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Reward and Relapse: Complete Gene-Induced Dissociation in an Animal Model of Alcohol Dependence

María E. Quintanilla, Lutske Tampier, Eduardo Karahanian, Mario Rivera-Meza, Mario Herrera-Marschitz, and Yedy Israel

Background: In animal models of continuous alcohol self-administration, in which physical dependence does not constitute the major factor of ethanol intake, 2 factors likely contribute to the perpetuation of alcohol self-administration: (i) the rewarding effects of ethanol and (ii) the contextual conditioning cues that exist along with the process of self-administration. Present studies are aimed at understanding the relative contribution of these factors on the perpetuation of heavy alcohol self-administration, as an indication of relapse.

Methods: Wistar-derived UChB high ethanol drinker rats were allowed access to 10% ethanol and water on a 24-hour basis. In initial studies, an anticatalase shRNA gene-coding lentiviral vector aimed at inhibiting acetaldehyde generation was administered into the ventral tegmental area (VTA) of the animals prior to ethanol access. In subsequent studies, the lentiviral vector was administered to animals, which had consumed ethanol on a 24-hour basis, or a 1-hour basis, after the animals had reached high levels of ethanol intake for 60 to 80 days. In final studies, quinine (0.01%) was added to the ethanol solution to alter the conditioning taste/smell cues of alcohol that animals had chronically ingested.

Results: Data indicate that the administration of an anticatalase vector into the VTA of naïve animals blocked reward and alcohol self-administration, while it was, nevertheless, inactive in inhibiting alcohol self-administration in rats that had been conditioned to ingest ethanol for over 2 months. The lack of inhibitory effect of the anticatalase vector on ethanol intake in animals that had chronically self-administered ethanol was fully reversed when the contextual conditioning cues of the alcohol solution were changed.

Conclusions: Data highlight the importance of conditioning factors in relapse and suggest that only abolishing or blunting it, along with long-lasting pharmacological treatment to reduce ethanol reward, may have protracted effects in reducing alcohol self-administration.

Key Words: Reward, Relapse, Lentiviral Vector, Catalase, Acetaldehyde.

IT IS WELL established that without psychotherapeutic or pharmacological treatment, and often both, alcohol- and drug-dependent individuals will generally fail to interrupt the use of the drug on which they are dependent. Classically 2 main elements are recognized as involved in the perpetuation of the addictive behavior (Koob and LeMoal, 1997): (i) the fact that the drug is rewarding and (ii) the relief of the drug withdrawal reaction. Volkow and Li (2005) have pointed out that physical dependence refers to adaptations that result in withdrawal symptoms when the drug is discontinued, which should be distinguished from addiction, which refers to the loss of control over the intense urge to take the drug. The latter state is prompted by mechanisms associated with environmental or contextual cues (including visual or olfactory cues) that may either reproduce the rewarding effects of the drug or may trigger a protracted withdrawal reaction. This additional conditioned component, referred to as craving in humans, is accepted as a third major constituent leading to relapse (Buccafusco and Shuster, 2009; Cunningham and Noble, 1992; Wikler, 1968).

The above 3 components have been partially dissociated in different animal models of alcohol self-administration, suggesting that they have different neurobiological mechanisms. Drugs that are known to block alcohol self-administration in animal models that aim at generating physical dependence do not block the self-administration of ethanol when available on a continued basis (see Simms et al., 2008). Alternatively, these drugs are either significantly less active (Gilpin and Koob, 2010) or may lack specificity (see Steensland et al., 2007). Conversely, low concentrations of a bitter substance such as quinine, added to the ethanol solutions, block self-administration when ethanol is freely available but do not influence the self-administration of ethanol in rat models

From the Program of Molecular and Clinical Pharmacology (MEQ, LT, MR-M, MH-M, YI), Faculty of Medicine, Institute of Biomedical Sciences, Santiago, Chile; Department of Pharmacological and Toxicological Chemistry (YI), Faculty of Chemical and Pharmaceutical Sciences, and Institute for Cell Dynamics and Biotechnology, University of Chile, Santiago, Chile; Center of Biomedical Research (EK), Faculty of Medicine, Universidad Diego Portales, Santiago, Chile; and Department of Pathology (YI), Anatomy and Cell Biology, Thomas Jefferson University, Philadelphia, Pennsylvania.

