# Reduction in Central $H_2O_2$ Levels Prevents Voluntary Ethanol Intake in Mice: A Role for the Brain Catalase-$H_2O_2$ System in Alcohol Binge Drinking

Juan Carlos Ledesma, Pablo Baliño, and Carlos M. G. Aragon

**Background:** Hydrogen peroxide ($H_2O_2$) is the cosubstrate used by the enzyme catalase to form Compound I (the catalase-$H_2O_2$ system), which is the major pathway for the conversion of ethanol (EtOH) into acetaldehyde in the brain. This centrally formed acetaldehyde has been shown to be involved in some of the psychopharmacological effects induced by EtOH in rodents, including voluntary alcohol intake. It has been observed that different levels of this enzyme in the central nervous system (CNS) result in variations in the amount of EtOH consumed. This has been interpreted to mean that the brain catalase-$H_2O_2$ system, by determining EtOH metabolism, mediates alcohol self-administration. To date, however, the role of $H_2O_2$ in voluntary EtOH drinking has not been investigated.

**Methods:** In the present study, we explored the consequence of a reduction in cerebral $H_2O_2$ levels in volitional EtOH ingestion. With this end in mind, we injected mice of the C57BL/6J strain intraperitoneally with the $H_2O_2$ scavengers alpha-lipoic acid (LA; 0 to 50 mg/kg) or ebselen (Ebs; 0 to 25 mg/kg) 15 or 60 minutes, respectively, prior to offering them an EtOH (10%) solution following a drinking-in-the-dark procedure. The same procedure was followed to assess the selectivity of these compounds in altering EtOH intake by presenting mice with a (0.1%) solution of saccharin. In addition, we indirectly tested the ability of LA and Ebs to reduce brain $H_2O_2$ availability.

**Results:** The results showed that both LA and Ebs dose-dependently reduced voluntary EtOH intake, without altering saccharin consumption. Moreover, we demonstrated that these treatments decreased the central $H_2O_2$ levels available to catalase.

**Conclusions:** Therefore, we propose that the amount of $H_2O_2$ present in the CNS, by determining brain acetaldehyde formation by the catalase-$H_2O_2$ system, could be a factor that determines an animal's propensity to consume EtOH.

**Key Words:** Ethanol Intake, Alpha-Lipoic Acid, Ebselen, $H_2O_2$, Catalase, Acetaldehyde.

HYDROGEN PEROXIDE ($H_2O_2$) is the cosubstrate used by catalase to form the catalase-$H_2O_2$ system (known as Compound I), which is the major pathway of brain ethanol (EtOH) metabolism (Zimatkin et al., 2006). It has been postulated that central EtOH metabolism by the activity of this enzymatic complex mediates some of the psychopharmacological effects produced by this alcohol (Deng and Deitrich, 2008). Previous research has demonstrated that individual differences in the activity of brain catalase in rats of the same strain correlate positively with their voluntary EtOH intake (Amit and Aragon, 1988; Aragon et al., 1985). Moreover, it has been shown that strains of rats with higher brain catalase activity drink more EtOH than other strains with a lower activity of this enzyme in the central nervous system (CNS) (Gill et al., 1996a). In another set of reports, it has been observed that the administration of the brain catalase inhibitor 3-amino-1,2,4-triazole (AT) prevents voluntary EtOH consumption in rats (Aragon and Amit, 1992; Tampier et al., 1995). Existing data also showed that rats treated with an anticatalase shRNA lentiviral vector, which inhibits catalase synthesis and expression in the CNS, consume lower amounts of EtOH than their control mates (Karahanian et al., 2011). Therefore, it has been proposed that differences in brain catalase activity correlate positively with the amount of EtOH intake and determine EtOH consumption in rats.

On the other side, it has been reported that strains of mice with a lower activity of this enzyme exhibited an increased preference to voluntarily consume EtOH. Thus, acatalasemic mice, which have lower levels of catalase (50%) in the brain, displayed a greater EtOH intake compared with normal mice (Aragon and Amit, 1993). Similarly, the C57BL/6J strain of inbred mice, which have a significantly lower activity of this enzyme in the brain, showed an increment in the amount of alcohol intake with regard to other strains with higher levels of catalase, thus suggesting a negative correlation between the expression of the enzyme catalase in the CNS of mice and EtOH self-administration (Correa et al.,

*From the Área de Psicobiologia (JCL, PB, CMGA), Universitat Jaume I, Castellón, Spain.*

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*Reprint requests: Carlos M. G. Aragon, Área de Psicobiologia, Universitat Jaume I, Avda. SosBaynat s/n, 12701-Castellón, Spain; Tel.: +34 964 729835; Fax: +34 964 729867; E-mail: aragon@psb.uji.es*



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2004; Gill et al., 1996b). However, other studies showed that mice treated with AT displayed a dose-dependent reduction in their volitional EtOH intake (Koechling and Amit, 1994). Overall, although the direction of the relationship between catalase activity and EtOH ingestion is still not clear, given all these data, it has been postulated that differences in brain catalase may be one of the factors determining EtOH consumption in alcohol naïve animals.

