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The σ-Receptor Antagonist BD-1063 Decreases Ethanol Intake and Reinforcement in Animal Models of Excessive Drinking

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σ-Receptors (SigRs) have been implicated in behavioral and appetitive effects of psychostimulants and may also modulate the motivating properties of ethanol. This study tested the hypothesis that SigRs modulate ethanol reinforcement and contribute to excessive ethanol intake. The effects of subcutaneous treatment with the potent, selective Sig-1R antagonist BD-1063 on operant ethanol self-administration were studied in two models of excessive drinking—Sardinian alcohol-preferring (sP) rats and acutely withdrawn ethanol-dependent Wistar rats—and compared to ethanol self-administration in nondependent Wistar controls. To assess the specificity of action, the effects of BD-1063 on self-administration of an equally reinforcing saccharin solution were determined in Wistar and sP rats. Gene expression of Sig-1R in reward-related brain areas implicated in ethanol reinforcement was compared between ethanol-naive sP and Wistar rats and withdrawn ethanol-dependent Wistar rats. BD-1063 dose dependently reduced ethanol self-administration in sP rats (3.3–11 mg/kg) and withdrawn, dependent Wistar rats (4–11 mg/kg) at doses that did not modify mean ethanol self-administration in nondependent Wistar controls. BD-1063 did not reduce concurrent water self-administration and did not comparably suppress saccharin self-administration, suggesting selectivity of action. BD-1063 also reduced the breakpoints of sP rats to work for ethanol under a progressive-ratio reinforcement schedule. Ethanol-naive sP rats and 24-h withdrawn, dependent Wistar rats showed reduced Sig-1R mRNA expression in the nucleus accumbens. The results suggest that SigR systems may contribute to innate or ethanol-induced increases in susceptibility to self-administer high ethanol levels, identifying a potential neuroadaptive mechanism contributing to excessive drinking and a therapeutic target for alcohol abuse and dependence.
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INTRODUCTION

Alcoholism is a multifactorial, chronic disorder of compulsive alcohol use (McLellan *et al*, 2000). Animal models that mimic alcoholism are unattainable, but syndrome components can be modeled (Koob *et al*, 1998). Excessive ethanol self-administration has been observed in rodents selectively bred for ethanol intake and in outbred rats withdrawn from ethanol following induction of ethanol dependence (McBride and Li, 1998; Rimondini *et al*, 2002; Roberts *et al*, 2000; Rogers *et al*, 1979). Use of such animal models has identified a role for opioid peptide systems in the acute reinforcing effects of ethanol and excessive drinking (Lovinger and Crabbe, 2005). Although much work has explored the role of opioid peptide systems and related peptides (eg nociceptin) on the reinforcing effects of ethanol, little work has examined the role of σ-receptors (SigRs).

SigRs were originally categorized as members of the opioid receptor family (Martin *et al*, 1976) and a high-affinity phencyclidine-binding site (Quirion *et al*, 1981). However, it is now known that SigRs are unique binding sites that differ from other known mammalian proteins (Gundlach *et al*, 1985; Walker *et al*, 1990). Two different SigR subtypes are known, Sig-1R and Sig-2R, differing in

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their binding profile and molecular weight (Hanner *et al*, 1996; Hellewell and Bowen, 1990; Moebius *et al*, 1993). The Sig-1R gene (Mei and Pasternak, 2001; Pan *et al*, 1998; Seth *et al*, 1998) encodes a 29 kDa polypeptide containing one or two putative transmembrane domains. Sig-1Rs are synthesized and expressed widely in rat brain, especially throughout limbic areas and brainstem motor structures. The highest levels of immunoreactivity are observed in the olfactory bulb, hypothalamus, and hippocampus, but the caudate putamen, septum, nucleus accumbens, and amygdala show moderately concentrated, intense labeling (Alonso *et al*, 2000; Bouchard and Quirion, 1997; Phan *et al*, 2003). The anatomical distribution of SigRs, which immunostain at synaptic contacts (Maurice *et al*, 2002; Alonso *et al*, 2000), suggests that SigRs may modulate motivationally relevant synaptic transmission, including that of drugs of abuse.

Accordingly, SigRs are implicated in actions of psychostimulants. SigR antagonists block cocaine-induced c-*fos* expression, locomotion, place preference conditioning, seizures, and lethality in rodents (Maurice and Romieu, 2004; McCracken *et al*, 1999; Menkel *et al*, 1991; Witkin *et al*, 1993). Likewise, the SigR antagonists BD-1063 and/or BD-1047 attenuated the locomotor stimulatory effects of methamphetamine and 3,4-methylenedioxymethamphetamine in mice (Brammer *et al*, 2006; Romieu *et al*, 2002), and MS-377 attenuated the development of behavioral sensitization to methamphetamine (Takahashi *et al*, 2000). A SigR antagonist also attenuated contextual reinstatement of cocaine-directed responding (Martin-Fardon *et al*, 2007). Many actions of SigR antagonists are shared by Sig-1R receptor antisense oligodeoxynucleotides (Brammer *et al*, 2006; Romieu *et al*, 2002). Furthermore, repeated passive cocaine (Romieu *et al*, 2002) and methamphetamine exposure (Itzhak, 1993) increased Sig-1R mRNA expression in several discrete brain regions, as did daily methamphetamine self-administration (Stefanski *et al*, 2004). Accordingly, activation and overexpression of Sig-1Rs are putative primary cellular responses to psychostimulants proposed to underlie addiction-associated neuroadaptations (Maurice and Romieu, 2004; Su and Hayashi, 2003; Takebayashi *et al*, 2002).

Few studies have examined SigR involvement in ethanol's actions. Although it often has been assumed that SigR antagonist-sensitive actions of cocaine and methamphetamine reflect direct molecular interactions, the psychostimulants in fact bind with only micromolar affinity to SigRs (Brammer *et al*, 2006; Nguyen *et al*, 2005; Sharkey *et al*, 1988). Thus, indirect SigR-mediated effects may exist for all substances of abuse, including ethanol. Indeed, the SigR antagonist BD-1047 dose dependently attenuated ethanol-induced locomotion and blocked ethanol-induced place and taste conditioning. Moreover, Sig-1R gene functional polymorphisms that correlate with alterations in receptor transcription were overrepresented in a Japanese population of alcoholic subjects (Miyatake *et al*, 2004). The purpose of this study was therefore to test the hypothesis that SigRs modulate reinforcing effects of ethanol. For this, we examined the effects of systemic administration of the SigR antagonist BD-1063 on operant oral ethanol self-administration in dependent Wistar rats withdrawn from repeated, intermittent ethanol vapor exposure and in genetically selected Sardinian alcohol-preferring (sP) rats (Colombo, 1997; Colombo *et al*, 1995). To assess the selectivity of action against high ('excessive') ethanol drinking, results were compared to those seen in nondependent, outbred Wistar rats and against similar rates of saccharin self-administration. Finally, gene expression of Sig-1R in several reward-related brain areas putatively implicated in ethanol reinforcement (eg nucleus accumbens, ventral tegmental area, central nucleus of the amygdala, basolateral amygdala; Vengeliene *et al*, 2008) were compared between ethanol-naive sP and Wistar rats and acutely withdrawn, dependent Wistar rats.