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Reprint requests: Yedy Israel, Laboratory of Gene Therapy, University of Chile, Sergio Livingstone 1007, Independencia, Santiago, Chile; Tel.: +11 562 978 2943; Fax: +11 562 737 7291; E-mail: yisrael@uchile.cl

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aimed at generating a major withdrawal reaction (likely involving physical dependence), or high concentrations of quinine are needed (Hopf et al., 2010; see also Spanagel et al., 1996). These results suggest that in animals allowed a continuous access to alcohol (which have blood ethanol levels generally low and minimal physical dependence), the motivation to drink alcohol is owing to the rewarding effects of ethanol and the contextual cues. When animals remain in their home cage, the contextual cues that are likely associated with the rewarding effects are the ethanol taste and smell. Taste has been shown to be highly associated with alcohol preference in animal studies (Blednov et al., 2010), and adulteration by addition of another taste (e.g., quinine) likely affects the contextual cues. Odor is also an important component in lower mammals, in which the olfactory cortex is comparatively large (Herrick, 1933). The fact that lower animals can smell ethanol present at low concentrations in fermented fruits in the wild has been proposed to have an evolutionary advantage (Dudley, 2000).

In most relevant recent studies in humans, Volkow and colleagues (2011) addressed the neurophysiological correlates of drug reward, withdrawal, and conditioned relapse. Cocaine is used as a model of a variety of addictive drugs, which include alcohol. The drug initially increases the levels of dopamine in the striatum (including the nucleus accumbens), while these increases in dopamine, as well as the rewarding effects perceived, are blunted in the addict. Most importantly, upon the presentation of drug-conditioned cues, addicted subjects show marked increases in dopamine in the striatum. The authors postulate that the conditioned effects (implied as the expectation of drug effects) along with the pharmacological effects are aimed at achieving the expected reward, thus leading to relapse and maintaining drug self-administration. The question arises as to which of these 2 mechanisms is most relevant to relapse. Such was the aim of this report, which utilized a recent methodology that allows to fully blocking the rewarding effects of alcohol.

Work by Karahanian and colleagues (2011) showed that a single injection of an anticatalase shRNA-coding lentiviral vector, designed to inhibit the metabolism of alcohol into acetaldehyde, a highly rewarding metabolite in the CNS (see Deitrich, 2011), into the ventral tegmental area (VTA) fully blocks (i) the self-administration of ethanol by rats bred for their high preference for alcohol and (ii) the alcohol-induced release of dopamine in the nucleus accumbens.

This study investigates whether the full abolition of the rewarding effects of ethanol shown in naïve animals by the administration of an anticatalase vector (Karahanian et al., 2011) also reduced alcohol intake in animals that have become dependent on ethanol following 60 days of alcohol self-administration (Ocaranza et al., 2008). The effect of the anticatalase viral vector was examined in 2 experimental conditions: (i) following the continuous self-administration of ethanol (10%) and water on a 24-hour basis in their home cage (i.e., no withdrawal period) and (ii) upon alcohol self-administration, when 10% ethanol was available for only 1 hour/day in their home cage (i.e., a 23-hour withdrawal period daily). Keeping constant the physical environment reduced the number of possible contextual variables.

Our hypothesis that reward was the most important element in eliciting relapse was proved wrong. Rather, data strongly indicate that relapse has neurobiological bases that are fully independent of the original rewarding effects that led to dependence and suggest that other mechanisms, such as cued conditioning (or a conditioned withdrawal reaction, as different from a noncued withdrawal reaction), likely play the most important role in alcohol relapse in this animal model. Only when these cues are blunted, reward plays again a major role in alcohol self-administration. Overall, the study suggests that in dependent animals under continuous access to alcohol, self-administration is abolished only when both behavioral and pharmacological modifications are combined.

MATERIALS AND METHODS

Generation of Lentiviral Vectors

A lentiviral vector expressing a rat catalase-targeting shRNA driven by the human U6 promoter (The RNAi Consortium, Broad Institute of MIT and Harvard TRCN0000120679) was packaged with the pPack Lentivector Packaging System (System Biosciences, Mountain View, CA) in HEK 293T cells, according to the manufacturer protocols. The control viruses were generated from the same vector but containing no shRNA sequences. The methods followed were those described by Karahanian and colleagues (2011).