In addition to EtOH intake, there are many other behaviors that could be modulated by alterations or differences in brain catalase. It has been shown that strains of mice with a lower activity of this enzyme in the CNS exhibited a decreased response to the locomotor-stimulating effects induced by EtOH (Aragon and Amit, 1993; Correa et al., 2004). Moreover, both rats and mice treated with the catalase inhibitor AT or with the anticatalase shRNA lentiviral vector displayed an attenuation of a wide range of EtOH-elicited behaviors such as locomotor stimulation (Escarabajal et al., 2000), conditioned place preference (Font et al., 2008), and EtOH-induced reward and relapse (Quintanilla et al., 2012) compared with their normal control mates. These results indicate that this enzyme participates in the mediation of some of the psychopharmacological effects caused by alcohol.

Nonetheless, the exact contribution of the catalase-$H_2O_2$ system to EtOH-induced behaviors has yet to be investigated in detail. In this respect, recent studies suggest that in addition to catalase, $H_2O_2$ could be a factor that modulates EtOH behavioral effects. Interestingly, it has been demonstrated that different treatments that boosted $H_2O_2$ central levels produced an increase in EtOH-induced locomotor stimulation in mice (Manrique et al., 2006; Pastor et al., 2002). In contrast, administration of $H_2O_2$ scavengers, which decrease $H_2O_2$ availability in the brain, reduced the locomotor stimulation evoked by an EtOH challenge (Ledesma and Aragon, 2012; Ledesma et al., 2012) and the acquisition and reconditioning of EtOH-induced conditioned place preference in mice (Ledesma and Aragon, 2013).

The aim of the present work was to evaluate the role of $H_2O_2$ on EtOH binge drinking following a drinking-in-the-dark (DID) procedure. Hence, we selected alpha-lipoic acid (LA) and ebselen (Ebs) as $H_2O_2$ scavengers. These compounds have been demonstrated to modulate brain $H_2O_2$ levels when administered systemically (Ledesma and Aragon, 2012, 2013; Ledesma et al., 2012). We hypothesized that the reduction in endogenous $H_2O_2$ levels by LA and Ebs would decrease the activity of the brain catalase-$H_2O_2$ system, and therefore voluntary EtOH consumption. We have also assessed whether these pharmacological tools were selective in reducing EtOH intake when compared to a sweetened solution. Moreover, the present investigation was also extended to study the ability of these agents to reduce the $H_2O_2$ levels in the brain. The results derived from the present work highlight the relevance of central $H_2O_2$ availability and thereby the functional role of catalase in volitional EtOH intake.

## MATERIALS AND METHODS

### *Subjects*

Male mice of the C57BL/6J strain, obtained from Charles River Laboratories (Madrid, Spain), were used. Animals were 5 weeks old at the time of arrival and were housed inside the quarantine room for 1 week under a 12-hour light/dark cycle (lights off at 6:00 AM). Following this period, they were housed 1 per cage and acclimated to the colony environment for at least 1 week before experiments began. The temperature and humidity of the colony room were maintained at 22 ± 1°C and 50 ± 5%, respectively. Food and water were provided ad libitum throughout the study, except during the restricted-access periods of EtOH drinking, in which water was not available. All experimental procedures complied with the European Community Council Directive (86/609/ECC) for the use of laboratory animal subjects.

### *Drugs*

LA (1,2-dithiolane-3-pentanoic acid), purchased from Sigma-Aldrich Química S.A. (Madrid, Spain), was dissolved in Dulbecco's phosphate-buffered saline (vehicle [Vh]) using a possible minimal amount of 1 M NaOH solution. The final pH of the resulting solution was 7 ± 1. LA was injected intraperitoneally (IP) at doses of 12.5, 25, and 50 mg/kg. Ebs [2-phenyl-1,2 benzoisosenazol-3 (2H-one)] (Sigma-Aldrich Química S.A., Madrid, Spain) was dissolved in Tween 80 at a concentration of 10% (Vh) and injected IP at doses of 6.25, 12.5, and 25 mg/kg. Cyanamide (Sigma-Aldrich Química S.A.) was diluted in physiological saline (9% NaCl [Sal]) and injected IP at a dose of 40 mg/kg. EtOH, obtained from Panreac Química S.A. (Madrid, Spain), was diluted in tap water from an initial stock of 96% EtOH, and presented in a glass cylinder at increasing concentrations from 2 to 10% v/v. Saccharin 0.1% w/v (Sigma-Aldrich Química S.A.) was diluted in tap water. All the drug doses and drinking solutions were selected based on previous reports (Aragon and Amit, 1993; Font et al., 2006; Ledesma and Aragon, 2012; Ledesma et al., 2012; Sanchis-Segura et al., 1999).