MATERIALS AND METHODS

Animals

Male Wistar rats ($n = 61$; Charles River, Raleigh, NC) and genetically selected TSRI sP rats ($n = 34$) were subjects. TSRI sP rats were generated from the 22nd to 24th generations of intraline breeding at The Scripps Research Institute from sP rats obtained after 32 generations of selection from Professor GL Gessa (University of Cagliari). Rats, 300 g at study onset, were group-housed (2–3 per cage) in a humidity- and temperature (22°C)-controlled vivarium on a 12-h light–dark cycle (lights off, 0800 hours) with water and chow (Harlan Teklad 7012) available *ad libitum*. Procedures adhered to the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee of The Scripps Research Institute.

Drugs

Ethanol (10% v/v) and saccharin solutions were prepared in tap water using 95% ethyl alcohol and saccharin sodium salt hydrate (Sigma-Aldrich, catalog number S1002), respectively. BD-1063 2 × HBr salt (1-(2-(3,4-dichlorophenyl)ethyl)-4-methylpiperazine dihydrobromide) was synthesized according to the previously reported procedure (de Costa *et al*, 1993). BD-1063 was solubilized in isotonic saline and injected subcutaneously (s.c. 1 ml/kg), 15 min before testing.

Oral Self-Administration Apparatus

The self-administration test chambers (Coulbourn Instruments, Allentown, PA) were located in sound-attenuating, ventilated environmental cubicles. Syringe pumps (Razel Scientific Instruments, Stamford, CT) dispensed ethanol or water into two stainless steel drinking cups mounted 4 cm above the grid floor in the middle of one side panel. Two retractable levers were located 4.5 cm to either side of the drinking cups. Fluid delivery and recording of operant responses were controlled by microcomputers.

Ethanol Self-Administration Procedure

Outbred Wistar rats. Wistar rats were allowed to press a lever for ethanol on a fixed ratio-1 (FR1) schedule of reinforcement via a modified Samson fading procedure (Funk *et al*, 2006). Briefly, rats first responded for 0.1 ml of a glucose (3% w/v)/saccharin (0.125% w/v) solution for 5 sessions.

Then, ethanol self-administration was initiated by adding ethanol (10% v/v) to the sweet solution for 4–5 sessions, followed by 4–5 sessions of 10% ethanol + 0.125% saccharin only. Finally, rats received operant access to the 10% ethanol solution alone. During training, subjects received a 30-min self-administration session 5 days per week.

sP rats. Sardinian alcohol-preferring rats were allowed to self-administer 10% v/v ethanol without a fading procedure under an FR1 reinforcement schedule, as previously described (Sabino *et al*, 2006). Lever presses had no scheduled consequences for 2.01 s after the activation of the pumps to avoid double responses (Sabino *et al*, 2006). For training, rats received a daily 60-min self-administration session 5 days per week until performance stabilized (<15% variation across three consecutive sessions). For both Wistar and sP rats, responses at the opposite lever produced water, with the lever that produced water or ethanol alternated daily during training.

Separate animals were allowed to self-administer 10% v/v ethanol under a progressive ratio (PR) schedule of reinforcement in which the number of responses required to produce successive ethanol deliveries increased per the exponential progression: response ratio $= 4 \times (e^{\text{no. of reinforcer} \times 0.1}) - 3.8$, rounded to the nearest integer. The session began upon completion of the first ratio with a maximum duration of 2 h. To avoid unintended session starts, the first reinforcement required three responses. Sessions ended when subjects did not complete a ratio for 15 min. The dependent measures were the following: (a) breakpoint, last ratio completed by a subject before the end of the session; (b) total responses, total number of reinforced and nonreinforced responses; (c) reinforcers, total number of reinforced responses. Testing, performed between 1500 hours and 1800 hours twice per week, began when stable responding was achieved (<15% variation across three consecutive sessions).

Saccharin Self-Administration Procedure

Rats were allowed 1–2 overnight (16-h) operant, 2-choice access sessions during which lever responses led to delivery of 0.1 ml saccharin (0.1% w/v) or water solutions under an FR1 schedule. Food was available *ad libitum*. All subsequent FR1 sessions were 60 min in duration, without food present, with saccharin concentrations reduced progressively from 0.1% w/v to 0.0035% w/v for Wistar rats and to 0.045% w/v for sP rats. These concentrations maintained response rates similar to those elicited by ethanol in dependent Wistar rats and sP rats, respectively.

Ethanol Vapor Exposure Procedure

To induce dependence, Wistar rats were housed within sealed, clear plastic chambers into which ethanol vapor was intermittently introduced, as described previously (Sabino *et al*, 2006). The chambers were connected to a timer that turned the ethanol vapor on (2000 hours) and off (1000 hours), for 14 h of daily ethanol exposure. Tail blood (0.05 ml) was sampled at vapor offset for blood alcohol level (BAL) determination twice during the first week and weekly thereafter. The plasma was extracted with trichloroacetic acid and assayed for ethanol content using the nicotinamide adenine dinucleotide–alcohol dehydrogenase enzyme spectrophotometric method (Sigma). Target BALs were 150–200 mg% across a 6-week exposure period. This paradigm induces physical dependence and increases operant ethanol self-administration during withdrawal (Funk *et al*, 2006; O'Dell *et al*, 2004; Sabino *et al*, 2006); control rats were kept under similar conditions without ethanol vapor exposure.

After 6 weeks of exposure, rats in the self-administration experiment were tested for operant ethanol self-administration twice weekly, beginning 6 h after vapor offset. The first four of these 30-min sessions allowed subjects to experience the potential negative reinforcing effects of ethanol during acute withdrawal, after which experimental testing was initiated. Rats in the qPCR experiment were instead anesthetized by isoflurane inhalation (6 or 24 h after vapor offset) and decapitated. Brains were sectioned coronally (2 mm slices) in a rat brain matrix. Brain regions of interest (nucleus accumbens, ventral tegmental area, central nucleus of the amygdala basolateral amygdala), chosen for their role in reward and reinforcement and moderate-to-high SigR expression (Alonso *et al*, 2000; Bouchard and Quirion, 1997), were punched using a 14-gauge needle, guided by atlas (Palkovits, 1988), and stored at −80°C until processing. Naive sP rats were sacrificed together with the 6 h withdrawal Wistar rat group.