Intracerebral Administration of Lentiviral Vectors

The studies were performed in Wistar-derived rats (UChB) bred for over 80 generations to ingest ethanol solutions in preference to water (Mardones and Segovia-Riquelme, 1983; Quintanilla et al., 2006). Rats (approximately 200 to 250 g) were anesthetized with a mixture of air and isoflurane administered by a mask fitted over the nose of the animal and placed in a Kopf stereotaxic frame with the skull oriented according to the atlas of Paxinos and Watson (1986). The skull was exposed, and a 2-μl Hamilton syringe with a conic tip (diameter at the insertion tip < 0.2 mm), filled with lentiviral vectors expressing a rat anticatalase shRNA or control vectors, was inserted into the VTA (coordinates: B-5.2; L-0.8; V-7.2, from the dura mater). Two minutes after syringe implantation, 1 μl of the corresponding solution (anticatalase-Lenti-shRNA 8×10^4 virus/μl, or the corresponding controls) was infused at the rate of 0.4 μl/min. The syringe was kept in place for an additional 2-minute period, before removing it slowly. The skin was then sutured, and the rat left to recover in the surgery station before being transferred to individual cages at the animal station.

Preparation of VTA Homogenates

Thirty days after a single injection of control or anticatalase-lentiviral vectors, alcohol-naïve UChB rats were killed, and their brains rapidly dissected out and placed onto a glass plate on ice. Coronal sections (200 to 300 μm) were cut on a cryostat. The VTA was identified according to Paxinos and Watson atlas (1986) and collected with a 1-mm diameter punch (Stille Kirurgiska Instrument, Solna, Sweden), according to Chen and colleagues (1997). These were rinsed with saline and blotted dry; the VTAs were weighed, and 0.8% homogenates were prepared in ice-cold 0.1 M potassium phosphate buffer (pH 7.4) containing 0.1% Triton. The samples were homogenized using a Potter-Elvehjem homogenizer (Corning, Inc., Lowell, MA) with a glass mortar and Teflon pestle.

Determination of Acetaldehyde Levels after the Incubation of VTA Homogenates with Ethanol

The level of acetaldehyde in VTA homogenates after incubation with 50 mM ethanol was measured by gas chromatography as described previously (Tampier et al., 1988; Zimatkin et al., 2001). An aliquot equivalent to 5 mg of wet tissue was incubated at 37°C in a Dubnoff shaker, using flask stopped with Mininert® valves (Sigma-Aldrich, Atlanta, GA). The incubation medium contained ethanol, 50 mM; glucose, 10 mM; potassium phosphate buffer (pH 7.4), 90 mM; final volume, 1.0 ml. After 30, 60, 90, and 120 minutes of incubation, 1 ml of the gas phase was removed with a Hamilton gas syringe and analyzed by headspace gas chromatography (Perkin Elmer SRI 8616; Waltham, MA) to measure acetaldehyde concentration. Nitrogen was used as the carrier gas at 65 ml/min through a stainless steel column packed with 5% Carbowax 20 M on 60/80 Carbopack (Sigma-Aldrich) at an oven temperature of 65°C and detected by flame ionization. Incubation of VTA homogenate with 10 mM 3-amino-1,2,4-triazole, a catalase inhibitor, inhibited (80%) the formation of acetaldehyde. The residual 20% formation is likely due to acetaldehyde generation by CYP2E1 (Zimatkin et al., 2006). Thus, this residual value was subtracted, and the difference in activity is referred to as VTA catalase activity. It is noted that no allowance is made for the possible oxidation of acetaldehyde into acetate, which is unlikely affected differentially by an anticatalase shRNA lentiviral vector versus a control vector.

Experiment 1: Effect of an Anticatalase Vector Injection into the VTA of Alcohol-Naïve Rats on Ethanol Intake

Four days after a single injection of control or anticatalase-lentiviral vectors into the VTA of alcohol-naïve rats ($n = 12$ rats per group), animals were allowed 24-hour continuous access to ethanol solution (10% v/v) and water for 59 days. Ethanol consumption was determined on a daily basis and expressed as g ethanol/kg body weight/d.