### *General Behavioral Procedures*

*Voluntary EtOH Intake.* Alcohol was always presented at the beginning of the third hour of the animals' dark cycle (at 9 AM). Prior to the initiation of the experiments, mice were subjected to a habituation period, for 10 days, in which exposure to EtOH started with increasing concentrations of EtOH that changed every 2 days by increments of 2% (from 2 to 10% w/v). This period of habituation to EtOH drinking has previously been used by other authors, and serves to accustom animals to the smell and flavor of EtOH. It also avoids the neophobia initially induced by the presentation of new fluids in rodents (Aragon and Amit, 1993; Correa et al., 2004; Font et al., 2006). During EtOH presentation, the water bottles were always replaced with 10-ml graduated glass cylinders containing an EtOH solution for 2 hours. When a concentration of EtOH of 10% was achieved, the baseline phase started, which consists in presenting a 10% w/v solution of EtOH every testing day until the intake of all mice reached stable levels (for 1 week, data not shown). In this baseline period, we followed the same schedule as in the experimental phase, with the exception that no injections were given. The methodology followed in the drinking procedures was a variation of that described by Rhodes and colleagues (2005) and Kamdar and colleagues (2007) known as *drinking-in-the-dark* (DID). Briefly, the 2-day DID procedure was repeated in the same animals twice a week (Monday-Tuesday, and Thursday-Friday, with Wednesday off) for 2 weeks (weekend off). On the first day (Monday and Thursday), all mice received Vh injections prior to EtOH presentation, and on the second day (Tuesday and Friday) of

each 2-day session, each mouse received an IP injection of one of the drug doses at the same time interval as on the preceding day. The order in which the drug doses were administered was counterbalanced across animals and days. Although the DID test is normally performed with 20% EtOH, in our experiments 10% EtOH is used because it is the most widely used concentration in pharmacological studies with EtOH (Aragon and Sternklar, 1985; Correa et al., 2004; Kamdar et al., 2007; Koechling and Amit, 1994; Quintanilla et al., 2012).

*Voluntary Saccharin Intake*. As in the procedure described above, the 2-day DID protocol was used to evaluate the effect of our manipulations on voluntary saccharin intake. The same experimental design was repeated with a new cohort of animals. Thus, before the DID tests started, mice underwent a 1-week habituation period to allow them to get accustomed to the flavor of saccharin and the experimental conditions. The concentration of the saccharin solution offered was always 0.1% w/v.

*Experiment 1: Effect of Several Doses of LA on Voluntary EtOH Intake*. This experiment evaluated the effect of LA on voluntary EtOH intake under the DID procedure outlined above. Thus, on the test days, mice ($n$ = 15) were injected with Vh or 1 of the LA doses (12.5, 25, or 50 mg/kg) 15 minutes prior to EtOH (10%) presentation. EtOH was available during 90 minutes, and then the water bottles were replaced again until the next testing session.

*Experiment 2: Effect of LA on Voluntary Saccharin Intake*. To test the selectivity of LA in modifying voluntary EtOH (10%) consumption, here the animals ($n$ = 16) were offered a (0.1%) solution of saccharin at the same time intervals as in Experiment 1. Thus, mice were treated with Vh or LA (25 or 50 mg/kg) 15 minutes before saccharin (0.1%) presentation.

*Experiment 3: Effect of Several Doses of Ebs on Voluntary EtOH Intake*. The following experiment was identical to Experiment 1, with the exception that in this case, mice ($n$ = 12) were treated with Vh or Ebs (6.25, 12.5 or 25 mg/kg) 60 minutes before placement of the EtOH (10%) cylinders. In addition, given that previous reports showed that the effects of Ebs on EtOH-induced behavior last longer than those of LA (Ledesma and Aragon, 2012; Ledesma et al., 2012), in this study, to establish the direct consequence of our treatments on alcohol intake more thoroughly, the EtOH drinking solution was offered for 180 minutes.

*Experiment 4: Effect of Ebs on Voluntary Saccharin Intake*. Similarly to Experiment 2, here, we evaluated the effect of Ebs on volitional saccharin (0.1%) intake. For this purpose, animals ($n$ = 14) were injected with Vh or Ebs (12.5 or 25 mg/kg) 60 minutes prior to offering them the (0.1%) saccharin solution for 180 minutes.

