Pharmacology, Biochemistry and Behavior 94 (2010) 431–437

Contents lists available at ScienceDirect

Pharmacology, Biochemistry and Behavior

journal homepage: www.elsevier.com/locate/pharmbiochembeh

# L-Cysteine reduces oral ethanol self-administration and reinstatement of ethanol-drinking behavior in rats

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ARTICLE INFO

*Article history:*
Received 18 June 2009
Received in revised form 10 October 2009
Accepted 14 October 2009
Available online 29 October 2009

*Keywords:*
Ethanol
Oral operant self-administration
Reinstatement
L-Cysteine
Wistar rat

ABSTRACT

Our previous findings have shown that L-cysteine, a non essential amino acid, prevented ethanol (EtOH) induced conditioned place preference. The aim of the present study was to examine the effect of L-cysteine on the acquisition and maintenance of oral EtOH self-administration and on the reinstatement of EtOH-drinking behavior in Wistar rats. Rats were pretreated intraperitoneally with saline or L-cysteine (20 and 40 mg/kg) 30 min before each acquisition trial, in an operant nose-poking paradigm where they were given the opportunity to orally self-administer tap water or EtOH (5–10% v/v). Further, to evaluate if L-cysteine reduces the acquired oral EtOH self-administration, we carried out an independent experiment in which rats were trained to self-administer EtOH (10%); after all groups of rats developed similarly stable oral EtOH self-administration, the effect of L-cysteine (0, 40, 60, 80 and 100 mg/kg) was tested. An additional group of rats was pretreated with saline or L-cysteine (80 mg/kg) and tested on reinstatement after EtOH extinction and, at the end of last reinstatement session, were utilized to measure blood and brain EtOH levels. The animals that had access to EtOH solution discriminated between the active and inactive nose-pokes and showed rates of active nose-pokes significantly higher than the tap water group. Furthermore, rats self-administering EtOH (10%) also demonstrated extinction behavior and gradually reinstated active nose-poke responding when EtOH was reintroduced. L-cysteine reduced both the acquisition and maintenance of oral EtOH self-administration. The reduced reinstatement of EtOH-drinking behavior was paralleled by a significant reduction of EtOH intake and correlated with blood and brain EtOH levels. The efficacy of L-cysteine on the various phases of alcohol drinking in rats, could represent an interesting pharmacological approach and could open a new line of research for the development of therapies to reduce EtOH intake in alcoholic patients.



## 1. Introduction

During the last decades, several novel pharmacological strategies have been introduced for the treatment of alcoholism. The clinical introduction of different drugs was based on results obtained from animal studies using various ethanol (EtOH) self-administration paradigms, thus underlying the contention that animal models have a predictive value in the search for novel pharmacological agents that may be therapeutically effective in the treatment of alcohol addiction in man (Heidbreder and Hagan, 2005). EtOH self-administration in rodents is among the most widely accepted preclinical test for alcohol abuse and alcoholism studies (Koob and Le Moal, 2006). Indeed, drug self-administration well resembles the aberrant behavior of self-administration in humans (Koob et al., 2004), permitting to model the various phases of the addiction cycle (Koob and Le Moal, 2006; Melis et al., 2005; Robinson and Berridge, 2001) such as acquisition and/or maintenance phases of EtOH-drinking behavior. Relapse to drug-seeking behavior is a primary manifestation of drug addiction, and reducing relapse is an important clinical index of a successful intervention (O'Brien and Gardner, 2005). Relapse is modeled in rodents by measuring the reinstatement of drug-seeking behavior in animals that have undergone extinction training (Epstein et al., 2006). This preclinical model has been shown to have face validity for human relapse based on the fact that the same factors that elicit relapse in humans (drug-associated cues) precipitate relapse in the reinstatement model (Knackstedt and Kalivas, 2009).