Effects of BD-1063 on Ethanol Self-Administration

Outbred Wistar rats. Rats (controls, $N = 11$; dependents, $N = 9$) were pretreated with BD-1063 (0, 4.4, 7, and 11 mg/kg of body weight, free base basis, s.c.) using a within-subject Latin square design.

sP rats. Rats ($N = 9$) were pretreated with BD-1063 (0, 3, 4.4, 7, and 11 mg/kg of body weight, free base basis, s.c.) using a within-subject Latin square design.

Effects of BD-1063 on Progressive-Ratio Ethanol Self-Administration

Sardinian alcohol-preferring rats ($N = 11$) were pretreated with BD-1063 (0, 3, 4.4, 7, and 11 mg/kg, s.c.) using a within-subject Latin square design.

Effects of BD-1063 on Saccharin Self-Administration

Rats were pretreated with BD-1063 (Wistar: 0, 4.4, 7, 11 mg/kg, $N = 8$; sP: 0, 3, 4.4, 7, 11 mg/kg, $N = 7$) using a within-subject Latin square design.

Sig-1R Gene Expression

Total RNA was prepared from each punch using the RNeasy mini kit (Qiagen, Valencia, CA) as recommended for animal tissue. Total RNA (1 μg), quantified by Ribogreen reagent (Molecular Probes, Invitrogen, Carlsbad, CA), was reverse transcribed with SuperScript First-Strand Synthesis System for RT–PCR (Invitrogen) in the presence of Oligo $(dT)_{12-18}$ per the manufacturer's instructions. For quantitative real-time PCR, Roche Light Cycler 480 Master-plus SYBR Green mix (Roche Applied Science, Indianapolis, IN) was used.

Reactions (20 μl) were carried out in a 96-well plate Realplex[4] machine (Eppendorf). The primers (0.5 μM final concentration; Valuegene, San Diego, CA), synthesized with a standard desalting purification, were for Cyclophilin A (Cyp), 5′-TATCTGCACTGCCAAGACTGAGTG-3′ and 5′-CTTCTTGCTGGTCTTGCCATTCC-3′, and for Sig-1R, 5′-GCTGCAGTGGGTGTTTGTGAACG-3′ and 5′-GGTGGAAAGTGCCAGAGATGATGGTA-3′. The Cyp sequence was amplified using a three-temperature protocol, which included an initial 5 min at 94°C to activate Taq polymerase, followed by 40 denaturation cycles at 95°C for 20 s, annealing at 58°C for 15 s, and extension at 72°C for 10 s. The Sig-1R sequence was amplified per a two-temperature protocol after an initial 5 min at 94°C: 40 cycles at 94°C for 15 s and at 68°C for 8 s. The primers for Sig-1R hybridize to sequences within exons 3 and 4 of the Oprs-1 transcript and therefore amplify the 'long' isoform of the protein, corresponding to the characterized receptor (Ganapathy *et al*, 1999). Standard curves were constructed using sequenced PCR products. Results were analyzed by second derivative methods and expressed in arbitrary units, normalized to Cyp expression levels. Standards and samples were run in duplicate, and all reactions for a given brain region were performed concurrently. Gene-specific amplification was determined by melting curve analysis as one peak at the expected melting temperature and by agarose gel electrophoresis.

Statistical Analysis

Ethanol responding data were analyzed by analyses of variance (ANOVAs) and expressed as mean ± SEM, normalized for body weight (ie ethanol, g/kg; water and saccharin, ml/kg). The effects of BD-1063 on ethanol and saccharin responding in sP rats were analyzed by separate one-way ANOVAs with dose of BD-1063 as a within-subject factor. The effects of BD-1063 on ethanol responding in Wistar rats were analyzed by a two-way ANOVA with ethanol history (dependent *vs* nondependent) as a between-subjects factor and dose of BD-1063 as a within-subject factor. Unless otherwise specified, Dunnett's test was used for pairwise comparisons after significant omnibus tests. Quantitative PCR data were analyzed by Student's *t*-test. The software package was Systat 11.0 (SPSS, Chicago, IL).

RESULTS

Effect of BD-1063 on Ethanol Self-Administration in Nondependent and Withdrawn, Dependent Wistar Rats

As shown in Figure 1 (top panel), treatment with the SigR antagonist BD-1063 reduced ethanol responding (treatment: $F(3, 54) = 5.41$, $P < 0.01$) in acutely withdrawn dependent Wistar rats, but not in nondependent rats (dependence × treatment: $F(3, 54) = 2.92$, $P < 0.05$). In dependent rats, BD-1063 reduced responding dose dependently as reflected in linear trend analysis (log-linear Dose effect: $F(1, 24) = 16.52$, $P < 0.01$). Pairwise *post hoc* comparisons revealed that the doses of 7 and 11 mg/kg significantly reduced intake, and all 9 dependent rats self-administered less ethanol after treatment with 11 mg/kg BD-1063 than after vehicle treatment. In contrast, BD-1063 did not alter water responding in either group (treatment: $F(3, 54) = 0.57$, n.s.; dependence × treatment: $F(3, 54) = 0.39$, n.s.; Figure 1, bottom panel).

Among the nondependent rats, subgroup analysis showed that BD-1063 (11 mg/kg) tended to decrease self-administration in high responders (38% reduction compared to vehicle, $P = 0.08$, $n = 4$, $0.80 + 0.06$ g/kg), but not in low responders ($n = 7$, $0.19 + 0.05$ g/kg). Among the dependent rats, median split subgroup analysis showed that BD-1063 treatment significantly and comparably reduced self-administration in low responders (49% reduction, $P = 0.0001$; $n = 5$, $0.84 + 0.14$ g/kg) *vs* high responders (34% reduction, $P = 0.02$; $n = 5$, $1.33 + 0.05$ g/kg). Thus, BD-1063 reduced ethanol responding in both nondependent and dependent rodents that had high mean baseline ethanol self-administration (~0.8 g/kg of ethanol). Above this threshold of intake, the relative suppression of ethanol self-administration by BD-1063 was unrelated to baseline responding, arguing against a rate-dependent effect.