*Experiments 5a and 5b: Determination of Brain Catalase Activity*. The goal of this study was to examine whether LA and Ebs were able to reduce central $H_2O_2$ levels available to catalase, and therefore to test the putative mechanism of action by which these pharmacological manipulations could interact with the metabolism of EtOH in the brain. Through 2 separate experiments, we evaluated the effect of these $H_2O_2$ scavengers on the inactivation of brain catalase activity induced by cyanamide. The rationale for this investigation was as follows: Cyanamide, like AT, is a non-competitive catalase inhibitor that only inactivates catalase when this enzyme reacts to $H_2O_2$ and forms Compound I (DeMaster et al., 1986). That is, the effect of cyanamide on catalase is $H_2O_2$-dependent (DeMaster et al., 1986). Therefore, the inactivation of the brain catalase activity of mice treated with cyanamide could be used as a measure of the amount of $H_2O_2$ produced in the brain (Ledesma et al., 2012; Pastor et al., 2002). In other words, changes in $H_2O_2$ levels could modify the inhibiting effect of cyanamide on brain catalase activity. Thus, the inactivation of cerebral catalase in animals treated with cyanamide may be used as an indirect indicator of the formation of Compound I in the CNS. We hypothesized here that the inhibition of brain catalase induced by cyanamide will decrease in mice treated with the $H_2O_2$ sequestering agents LA and Ebs (Bilska et al., 2007; Johnsen-Soriano et al., 2007). To test this, EtOH-naïve animals ($n$ = 6 per group) were treated with Vh or LA (50 mg/kg; Experiment 5a) and with Vh or Ebs (25 mg/kg; Experiment 5b); simultaneously, they were also treated with Sal or cyanamide (40 mg/kg). Four hours after these treatments, mice were perfused through the heart using 50 ml of a heparinized (1,000 IU/l) isotonic saline solution. The whole brain was removed and homogenized in phosphate buffer (50 mM; pH 7.0) with digitonin (0.01%). Brain homogenates were centrifuged at $5{,}000\times g$ for 15 minutes in an Eppendorf microcentrifuge. Supernatant aliquots were used to determine brain catalase levels. Catalase activity was assayed spectrophotometrically by measuring the decrease in absorbance of $H_2O_2$ at 240 nm ($\epsilon_{240}$ = 0.00394 $mmol^{-1} \times mm^{-1}$). Protein levels were determined from supernatants in accordance with the method described by Bradford (1976).

### *Statistical Analyses*

One- or 2-way analysis of variance (ANOVA), following post hoc comparisons with Duncan's post hoc test, was applied when appropriate. The Statistica 6.1 (StatSoft, Tulsa, OK) software package was used.

## RESULTS

### *Experiment 1: Effect of Several Doses of LA on Voluntary EtOH Intake*

Figure 1*A* shows the consequence of LA administration (0, 12.5, 25, or 50 mg/kg) on EtOH (10%) consumption. Results from a 1-way ANOVA indicated a significant effect of the LA dose, $F(3, 56) = 2.99$, $p < 0.05$. Post hoc Duncan's comparisons revealed that animals drank less EtOH when they were treated with the LA doses of 25 and 50 mg/kg ($p < 0.05$).

### *Experiment 2: Effect of LA on Voluntary Saccharin Intake*

As can be seen in Fig. 1*B*, LA (25 or 50 mg/kg) had no effect on voluntary saccharin intake. This conclusion was confirmed by a 1-way ANOVA, $F(2, 45) = 0.77$, $p > 0.05$.

### *Experiment 3: Effect of Several Doses of Ebs on Voluntary EtOH Intake*

Figure 2*A* represents the volitional EtOH (10%) ingestion of mice previously treated with Ebs (0, 6.25, 12.5, and 25 mg/kg). The 1-way ANOVA showed a significant effect for the Ebs dose, $F(3, 44) = 13.66$, $p < 0.01$. Duncan's post hoc test revealed that EtOH intake was statistically reduced when animals were injected with the doses of Ebs of 12.5 and 25 mg/kg ($p < 0.01$).