Experiment 2: Inhibition of Catalase Activity in the VTA Following the Administration of an Anticatalase Vector

Twenty-four adult (2-month-old) female alcohol-naïve UChB rats were divided into 2 groups ($n = 12$ rats per group), anesthetized, and injected with 1 μl of anticatalase-Lenti-shRNA 8×10^4 virus/μl, or the corresponding controls, as described previously. Rats were returned to their home cage with food and water ad libitum. After 30 to 35 days, rats were killed and the VTA was dissected for catalase activity determination as described.

Experiment 3: Effect of Anticatalase Vector Injection into the VTA and of Alcohol Deprivation on Ethanol Intake of Dependent Animals

Following 2 months of 24-hour continuous access to ethanol solution (10% v/v) and water, rats were deprived of alcohol for 17 hours prior to injecting the control or anticatalase-lentiviral vectors in the VTA ($n = 10$ rats per group). Thereafter, they were transferred to their home cage and allowed 24 hours of continuous access to ethanol solution (10% v/v) and water. Following 18 days of continuous voluntary ethanol intake, rats were deprived of alcohol for 4 weeks. After this deprivation phase, all rats were reexposed to the continuous ethanol intake paradigm for 18 days.

Experiment 4: Effect of an Anticatalase Vector Injection into the VTA on Ethanol Self-Administration in Dependent Animals Before and After Adulteration of the Ethanol Solution with Quinine

Following 2 months of continuous 24-hour access to ethanol solution (10% v/v) and water, rats were deprived of alcohol for 17 hours before injecting the control or anticatalase-lentiviral vectors into the VTA ($n = 10$ rats per group). Thereafter, they were transferred to their home cage and allowed continuous 24-hour access to ethanol solution (10% v/v) and water for 18 days. After this time, the rats were deprived of alcohol for 3 days and then ethanol access was restricted to 1 hour every day (from 14:00 to 15:00 hours), while food and water were freely available. After 11 days of limited access, quinine hydrochloride (Sigma-Aldrich) (0.01%) was added to the ethanol solution, but not to the water, and the ethanol consumption under the 1-hour limited access was registered every day for 17 days.

Statistical Analyses

Data are expressed as means ± SEM. Statistical differences were analyzed by Student's *t*-test or ANOVA for repeated measures for the time factor, with a post hoc test (Student–Newman–Keuls) when required. A level of $p < 0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

Figure 1 shows the robust effect of the anticatalase vector in inhibiting alcohol self-administration of naïve rats bred for their high ethanol preference. The control and the active anticatalase viral vectors were injected into the VTA 4 days prior to placing the animals in individual cages and offering 10% ethanol and water (shown as day 0 in Fig. 1). A maximal inhibition of ethanol intake (about 95%) was found after 7 weeks of the active viral vector administration, while the effect at intermediate times was of the order of 70 to 75%. As suggested earlier (Karahanian et al., 2011), the lesser inhibitory effect seen at the intermediate times may relate to the fact that for lentiviral vectors, the initial gene expression occurs in the episomal state, generated by reverse transcription, while