Effect of BD-1063 on Saccharin Self-Administration in Wistar Rats

As shown in Figure 2, treatment with BD-1063 did not reliably affect responding for saccharin in Wistar rats (treatment: $F(3, 21) = 0.65$, n.s.). Moreover, no significant linear contrast effect of dose was observed. As desired, the saccharin solution maintained levels of responding under vehicle conditions that did not differ from those observed in withdrawn, ethanol-dependent rats. BD-1063 treatment also did not affect concurrent responding for water (treatment: $F(3, 21) = 0.13$, n.s.), with no significant linear contrast effect of dose observed.

Effect of BD-1063 on Ethanol Self-Administration in sP Rats

As shown in Figure 3 (top panel), systemic treatment with the SigR antagonist BD-1063 reduced ethanol responding of sP rats (treatment: $F(4, 32) = 11.63$, $P < 0.001$), in a dose-dependent manner (log-linear Dose effect: $F(1, 32) = 44.90$, $P < 0.001$). *Post hoc* comparisons revealed that the 4.4, 7, and 11 mg/kg doses significantly reduced responding relative to vehicle conditions; all nine sP rats showed reduced ethanol self-administration following the 11 mg/kg dose. In contrast, responding for water was not significantly affected by BD-1063 (treatment: $F(4, 32) = 0.45$, n.s.; Figure 3, bottom panel). Figure 4 shows the time course of BD-1063 action, with the drug reducing ethanol intake within the first 5 min of the session and the cumulative reduction persisting for the duration of the session (60 min). Median split analysis again showed that BD-1063 achieved a similar relative suppression of ethanol self-administration in low responders (42% reduction, $P = 0.04$; $n = 4$, $0.92 + 0.05$ g/kg) *vs* high responders (41% reduction, $P < 0.01$; $n = 5$, $1.30 + 0.11$ g/kg).

Effect of BD-1063 on Saccharin Self-Administration in sP Rats

Treatment with BD-1063 did not reliably affect saccharin self-administration in sP rats (treatment: $F(4, 24) = 1.90$, n.s.), as shown in Figure 5. Though a linear contrast effect of Dose was observed ($F(1, 24) = 5.24$, $P = 0.03$), pairwise

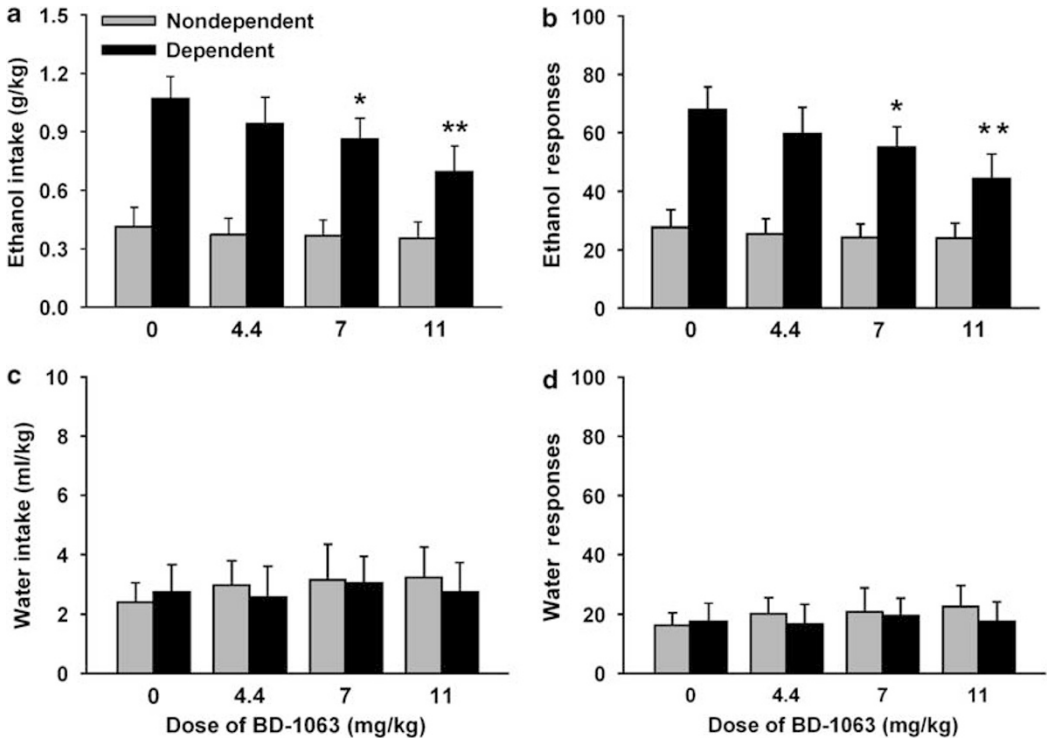

Figure 1 Effect of acute subcutaneous pretreatment (−15 min) with the σ-receptor (SigR) antagonist BD-1063 on ethanol (a and b) and water (c and d) self-administration on a fixed ratio-1 schedule of reinforcement. Subjects were ethanol-dependent Wistar rats ($n=9$), tested 6-h into withdrawal from ethanol vapor, and nondependent Wistar rats ($n=11$). Data represent mean + SEM intake, normalized for body weight (a and c), or number of lever press responses (b and d). $^{*}p<0.05$, $^{**}p<0.01$ *vs* vehicle-treated group (Dunnett's test).

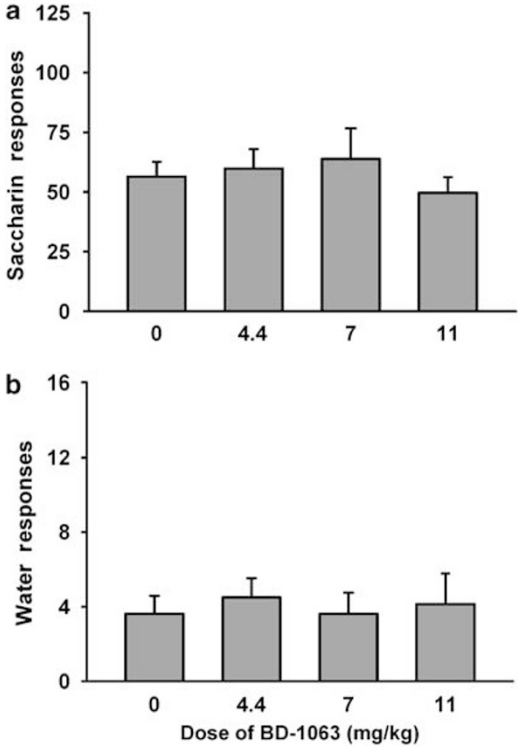

Figure 2 Effect of acute subcutaneous pretreatment (−15 min) with the σ-receptor (SigR) antagonist BD-1063 on saccharin (a) and water (b) self-administration on a fixed ratio-1 schedule of reinforcement in Wistar rats ($n=8$). Data represent mean + SEM number of lever press responses.

comparisons showed that no dose reduced responding relative to vehicle conditions. Moreover, the relative reduction in ethanol responding in sP rats elicited by BD-1063 (11 mg/kg) was greater in sP rats responding for ethanol than in those responding for saccharin (42 *vs* 15%, respectively). As desired, the saccharin solution elicited levels of responding under vehicle conditions that were similar to sP rats responding for ethanol. BD-1063 treatment did not significantly affect water intake ($F(4,24)=1.42$, n.s.), and no significant linear contrast effect of dose was observed.