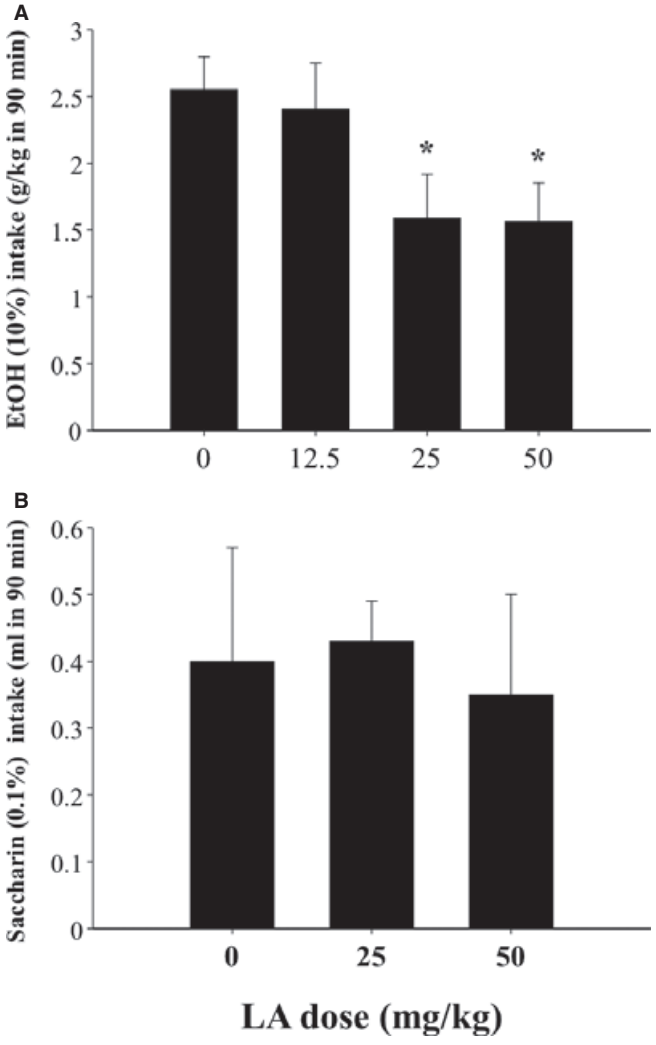


**Fig. 1.** Effect of different doses of alpha-lipoic acid (LA) on ethanol (EtOH) (**A**) and saccharin (**B**) intake for 90 minutes. Animals were offered EtOH (10%; **A**) and saccharin (0.1%; **B**) 15 minutes after LA (0, 12.5, 25, or 50 mg/kg) administration following the described drinking-in-the-dark procedure (see Materials and Methods section). Bars depict mean ± SEM of total EtOH (g/kg; **A**) and saccharin (ml; **B**) consumption (*$p < 0.05$ significantly different from LA 0 control group).

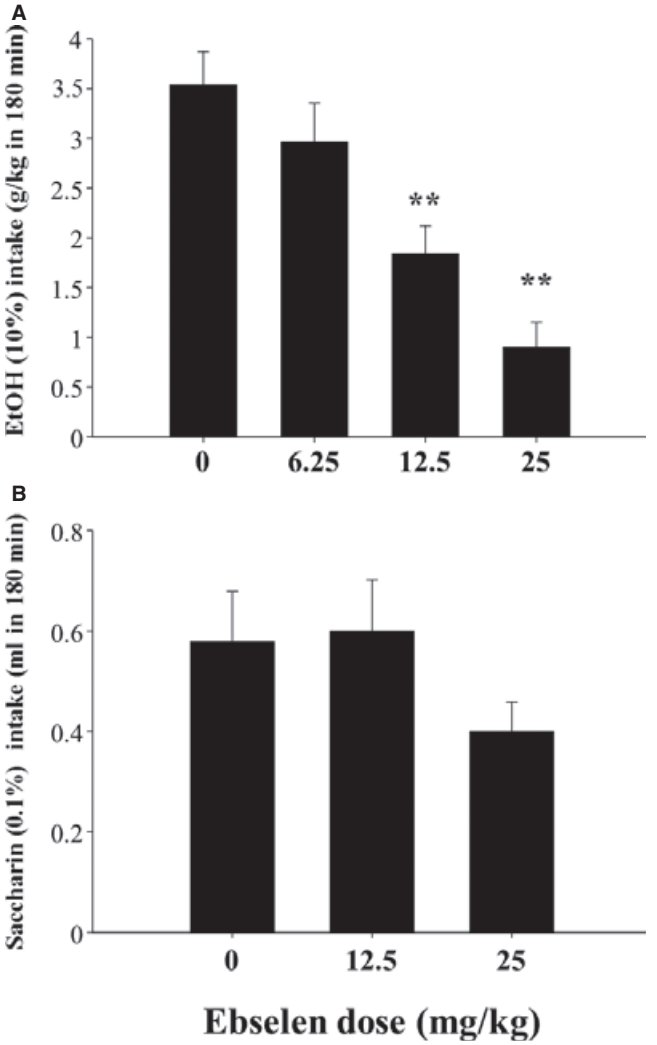


**Fig. 2.** Effect of different doses of ebselen (Ebs) on ethanol (EtOH) (**A**) and saccharin (**B**) intake for 180 minutes. Animals were offered EtOH (10%; **A**) and saccharin (0.1%; **B**) 60 minutes after Ebs (0, 6.25, 12.5, or 25 mg/kg) administration following the described drinking-in-the-dark procedure (see Materials and Methods section). Bars depict mean ± SEM of total EtOH (g/kg; **A**) and saccharin (ml; **B**) consumption (**$p < 0.01$ significantly different from Ebs 0 control group).

*Experiment 4: Effect of Ebs on Voluntary Saccharin Intake*

Figure 2*B* depicts the result of Ebs (12.5 or 25 mg/kg) administration on saccharin consumption. The 1-way ANOVA demonstrated that this compound did not modify saccharin ingestion, $F(2, 39) = 1.522$, $p > 0.05$, under these experimental conditions.