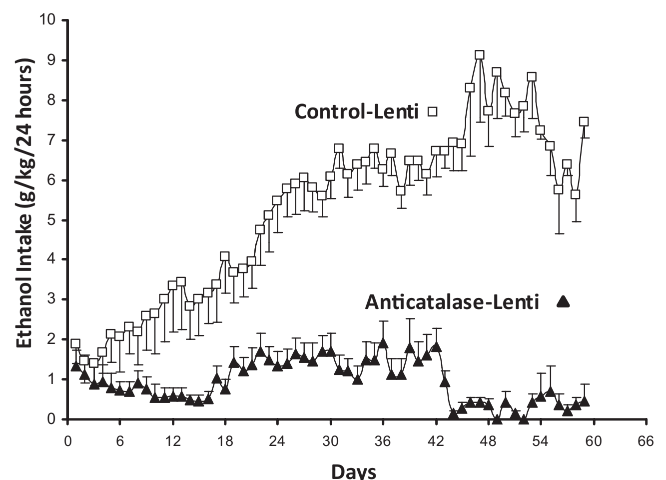

Fig. 1. Anticatalase lentiviral vector administration into the ventral tegmental area (VTA) reduces voluntary alcohol intake in alcohol-naïve UChB rats. Alcohol-naïve UChB rats significantly [ANOVA; $F(1, 117) = 221.23$, $p < 0.001$] reduced their alcohol intake when injected a lentiviral vector coding for an shRNA against catalase (anticatalase-Lenti) into the VTA, compared to alcohol-naïve rats that received an injection of an empty lentiviral vector (control-Lenti). Animals were allowed free availability of 10% (v/v) ethanol and water 4 days after the administration of a single dose (8×10^4 virus) of anticatalase-Lenti-shRNA vector ($n = 12$) or an empty lentiviral vector (control-Lenti) ($n = 12$). See Experiment 1 in Materials and Methods. Abscissa: days of ethanol availability. Data shown are means ± SEM.

the subsequent long-term expression is owing to its permanent insertion into the genome, with a likely period of reduced expression between the 2 processes (see Haas et al. 2000). The inhibitory effect of the lentiviral vector anticatalase administration on VTA catalase activity determined 30 to 35 days following viral administration (a time chosen from earlier studies; Karahanian et al., 2011) was of the order of 70 to 80% (Fig. 2).

Figure 3 shows the alcohol intake of naïve rats allowed 10% alcohol and water for 60 days, which attained alcohol consumption levels similar to those obtained earlier (7 to 8 g ethanol/kg/d). At such time, animals were divided into 2 groups, matched according to their average ethanol intake, and the anticatalase or control vectors were administered. As can be observed, except for the first day after the viral vector injection under anesthesia, no reduction in ethanol intake was observed in the groups following 18 days. Thus, the anticatalase vector was fully inactive in preventing alcohol self-administration in chronically alcohol-consuming animals. These data fully contrast with the powerful inhibitory effects on ethanol consumption observed in naïve animals (see for example 12 to 18 days in Fig. 1) following the administration of the anticatalase vector. For these animals, conditioning cues of ethanol smell and/or taste may be relevant to the continuation of intake, as given the continued presence of ethanol a withdrawal reaction can be ruled out. Thereafter, the experiment proceeded with a 4-week period of ethanol deprivation aimed at blunting the mechanisms that prompted animals to continue consuming ethanol, after which the choice of 10% ethanol (water was always available throughout the study) was again freely available on a 24-hour basis. Initially, both the control and the active viral vector groups consumed high levels of ethanol, but the animals that had received the anticatalase vector started reducing their consumption in time while stabilizing only at one-half of consumption levels of the control animals. This time-dependent limited reduction in alcohol self-administration after the 4-week period of ethanol deprivation is likely due to the lack of reward in animals administered with the anticatalase vector following a limited extinction of the conditioning to the ethanol smell/taste. This reduction was clearly not seen in animals administered with the control viral vector in which the rewarding mechanisms were intact. Thus, rewarding mechanisms appear necessary to maintain the reconsolidation of conditioning following a short period of extinction.

In a subsequent experiment (Fig. 4), rats were allowed access to 10% ethanol and water for 60 days time at which time the animals were divided into 2 groups that were administered into the VTA either the anticatalase or the control vector. Again, under a continued 24-hour ethanol access, alcohol self-administration was not altered by the anticatalase vector for the 18 days postvector tested (Fig. 4 inset). Thereafter, alcohol was removed for 3 days, and animals were allowed access to 10% ethanol (and water) for only 1 hour while deprived of ethanol for 23 hours each day. Under this condition, alcohol intake was very high in both groups, independently of the pretreatment with anticatalase or the control viral vector, reaching consumption levels of 1.6 to 1.8 g ethanol/kg/h (i.e., some 5-fold higher that the average hourly consumption in 24 hours). This oral consumption would