Effect of BD-1063 on Progressive Ratio Responding for Ethanol in sP Rats

As shown in Figure 6, systemic treatment with the SigR antagonist BD-1063 reduced the break point of sP rats responding for ethanol under a PR schedule of reinforcement (treatment: $F(4,40)=8.28$, $P<0.001$), in a dose-dependent manner (log-linear dose effect: $F(1,40)=22.77$, $P<0.001$). BD-1063 treatment also dose dependently reduced the total number of responses emitted for ethanol (treatment: $F(4,40)=8.56$, $P<0.001$; log-linear Dose effect: $F(1,40)=23.46$, $P<0.001$) as well as the total number of ethanol reinforcers earned (treatment: $F(4,40)=7.88$, $P<0.001$; data not shown). *Post hoc* comparisons revealed that the 4.4, 7, and 11 mg/kg doses of BD-1063 significantly reduced each of the above measures. Responding at the inactive lever was not altered by BD-1063 treatment ($F(4,40)=2.57$, n.s.).

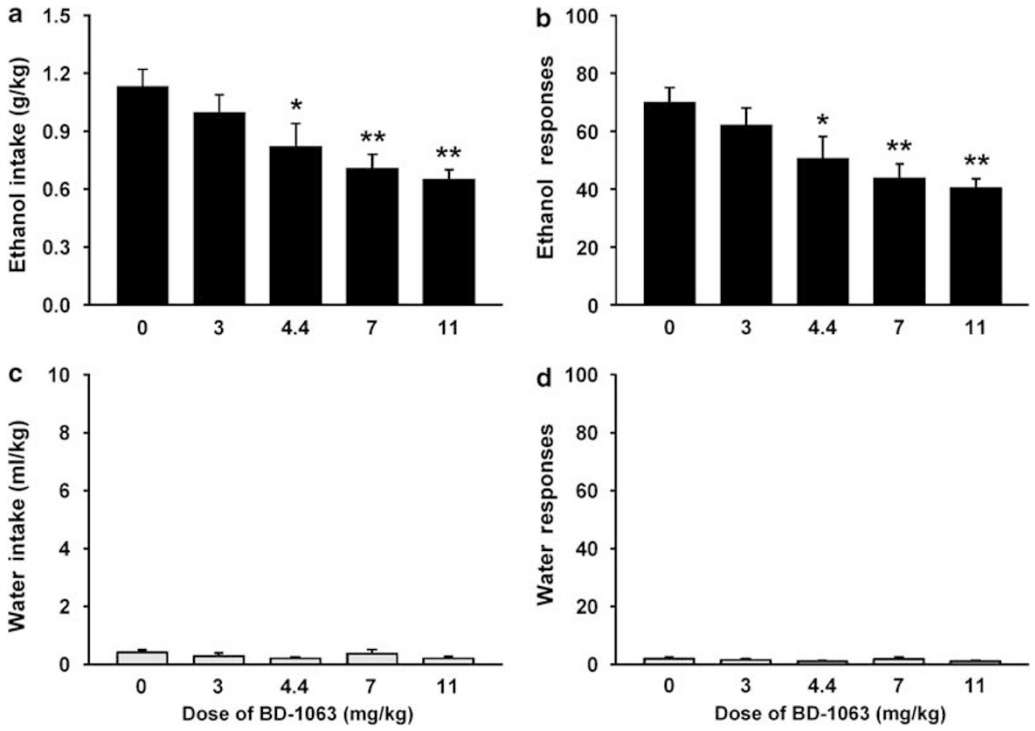

Figure 3 Effect of acute subcutaneous pretreatment (−15 min) with the σ-receptor (SigR) antagonist BD-1063 on alcohol (a and b) and water (c and d) self-administration on a fixed ratio-1 schedule of reinforcement in sP rats ($n=9$). Data represent mean + SEM intake normalized for body weight (a and c), or number of lever press responses (b and d). *$p<0.05$, **$p<0.01$ *vs* vehicle-treated group (Dunnett's test).

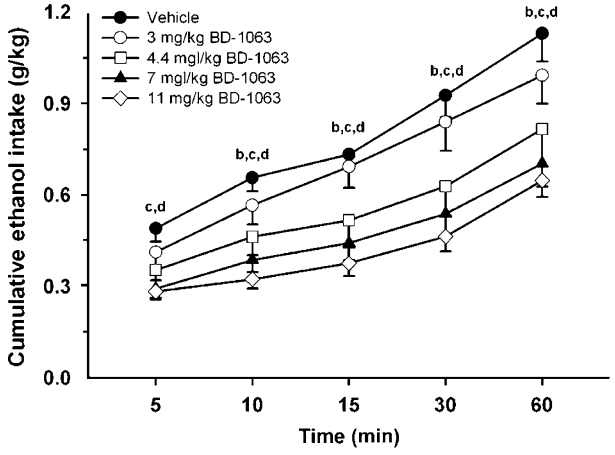

Figure 4 Time-course of the reduction of cumulative ethanol intake resulting from subcutaneous pretreatment with BD-1063 in sP rats ($n=9$). Graph shows the cumulative ethanol intake at 5, 10, 15, 30, and 60 min into the session of subjects shown in Figure 3. Data represent mean−SEM. b = dose of 4.4 mg/kg, c = dose of 7 mg/kg, d = dose of 11 mg/kg, significantly different from vehicle (Dunnett's test).