*Experiment 5a and 5b: Determination of Brain Catalase Activity*

Figure 3 shows the effect of the combined treatments with LA (0 or 50 mg/kg; Fig. 3*A*) or Ebs (0 or 25 mg/kg; Fig. 3*B*) and cyanamide (0 or 40 mg/kg) on brain catalase activity. In Experiment 5a (Fig. 3*A*), a 2-way ANOVA LA treatment (0 or 50 mg/kg) × cyanamide treatment (0 or 40 mg/kg) yielded a significant effect for the cyanamide treatment, $F(1, 20) = 10.30$, $p < 0.01$, and a significant interaction between factors, $F(1, 20) = 7.11$, $p < 0.05$. Post hoc tests indicated that the brain catalase activity of mice treated with Vh and cyanamide (Vh-Cyanamide 40 mg/kg group) was significantly lower than that of animals injected with Vh and Sal (Vh-Cyanamide 0 mg/kg group) ($p < 0.01$). In contrast, LA protected catalase against cyanamide inhibition because the central catalase activity of mice treated with LA (50 mg/kg) and cyanamide (40 mg/kg) (LA-Cyanamide 40 mg/kg group) was not altered when this group was compared with the Vh-Cyanamide 0 mg/kg group ($p > 0.05$). Moreover, the brain catalase activity of the LA-Cyanamide 40 mg/kg group was significantly higher than that of the Vh-Cyanamide 40 group ($p < 0.05$).

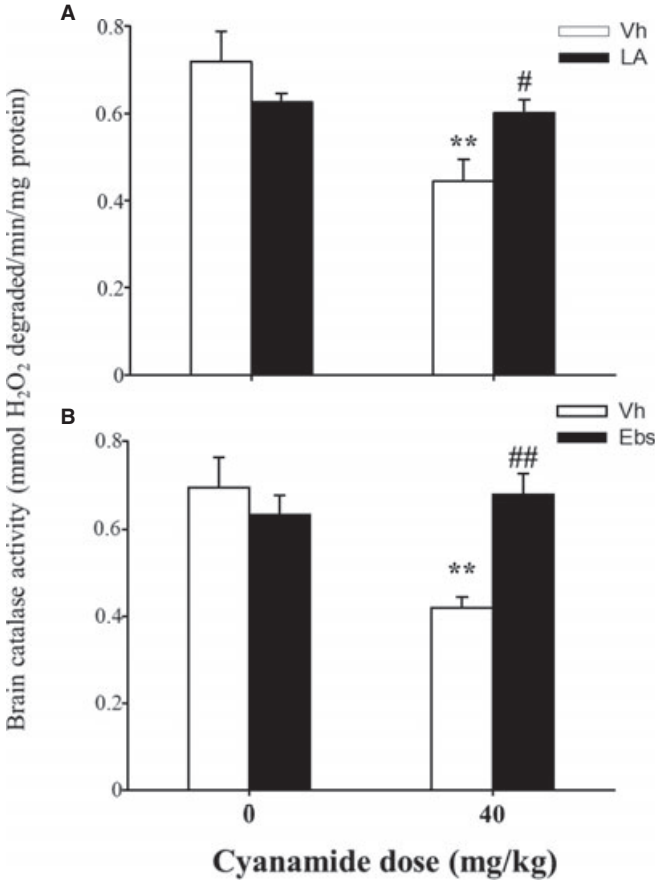


**Fig. 3.** Effect of alpha-lipoic acid (LA) (**A**) and ebselen (Ebs; **B**) treatment on the inhibition of brain catalase activity induced by cyanamide. Bars depict mean ± SEM of brain catalase activity (mmol $H_2O_2$ degraded/min/mg protein) ($n$ = 6 per group). LA (0 or 50 mg/kg; **A**) and Ebs (0 or 25 mg/kg; **B**) were administered simultaneously with cyanamide (0 or 40 mg/kg) treatment and 4 hours later catalase activity was analyzed (**$p < 0.01$ significantly different from its respective Vh-Sal control group; ##$p < 0.01$, #$p < 0.05$ significantly different from its Vh-Cyanamide 40 group). $H_2O_2$, hydrogen peroxide, Vh, vehicle, Sal, saline.

Similarly, in Fig. 3*B*, we can observe that Ebs (25 mg/kg) impeded the impairment of brain catalase mediated by cyanamide (40 mg/kg) injection. This conclusion was reported by a 2-way ANOVA Ebs treatment (0 or 25 mg/kg) × cyanamide treatment (0 or 40 mg/kg), which found a significant effect for the cyanamide treatment, $F(1, 20) = 5.52$, $p < 0.05$, and a significant interaction between the 2 factors, $F(1, 20) = 10.97$, $p < 0.01$. Again, Duncan's post hoc testing revealed that the brain catalase activity of mice treated with Vh and cyanamide (Vh-Cyanamide 40 mg/kg group) was significantly lower than that of animals injected with Vh and Sal (Vh-Cyanamide 0 mg/kg group) ($p < 0.01$). In contrast, as occurred with LA, Ebs (25 mg/kg) treatment blocked the catalase impairment induced by cyanamide administration, because catalase activity in the brains of mice treated with Ebs (25 mg/kg) and cyanamide (40 mg/kg) (Ebs-Cyanamide 40 mg/kg group) was not altered when this group was compared with the Vh-Cyanamide 0 mg/kg group ($p > 0.05$). In addition, post hoc comparisons demonstrated that catalase activity was significantly higher in the Ebs-Cyanamide 40 mg/kg group with regard to the Vh-Cyanamide 40 mg/kg group, thus suggesting that Ebs protects catalase against cyanamide inhibition ($p < 0.01$).