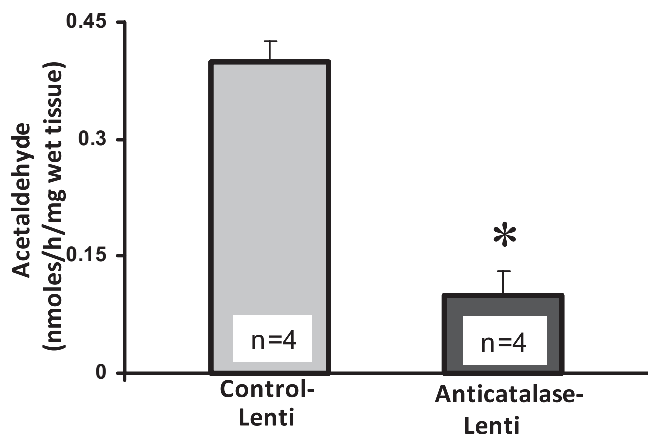

Fig. 2. Anticatalase-lentiviral vector injection into the ventral tegmental area (VTA) inhibited acetaldehyde generation by VTA homogenates. VTA homogenates from UChB rats pretreated with a single dose (8×10^4 virus) of anticatalase-Lenti-shRNA vector injected locally into the VTA, 30 days before the experiment, produced a significantly ($p < 0.001$) lower concentration (-75%) of acetaldehyde via ethanol oxidation than VTA homogenates from rats pretreated with empty lentiviral vector (control-Lenti). Incubations were carried out for 60 minutes at 37°C in the presence of 50 mM ethanol, 10 mM glucose, and VTA homogenate (5-mg wet tissue). At 10 mM, 3-amino-1,2,4-triazole (AT), a catalase inhibitor, blocked by 80% the formation of acetaldehyde by the homogenates. Acetaldehyde generated was therefore adjusted to reflect only that owing to catalase activity. See Experiment 2 in Materials and Methods. Data shown are means ± SEM. Significant (*$p < 0.001$).

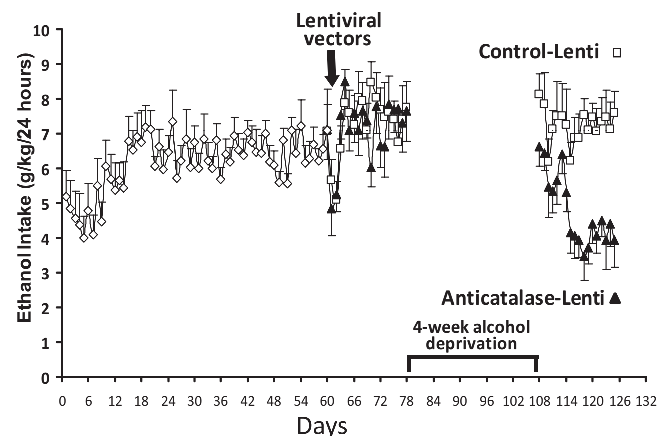

Fig. 3. A period of alcohol deprivation is required to reduce voluntary alcohol intake following the anticatalase-lentiviral vector administration into the ventral tegmental area (VTA). UChB rats ($n = 10$) allowed to access 10% ethanol and water on a 24-hour basis for 2 months did not change their voluntary alcohol intake when these were subsequently injected into the VTA a single dose of a lentiviral vector coding for an shRNA against catalase (anticatalase-Lenti), but significantly [ANOVA; $F(1, 33) = 111.54$, $p < 0.001$] reduced (50%) their alcohol intake following 4 weeks of alcohol deprivation when compared to the ethanol intake of animals ($n = 10$) that received a control lentiviral vector (control-Lenti). After the alcohol deprivation period, the animals were returned to a free access of 10% ethanol and water on a 24-hour basis. See Experiment 3 in Materials and Methods. Abscissa: days of ethanol availability. Data shown are means ± SEM.