Sig-1R Gene Expression in sP Rats and in Acutely Withdrawn Dependent Wistar Rats

Quantitative real-time PCR showed, as depicted in Figure 7, that Sig-1R mRNA levels were significantly lower in the nucleus accumbens of ethanol-naive sP rats ($t(14)=2.60$, $p<0.05$) and 24-h withdrawn, ethanol-dependent rats ($t(16)=1.76$, $p<0.05$) compared with ethanol-naive outbred Wistar controls. No significant differences were observed in the nucleus accumbens of 6-h withdrawn, ethanol-dependent rats ($t(14)=0.55$, n.s.). No differences were observed among groups in expression of Sig-1R mRNA within the ventral tegmental area, central nucleus of the amygdala or basolateral amygdala (data not shown).

DISCUSSION

This study shows that the preferential Sig-1R antagonist BD-1063 dose dependently and selectively reduced oral ethanol self-administration in two animal models of excessive ethanol drinking—acutely withdrawn, ethanol dependent outbred Wistar rats and genetically selected, alcohol-preferring sP rats. BD-1063 also reduced the motivation of sP rats to work to obtain alcohol. In contrast, BD-1063 did not reduce ethanol self-administration in nondependent control Wistar rats or comparably reduce self-administration of water or of an equally reinforcing saccharin solution. Both ethanol-naive sP rats and acutely withdrawn (24 h), ethanol-dependent Wistar rats showed decreased Sig-1R mRNA expression in the nucleus accumbens compared with ethanol-naive control Wistar rats. The results collectively support an endogenous role for brain Sig-1Rs in the modulation of excessive ethanol intake and reinforcement.

The highest dose of BD-1063 administered (11 mg/kg of free base) decreased fixed-ratio operant responding for ethanol, a measure of ethanol's reinforcing properties with predictive validity for medications used to treat alcoholism (Altshuler *et al*, 1980; Czachowski *et al*, 2001; Johnson and

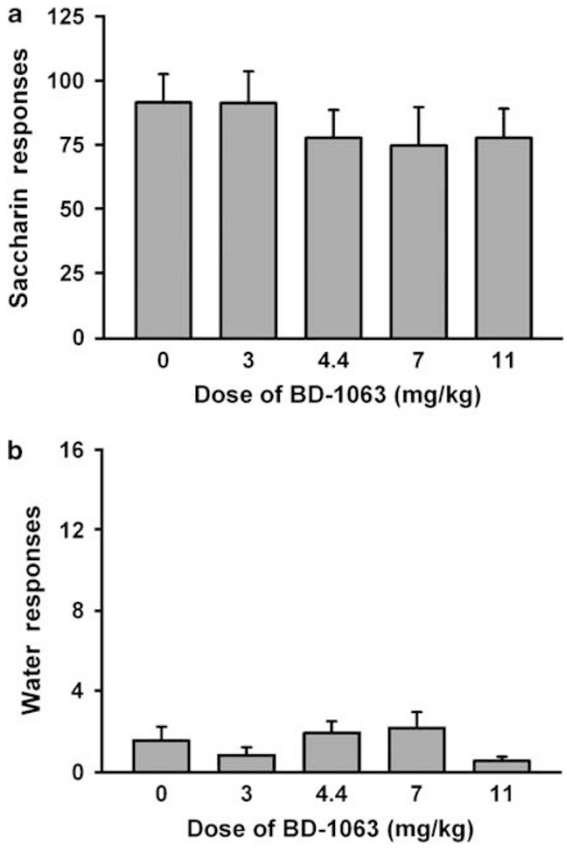

Figure 5 Effect of acute subcutaneous pretreatment (−15 min) with the σ-receptor (SigR) antagonist BD-1063 on saccharin (a) and water (b) self-administration on a fixed ratio-1 schedule of reinforcement in sP rats ($n = 8$). Data represent the mean + SEM number of lever press responses.

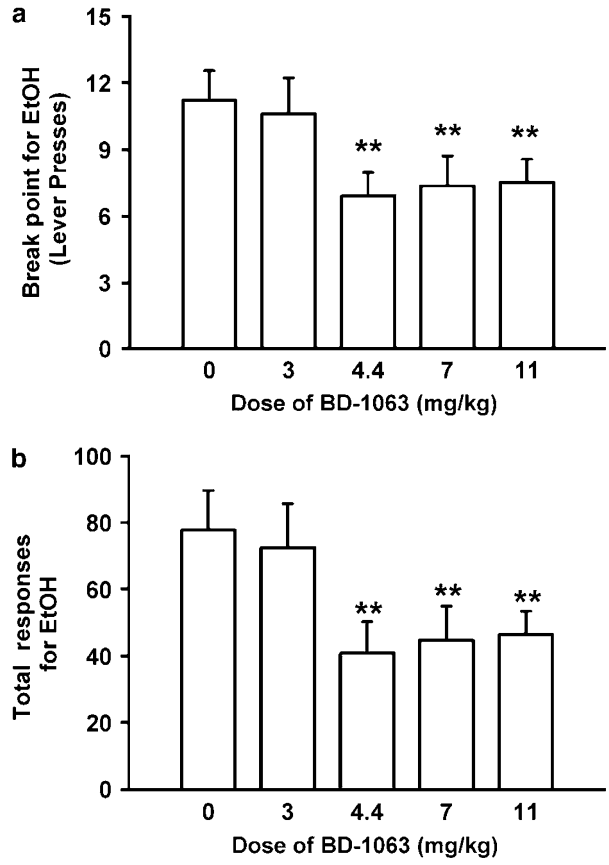

Figure 6 Effect of acute subcutaneous pretreatment (−15 min) with the σ-receptor (SigR) antagonist BD-1063 on break point (a) and total responses (b) for ethanol in sP rats ($n = 11$) tested under a progressive ratio schedule of reinforcement. Data represent mean + SEM. $^{**}p < 0.01$ *vs* vehicle-treated group (Dunnett's test).

Ait-Daoud, 2000), by 37 and 42% in dependent Wistar and sP rats, respectively. Several control measures demonstrated the specificity of Sig-1R antagonist action on excessive ethanol self-administration, including no effect on mean ethanol self-administration of nondependent rats, on concurrent water self-administration, or on self-administration of an equally reinforcing saccharin solution. Also, arguing against a rate-dependent interpretation of BD-1063 action, median split analysis showed that BD-1063 similarly reduced ethanol self-administration of high *vs* low responding ethanol-dependent and sP rats. Thus, BD-1063 did not reduce general behavior or produce rate-suppressant effects, consistent with previous reports showing lack of locomotor suppressant effect of BD-1063, similar to the chemically related BD-1047, at doses up to 30 mg/kg (Brammer *et al*, 2006; Maurice *et al*, 2003; McCracken *et al*, 1999; Nguyen *et al*, 2005).