## DISCUSSION

In the present study, a systemic injection of LA and Ebs diminished voluntary EtOH intake in mice. Furthermore, these compounds did not modify saccharin ingestion, at least at the times and doses tested in our experiments. In addition, we have found that these treatments were able to reduce brain $H_2O_2$ levels, as measured by the $H_2O_2$-dependent inactivation of endogenous brain catalase activity following an injection of cyanamide. Therefore, we suggest that a reduction in central $H_2O_2$ levels could alter the motivational properties of EtOH that lead to alcohol binge drinking.

In the DID experiments, LA and Ebs administration dose-dependently decreased voluntary EtOH consumption. Mice treated with LA (25 or 50 mg/kg) or Ebs (12.5 or 25 mg/kg) displayed a significant reduction in their voluntary EtOH intake. The DID method represents a model of animal EtOH binge drinking that could be used to explore the mechanisms underlying high rates of alcohol drinking and to screen new compounds capable of limiting its excessive intake (Kamdar et al., 2007; Rhodes et al., 2005). This behavioral procedure captures important features of alcoholism, such as drinking to the point of intoxication and rapid initiation to high levels of drinking (Rhodes et al., 2005). In our experiments, DID EtOH intakes were consistent with the available literature demonstrating that the DID model consistently yields high EtOH consumption over a relatively short time period (Kamdar et al., 2007; Rhodes et al., 2005). Considering that binge drinking is a substantial risk factor predicting the development of alcohol use disorders (Crabbe et al., 2011), our data are significantly relevant as they represent a validation of 2 new pharmacological tools to impair EtOH binge drinking.

Both LA and Ebs are $H_2O_2$ scavengers that are able to decrease the production and the concentrations of reactive oxygen species (ROS) in the CNS, including $H_2O_2$. LA is an essential cofactor for mitochondrial enzymes that has been described as a novel biological antioxidant and a potent free radical scavenger (Somani et al., 2000). This agent participates in the recycling of vitamins C and E, increases levels of glutathione, and suppresses nonenzymatic glycation (Suzuki et al., 1992). Acute administration of LA enhances glutathione peroxidase activity in different regions of the rat brain and decreases the concentration of ROS, especially $H_2O_2$ (Bilska et al., 2007). Similarly, Ebs is a seleno–organic compound with radical-scavenging activity (Müller et al., 1984). This compound has been shown to be involved in a number of antioxidant activities both in vitro and in vivo (Moretto et al., 2004). Ebs is able to increase the rate of elimination of $H_2O_2$ because it catalyzes the thiol- peroxidase reaction in a

mechanism that appears to be kinetically identical to the mechanism of glutathione peroxidase (Parnham and Sies, 2000). In the second set of experiments, we focused on the effect of LA and Ebs treatment on the inhibitory effect that sodium cyanamide causes in brain catalase. Cyanamide is a suicide inhibitor of the enzyme catalase (DeMaster et al., 1986; Sanchis-Segura et al., 1999). Interestingly, this drug only inhibits catalase activity when this enzyme reacts to $H_2O_2$ by forming Compound I (Cederbaum and Dicker, 1985; DeMaster et al., 1986).Therefore, the effect of cyanamide on catalase activity may be used as an indirect measure of the capacity of this enzyme to form Compound I, because the cyanamide-mediated inactivation of brain catalase is $H_2O_2$-dependent. Thus, based on the suggestion that both LA and Ebs are capable of reducing endogenous $H_2O_2$ in rodents, in Experiments 5a and 5b, we hypothesized that the inhibition of cerebral catalase induced by cyanamide will be decreased in mice treated with these compounds. In agreement with this idea, our results showed that brain homogenates of mice treated with LA (50 mg/kg)-Cyanamide (40 mg/kg) and Ebs (25 mg/kg)-Cyanamide (40 mg/kg) have a higher catalase activity than Vh-Cyanamide (40 mg/kg)-treated mice. This indicates that, by reducing $H_2O_2$ availability in the CNS, both LA and Ebs treatments could reduce the formation of Compound I in the brain. These findings are in agreement with previous data obtained in our laboratory in which it has been also showed that both LA and Ebs reduced the amount of $H_2O_2$ available for brain catalase, as measured by the $H_2O_2$-dependent inactivation of central catalase activity following an injection of AT (Ledesma and Aragon, 2012, 2013; Ledesma et al., 2012).