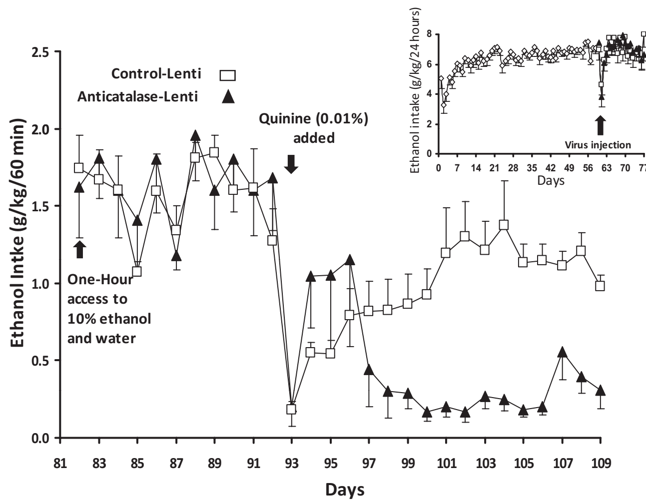

Fig. 4. Anticatalase-lentiviral vector administration into the ventral tegmental area (VTA) plus adulteration of ethanol solution with quinine reduces voluntary alcohol intake in long-term alcohol-drinking rats. Alcohol (10%)- and water-preexposed UChB rats for 2 months under a 24-hour access schedule were injected into the VTA a single dose of lentiviral vector coding for an shRNA against catalase (n = 10) or a control lentiviral vector (n = 10). Thereafter, animals were transferred to their home cage and returned to continuous 24-hour access to a 10% ethanol and water for 18 days (inset). Following this time, rats were deprived of alcohol for 3 days, and ethanol (10%) access was restricted to 1 hour every day (from 14:00 to 15:00 hours on a normal circadian light–dark cycle) in their home cage. After 11 days of limited alcohol access, quinine hydrochloride (Sigma-Aldrich) (0.01%) was added to the 10% ethanol solution, and ethanol consumption under the 1-hour limited access was registered every day for 17 days. Following the quinine addition, animals that received the anticatalase viral vector significantly reduced their alcohol intake [ANOVA; $F(1, 53) = 6.94$, $p < 0.01$] compared to controls injected with empty lentiviral vector (control-Lenti). See Experiment 4 in Materials and Methods. Data shown are means ± SEM.

reach blood alcohol levels of the order of 150 mg/dl (0.15%), levels never observed in animals allowed access to 10% ethanol and water on a 24-hour basis (unpublished data). Under this condition, consumption upon readministration may be driven by (i) a nonconditioned withdrawal reaction, resulting in relief by negative reinforcement, or (ii) a conditioned (alcohol taste/smell) reaction, as it has been proposed, which might also occur in animals and humans experiencing the contextual cues of the drug to which they have been chronically exposed (Buccafusco and Shuster, 2009; Volkow et al., 2011). To test whether the contextual cues were an important factor leading to a high alcohol self-administration, a low concentration of quinine (0.01%) was permanently added to adulterate the 10% ethanol solution while the 1-hour daily administration of 10% ethanol (and 23-hour deprivation) was maintained. Data show that upon adulteration of ethanol with quinine, both groups greatly reduced their alcohol intake, suggesting that a contextual taste cue was important for the drinking in both groups, whether having received the control viral vector or the anticatalase vector. The quinine adulteration was maintained until the end of the experiment, such that a new cue for ethanol would be constantly present. It is noteworthy that animals treated with the control vector returned to a high 1-hour consumption of ethanol, suggesting that in these animals, reward continued to play a role in alcohol self-administration. In contrast, animals treated with the anticatalase vector remained at a low consumption level. After ethanol adulteration, the overall inhibition of ethanol–quinine intake exerted by the anticatalase viral vector was 80 to 85%, compared to the intake of animals that received the control lentiviral vector.

On the basis of long-term studies in animal models of acute ethanol intoxication and repeated withdrawal, where physical dependence is likely generated, in which quinine does not greatly affect alcohol self-administration (Hopf et al., 2010; Spanagel et al., 1996), the present findings suggest that contextual cues (the taste/odor of ethanol) rather than physical dependence play a more important role in the continued alcohol consumption model. For the animals treated with the control virus, addition of quinine to the ethanol solution appears akin to resetting the contextual drug cues versus drug reward. For these animals, in which the mechanism of reward is intact, a new association (conditioning) between the novel cues (ethanol–quinine) and reward is likely, while this was clearly not observed in animals given the anticatalase vector, in which the rewarding effects of ethanol were blocked.

Overall, data suggest that abolishing or blunting of conditioning, added to appropriate pharmacological means to reduce ethanol reward, appears to have optimum effects in reducing relapse. The studies may have relevance to the treatment of alcoholics.

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