To address further the alternative explanation that the selective action of BD-1063 in models of excessive drinking was a rate-suppressive effect on the higher baseline responding of ethanol-dependent and sP rats, the effects of BD-1063 also were evaluated under a PR schedule of reinforcement. Under PR reinforcement schedules, ratio requirements increase with subsequent reinforcer deliveries, and the influence of local response rates on performance are reduced. BD-1063 potently (4.4 mg/kg) reduced the break point, an objective measure of the effort an animal will expend to obtain a reinforcer that is sensitive both to the subjects' incentive state and to the reinforcers' stimulus properties (Walker *et al*, 2008). The ability of BD-1063 to reduce break point also counters the alternative explanation that the drug acted by altering ethanol absorption or metabolism because the total amount of ethanol self-administered by rats under this schedule (0.18 g/kg under vehicle treatment) would not result in significant BALs (personal observation). Thus, the SigR antagonist reduced not only ethanol intake, but also the work that rats would emit to obtain ethanol.

These results are broadly consistent with previous findings that the Sig-1R antagonist BD-1047 attenuated several behavioral and motivational effects of acute passive ethanol administration in mice (Maurice *et al*, 2003) including ethanol-induced locomotor activation and ethanol-induced place preference and taste aversion conditioning. Here, a different Sig-1R antagonist selectively reduced responding for ethanol in two distinct models of excessive ethanol self-administration, supporting the hypothesis that endogenous SigR signaling contributes to oral ethanol's reinforcing effects. However, BD-1063 did not reduce ethanol self-administration to control-like levels. Perhaps higher doses of BD-1063 could have eliminated the excess component of ethanol self-administration altogether. Alternatively, other transmitter systems likely independently

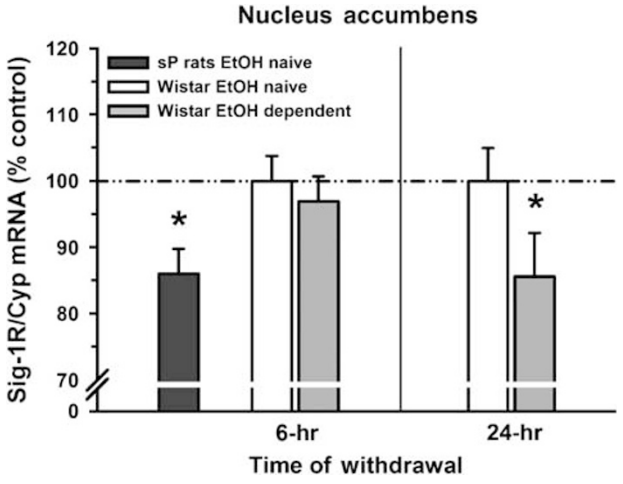

Figure 7 Sig-1R mRNA expression in the nucleus accumbens of ethanol-naive Sardinian alcohol-preferring (sP) rats, ethanol-naive outbred Wistar control rats, and acutely withdrawn (6-h) Wistar rats previously made ethanol-dependent through intermittent exposure to ethanol vapors for 6 weeks (left panel, $n=7$, 9, and 7, respectively) and ethanol-naive outbred Wistar control rats, and acutely withdrawn (24-h) Wistar rats (right panel $n=9$ and 7, respectively). Data represent mean + SEM expressed as percent of the control group. $^*p<0.05$ *vs* outbred control rats (Student's *t*-test).

contribute to the excess ethanol intake in these models (Funk *et al*, 2006; Walker *et al*, 2008; Roberto *et al*, 2008).

Previous studies have demonstrated that SigR blockade can attenuate several neurobehavioral effects of cocaine and methamphetamine, including the drugs' subjective (Katz *et al*, 2003), psychomotor stimulant (Liu and Matsumoto, 2008; Menkel *et al*, 1991; Ujike *et al*, 1992), rewarding (Romieu *et al*, 2000), and toxic (Matsumoto *et al*, 2001b; Maurice *et al*, 2002) effects. A recent study, however, observed that the SigR antagonist BD-1047 did not reduce nondependent intravenous cocaine self-administration (Martin-Fardon *et al*, 2007), unlike this study in which we observed suppression of oral ethanol self-administration by BD-1063. Several procedural differences may explain the discrepant findings, including major ones such as the substance of abuse under study, the antagonists and route of drug intake used, and the session duration. The subjects under study also may be critical because ethanol self-administration was only reduced here in models of excessive self-administration and not in nondependent controls, a group more analogous to those of the previous cocaine self-administration study. Interestingly, BD-1047 did reduce reinstatement of cocaine-directed responding elicited by a drug-taking contextual stimulus (Martin-Fardon *et al*, 2007); it remains to be seen whether Sig-1R antagonists similarly suppress reinstatement of ethanol-directed responding.

The greater efficacy of the SigR antagonist in the two models of excessive drinking raises the possibility that withdrawn, ethanol-dependent rats and sP rats might both carry alterations in the expression or function of SigR sites or endogenous ligands, leading to an increased reinforcing efficacy of ethanol. In this study, quantitative real-time PCR showed that Sig-1R mRNA levels were modestly (15%) but significantly lower in the nucleus accumbens of both sP rats and 24-h withdrawn, dependent Wistar rats. Similar to previous studies (Economidou *et al*, 2008; Hansson *et al*, 2007), outbred Wistar rats were used as the control group for both sP rats and outbred Wistar dependent rats in this study to allow a more direct comparison between these two animal models of excessive drinking. Using alcohol non-preferring rats as a control group for sP rats also might have left it more ambiguous as to whether observed differences were associated with excessive drinking as opposed to ethanol aversion, which can be associated with molecular changes distinct from those which drive excess intake (Saba *et al*, 2001).

