability. However, we did not find this to be the case, as disulfiram did not alter serum cocaine concentrations in wild-type mice. Interestingly, nepicastat actually tended to inhibit CIS in *Dbh* −/− mice. Nepicastat does not chelate copper and is a direct, potent inhibitor of DBH, and initial screening did not reveal any other high-affinity targets aside from DBH. It appears that the lack of DBH in *Dbh* −/− mice revealed a secondary anticonvulsant target for nepicastat.

Because pharmacological DBH inhibition increased CIS frequency, *Dbh* −/− mice would be expected to demonstrate more frequent CIS. We did not find this to be the case. One possible explanation is that compensatory changes in monoamine neurotransmitters arise following chronic and complete knockout of DBH and NE function. One likely compensatory candidate is the serotonin (5-HT) system. Serotonergic activity modulates seizure activity in response to cocaine and seems to have proconvulsant effects. Selective 5-HT reuptake inhibitors, such as fluoxetine, citalopram, paroxetine and the tricyclic antidepressant imipramine, all facilitate CIS (O'Dell et al., 1999; Ritz and George, 1997), whereas 5-HT_2 receptor antagonists decrease CIS (O'Dell et al., 1999, 2000a,b; Ritz and George, 1997; Schechter and Meehan, 1995). Since cocaine is known to inhibit 5-HT transporters (SERT), increases in 5-HT following cocaine administration can lead to accumulation of serotonin in synapses, which in turn can increase seizure activity via 5-HT_2 receptor activation. The serotonergic raphe nuclei receive dense innervation from brainstem noradrenergic nuclei (Baraban and Aghajanian, 1981; Marcinkiewicz et al., 1989; Fritschy and Grzanna, 1990; Peyron et al., 1996), and activation of α_1-adrenergic receptors increases tonic excitatory activity in the dorsal raphe nucleus (Baraban and Aghajanian, 1980; Vandermaelen and Aghajanian, 1983; Hertel et al., 1998; Pudovkina et al., 2003; Judge and Gartside, 2006). Therefore, *Dbh* −/− mice should have lower levels of extracellular 5-HT, because they lack the noradrenergic excitatory drive on the serotonergic system. Indeed, when compared with control mice, *Dbh* −/− mice have decreased 5-HT release in the nucleus accumbens following amphetamine treatment (D. Weinshenker and S. Puglisi-Allegra, unpublished observations), as well as in the hippocampus following fluoxetine administration (Cryan et al., 2004). This decreased concentration of the proconvulsant 5-HT in *Dbh* −/− mice may explain their "normal" CIS susceptibility at baseline.

Because disulfiram acts as a copper chelator, this drug is relatively nonspecific and inhibits two cocaine-metabolizing enzymes, cholinesterase and carboxylesterase (Zemaitis and Greene, 1976; Nousiainen and Törrönen, 1984; Savolainen et al., 1984). Disulfiram treatment increased cocaine plasma levels and decreased cocaine clearance in humans following intranasal cocaine administration (McCance-Katz et al., 1998a, b; Hameedi et al., 1995; Baker et al., 2007). Thus, it is possible that the disulfiram-induced increase in CIS was a direct result of decreased cocaine metabolism. However, disulfiram did not alter peak serum cocaine levels in most cases in our study. The single exception was an interaction between disulfiram treatment and genotype; the higher dose of cocaine (60 mg/kg) significantly increased cocaine plasma levels in *Dbh* −/− mice only. The mechanism underlying this synergy is unclear. One possibility is that NE limits the spread of cocaine through the bloodstream via its vasoconstrictive properties, while cholinesterase and carboxylesterase are responsible for its metabolism. Perhaps at low doses of cocaine, either mechanism alone is sufficient to maintain "normal" peak cocaine serum levels, but at high doses, the impairment of both noradrenergic function and cocaine metabolic enzymes results in increased serum levels. It is not clear why disulfiram did not increase peak serum cocaine levels in wild-type mice. The differences between the effects of disulfiram on serum cocaine levels in humans and rodents may be due to different routes of cocaine administration (intranasal in humans vs. intraperitoneal in mice) or to species differences in cocaine metabolism.

Our results show that acute disulfiram administration increases the probability and frequency of CIS, which may present a clinical problem during cocaine addiction treatment with disulfiram pharmacotherapy. It should be noted that, while CIS have not been reported during cocaine dependence clinical trials examining disulfiram efficacy, there have been several reports of individuals without a history of epilepsy developing seizures following treatment with therapeutic doses of disulfiram (Liddon and Satran, 1967; Price and Silberfarb, 1976a,b; McConchie et al., 1983; Daniel et al., 1987). Thus, clinicians should be cautious when considering disulfiram as a cocaine pharmacotherapy, particularly in patients with a history of epilepsy or cocaine overdose. The more selective DBH inhibitor nepicastat may be a safer alternative to disulfiram for treating cocaine dependence, as it does not increase CIS probability and is in fact anticonvulsant in *Dbh* −/− mice. DBH activity is genetically controlled and highly variable in humans (Weinshilboum, 1978; Zabetian et al., 2001). The haplotype associated with low DBH activity in humans is also associated with more cocaine-induced paranoia (Cubells et al., 2000; Kalayasiri et al., 2007). This increase in one of the aversive properties of cocaine may underlie the effectiveness of DBH inhibition via disulfiram in curbing cocaine intake. Given that the proconvulsant effect of disulfiram on CIS frequency is absent in *Dbh* −/− mice, disulfiram pharmacotherapy might perhaps be safer for cocaine addicts with low DBH activity. Preliminary data suggest that disulfiram is most effective for these individuals

(R. Schottenfeld and J. Cubells, personal communication), possibly due to their enhanced aversion to cocaine. Our results indicate that they may also be resilient to disulfiram-induced exacerbation of CIS and possibly other toxic effects of cocaine.

Acknowledgements

We thank Dainippon Sumitomo Pharma, Co., Ltd. and Roche Pharmaceuticals for their generous donation of DOPS and nepicastat, respectively. This work was funded in part by the National Institute of Drug Abuse (DA017963 and DA015040).

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