As outlined above, we propose that both LA and Ebs reduce the formation of Compound I by decreasing $H_2O_2$ levels. As a result, when animals are exposed to EtOH, the rate of brain-EtOH metabolism would be decreased, thus preventing voluntary EtOH drinking. As noted earlier, the first product coming from brain-EtOH metabolism by the activity of Compound I is acetaldehyde. Acetaldehyde is a psychoactive molecule that, when administered centrally, exerts a broad range of behavioral (Arizzi-LaFrance et al., 2006; Rodd-Henricks et al., 2002; Smith et al., 1984) and neurochemical (Diana et al., 2008; Enrico et al., 2009; Foddai et al., 2004; Sirca et al., 2011) effects that are similar to those induced by EtOH in rodents. For this reason, it has been proposed that brain-EtOH-derived acetaldehyde could play a role in some of the psychopharmacological effects caused by EtOH. In agreement to this, it has been observed that administration of acetaldehyde chelating agents, such as D-penicillamine and L-cysteine, prevents a wide range of EtOH-elicited behaviors, including EtOH intake (Font et al., 2005, 2006; Martí-Prats et al., 2010; Pautassi et al., 2010; Peana et al., 2010). The results obtained in this study indicated that $H_2O_2$ levels are critical to EtOH intake. We suggest that in addition to the levels of catalase present in the organisms, another factor that modulates EtOH-induced behaviors might be the availability of $H_2O_2$, which would determine the rate of acetaldehyde production at the time of EtOH administration.

It has been suggested that the mesocorticolimbic dopamine (Da) is involved in EtOH drinking. Various reports have demonstrated that manipulations that reduce the activity of this neurotransmitter system decrease voluntary EtOH intake in rodents (Nguyen et al., 2007; Thanos et al., 2005). Moreover, acetaldehyde has been shown to be capable of stimulating the transmission of mesolimbic Da (Deehan et al., 2013; Deng and Deitrich, 2008; Enrico et al., 2009; Foddai et al., 2004; Sirca et al., 2011). Several authors have proposed that acetaldehyde mediates the EtOH-induced stimulation of mesolimbic Da transmission. It has been reported that both acetaldehyde sequestration with D-penicillamine and L-cysteine, or the inhibition of brain catalase activity by AT prevent the activation of this brain circuitry evoked by EtOH (Deng and Deitrich, 2008; Enrico et al., 2009; Sirca et al., 2011). Brain catalase has been shown to be highly active in aminergic neurons and along the mesolimbic DA pathway (Deng and Deitrich, 2008; Zimatkin and Lindros, 1996). Therefore, given the above studies, we suggest that the reduction in EtOH self-administration observed in our experiments could be due to a decreased activation of this neurotransmitter in the mesolimbic DA pathway system.

Alternative explanations should also be considered. That is, this reduction in volitional EtOH consumption could be interpreted as being produced by an unspecific alteration of these pharmacological tools in sensitivity to taste, or an impairment of some brain areas or neurotransmitter circuitry related to reinforcement and motivation. However, here, we showed that voluntary saccharin intake was not affected by these treatments. Therefore, if the reduction in EtOH drinking was only the result of a general alteration of these compounds in motivated behavior, we should expect these drugs to have similar consequences on saccharin ingestion. Our results demonstrated that these treatments selectively affected EtOH consumption because saccharin intake remained unaltered after LA and Ebs administration, thus ruling out that possibility. As support to this notion, previous data also showed that the effects of these compounds are specifically selective for other EtOH-induced behaviors (Ledesma and Aragon, 2012, 2013; Ledesma et al., 2012).

Another possibility that should be contemplated is that the capacity of these $H_2O_2$ scavengers to diminish volitional EtOH drinking might be mediated by a modulation in spontaneous locomotion or in blood EtOH levels after its administration. Nonetheless, data obtained in other studies demonstrated that these compounds did not modify the baseline motor activity or blood EtOH concentrations in mice (Ledesma and Aragon, 2012, 2013; Ledesma et al., 2012).

In conclusion, we propose here a reduction in the activity of the catalase-$H_2O_2$ system as the main mechanism responsible for the interaction between LA and Ebs in modulating voluntary EtOH self-administration. The current findings

represent an extension of previous research in which it has also been suggested that modifications of $H_2O_2$ levels in the CNS could alter some EtOH-induced behaviors (Ledesma and Aragon, 2012, 2013; Ledesma et al., 2012; Manrique et al., 2006; Pastor et al., 2002). What is most important here is that the brain peroxidative status of the organism could modulate the acute psychopharmacological effects of EtOH. It seems that variations in the physiological conditions, that is, in the levels of $H_2O_2$ present in the CNS in a particular moment in the same organism, could determine some effects induced by EtOH. These data are particularly relevant to be able to understand the mechanisms underlying the causes of EtOH binge drinking. Because EtOH binge drinking is a feature of alcoholism, further research is needed to test the implication of the catalase-$H_2O_2$ system in the EtOH-dependent brain, for example, by performing the same experiments described here in EtOH-dependent rats. This should open up the way to the development of new pharmacological tools to reduce chronic alcohol use.

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