Reduced Sig-1R mRNA expression was not observed 6 h after cessation of ethanol vapor suggesting that the decrease may be a delayed transcriptional consequence of ethanol withdrawal, rather than of the ethanol exposure history *per se*. A related question is why differences in Sig-1R mRNA levels were not observed between Wistar ethanol-naive and ethanol-dependent rats during early withdrawal (6 h), a time point at which differences in ethanol self-administration and sensitivity to BD-1063 already were present. Perhaps Sig-1R activity is already altered at the 6-h time point at the ligand or receptor protein level due to the early stages or previous cycles of withdrawal, but a change in Sig-1R transcription takes longer to manifest. Such a delay might result if the decreased receptor mRNA expression occurs in response to heightened SigR ligand activity or per the slower time course of transcription *vs* ligand release/binding. Testing this hypothesis in future studies could involve measuring Sig-1R protein or endogenous ligands (once identified) during intoxication and 6-h withdrawal. In addition, differences in the experimental paradigms used for the behavioral *vs* molecular studies may be relevant. Perhaps rats with a history of self-administering ethanol during withdrawal might show changes in Sig-1R mRNA levels at the earlier 6-h time point. To identify innate differences in sP rats, we measured Sig-1R mRNA levels in ethanol-naive rats in this study, rather than in rats that were acutely or historically self-administering ethanol. Ethanol drinking can differentially affect ethanol-naive alcohol-preferring rats, eliminating or introducing neurochemical and behavioral differences (Hansson *et al*, 2007; Katner and Weiss, 2001; Weiss *et al*, 1993), and it would be valuable in future studies also to examine differential changes in SigR systems that result from ethanol drinking in the groups studied here. As a first-priority study relevant to the current hypothesis, however, the present experiments identified the presence of innate differences in the SigR system of sP rats in relation to their outbred stock and showed that withdrawal from repeated ethanol exposure yielded an environmental phenocopy of this molecular correlate of the genetic propensity to drink ethanol.

Similar changes were not observed in the central or basolateral amygdala or ventral tegmental area, suggesting regional specificity. The nucleus accumbens, intimately related to reward-related processes, integrates limbic information related to memory, drive, and motivation with the generation of goal-directed behaviors (Carelli and Wightman, 2004; Pecina *et al*, 2006; Pennartz *et al*, 1994). The nucleus accumbens also is implicated in excessive alcohol drinking and the pathophysiology of dependence (Koob, 2003; Tupala and Tiihonen, 2004). Further studies

are needed to determine the functional significance of the molecular difference shared by sP and withdrawn dependent rats. The decreased Sig-1R mRNA expression might represent a compensatory response to a higher availability of neuroactive steroids, putative endogenous SigR ligands (for a review see Maurice, 2004). Such an interpretation is consistent with the observed higher sensitivity to pharmacological blockade of SigR by BD-1063. Studies that localize the decrease in Sig-1 mRNA to the shell vs core nucleus accumbens subregions, that potentially relate observed mRNA differences to protein expression changes, that explore corresponding changes in putative endogenous SigR ligands, and that investigate the effects of brain site-specific administration of Sig-1R ligands on the reinforcing effects of ethanol will be required.

Ethanol has not been shown to interact directly with SigRs, but SigRs ligands may alter reinforcing actions of ethanol by indirectly modulating activity of other ethanol-sensitive transmitter systems; for example, SigR ligands affect synthesis, release, and uptake of dopamine (Bastianetto *et al*, 1995; Booth and Baldessarini, 1991; Weatherspoon and Werling, 1999; Weiser *et al*, 1995). The SigR agonists (+)pentazocine, (+)-SKF 10,047, and DUP 734 increased extracellular dopamine concentration and dopamine metabolism in the striatum (Iyengar *et al*, 1990), facilitating release through a nontransporter-mediated mechanism. In addition (+)pentazocine enhanced amphetamine-stimulated DA release in striatal slices (Izenwasser *et al*, 1998). Electrophysiological studies also have shown a modulatory role of SigRs on the firing activity of both the A9 and the A10 dopaminergic pathways, with an activation of SigRs generally resulting in an increase in firing (Engberg and Wikstrom, 1991; Minabe *et al*, 1999; Sanchez-Arroyos and Guitart, 1999; Steinfels *et al*, 1989; Zhang *et al*, 1992). In addition, the SigR agonist PRE-084 dose dependently increased the functional coupling of dopamine receptors with G-proteins in dopaminergic terminal brain areas (Peeters *et al*, 2004). Based on these observations, SigR antagonists might attenuate ethanol reinforcement by influencing dopaminergic transmission, directly or indirectly, within the mesolimbic pathway.

Alternatively, the drive to consume ethanol and other drugs of abuse may interfere with the activity of one of the endoplasmic reticulum (ER) proteins recently shown to be associated with SigRs (Aydar *et al*, 2002; Hayashi and Su, 2001; Yamamoto *et al*, 2002). SigRs, subcellularly localized within perikarya and dendrites, are compartmentalized to lipid-enriched globular membranes (Alonso *et al*, 2000; Phan *et al*, 2003), such as mitochondria-associated membranes of the smooth ER. In the ER, Sig-1Rs associate with proteins such as inositol 1,4,5 triphosphate receptor type 3 (IP3R3), K^+ channel subunits, or the ER chaperone BiP (GRP78) and serve as chaperone molecules in response to Ca^{2+} depletion (Hayashi and Su, 2001, 2007). Sig-1Rs translocate following cellular stimuli, redistributing to the entire ER or to cell components, events that alter intracellular communication (Hayashi *et al*, 2000; Hayashi and Su, 2003a, b, 2007; Monnet *et al*, 2003; Morin-Surun *et al*, 1999). Thus, SigRs are putative sensors and/or modulators of neuronal intracellular Ca^{2+} mobilization, and consequently of extracellular Ca^{2+} influx. Such action could explain the broad neuromodulation of SigR on several neurotransmitter systems by SigRs (Maurice *et al*, 2002). Based on these observations, a SigR antagonist may attenuate ethanol reinforcement by interfering with the putative activation of intracellular calcium mobilization associated with the drive to excessively consume ethanol.

BD-1063 has preferential, nanomolar affinity for Sig-1Rs, being 30-fold selective for Sig-1R *vs* Sig-2R sites (Brammer *et al*, 2006; Matsumoto *et al*, 2001a). Still, it is possible that at the systemic doses administered here, BD-1063 binds both SigR subtypes. Although most studies implicate the Sig-1R subtype in the actions of drugs of abuse, a few lines of pharmacological evidence suggest that some actions may also (or alternatively) involve Sig-2Rs (Mach *et al*, 1999; Matsumoto and Mack, 2001; Matsumoto *et al*, 2001b, 2007; Nuwayhid and Werling, 2006). As the Sig-2R gene has not been cloned or predicted from homology, this study could only investigate changes in Sig-1R mRNA, with the results pointing to a role for the Sig-1R subtype in excessive drinking. Further studies can better define the relative roles of each subtype in ethanol reinforcement. In summary, a selective SigR antagonist preferentially diminishes ethanol reinforcement in models of excessive drinking. Control procedures rule out a nonspecific or rate-dependent suppression in responding or a generalized action on all reinforcers. The selective effects of blocking SigRs on excessive drinking suggest a potential mechanism for neuroadaptation that leads to excessive drinking and a potential target for medication development for treatment of alcoholism.

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DISCLOSURE/CONFLICTS OF INTEREST

The authors declared no conflicts of interest.

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