A variety of neurotransmitter, spanning from dopamine, GABA, serotonin, corticotropin-releasing factor, opioids, glutamate, acetylcholine, adenosine as well as endocannabinoids systems have been shown to be involved in the neurobiological underpinnings of alcohol

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doi:10.1016/j.pbb.2009.10.005

abuse and alcoholism. These neurotransmitters have been reported to modulate oral operant EtOH self-administration behavior in mice (Miczek and de Almeida, 2001; Heidbreder et al., 2007) or in rats (Buczek et al., 1997; Cippitelli et al., 2007; Doyon et al., 2006; Thorsell et al., 2007; Funk and Koob, 2007; Adams et al., 2008; Economidou et al., 2006; Kuzmin et al., 2009). Further, recent rodent studies indicated that acetaldehyde (ACD), the first metabolite of EtOH, is involved in EtOH's psychoactive effects through its own motivational properties (Quertemont et al., 2005; Font et al., 2005, 2006a; Enrico et al., 2009; Foddai et al., 2004; Melis et al., 2007; Peana et al., 2008, 2009). Our previous findings have shown that L-cysteine, a non essential amino acid, prevented EtOH and ACD-induced conditioned place preference in Wistar rats (Peana et al., 2009). Since L-cysteine interacts with the high reactivity carbonyl carbon atom of the ACD to form stable adducts (Kera et al., 1985; Nagasawa et al., 1984), the aim of the present study was to examine the effect of this amino acid on acquisition and maintenance of oral operant EtOH self-administration as well as on reinstatement for nose-poking induced by oral EtOH relapse after extinction. In parallel, we measured blood and brain ACD levels in rats pretreated with saline or L-cysteine that self-administered oral EtOH.

## 2. Materials and methods

The study was carried out in accordance with Italian law D.L. 116, 1992, which allows experiments on laboratory animals only after submission and approval of a research project to the Minister of health (Italy), and in strict accordance with the "Raccomandazioni della Commissione dell'Unione Europea" (n. 2007/526/CE). All possible efforts were made to minimize animal pain and discomfort and to reduce the number of experimental subjects.

### 2.1. Animals

Male Wistar rats (Harlan, Udine, Italy) weighing 175–225 g were housed in groups of two/three and maintained under controlled environmental conditions (temperature 22 ± 2 °C; humidity 60–65%) on a reverse 12-h light/dark cycle (light on 18:00 h; off 06:00 h). Standard laboratory rat chow and tap water were available *ad libitum* in the home cage, except as noted below. All training and experimental sessions were conducted during the dark phase of the cycle all weekdays. Rats were housed in the same room where are located the operant chambers (Testa and Badiani, 2008).

### 2.2. Apparatus

Training and testing were conducted in four modular operant chambers located in sound attenuating, environmentally ventilated cubicles (Med Associates Inc, USA, Basile, Italy). Each chamber was equipped with a drinking reservoir, not retractable (capacity 0.50 ml), corresponding to 5 infusions. The drinking cup is positioned above the floor in the center of the front panel of the chamber, and two nose-poke holes, located at 3 cm to the left and right of the drinking receptacle. A white light (on) was placed over the active and a red light (on) over the inactive one as discriminative stimulus. Only the active nose-poke activated the dipper and delivered a solution in (0.1 ml) 3.05 s. Explorations on the other nose-poke (inactive nose-poke) were recorded, but not reinforced, and served as a control for specificity of activity in the operant chamber. The availability of liquid was signaled by a white light placed near the top of the operant cage on the wall in front of the liquid delivery system and was on during drug delivery (3.05 s). Following each liquid delivery there was a 2.00 s time out period (white light over active hole was off) during which responding had no programmed consequences. Chambers were interfaced to a PC equipped with software for programming sessions as well as data recording (active and inactive nose-pokes and controlled stimulus presentation and reinforce delivery). Behavior was monitored directly, by visual observation through the peep-hole of the self-administration boxes.

### 2.3. Training procedure for acquisition of oral ethanol self-administration

Rats were i.p. pretreated with saline (1 ml/kg) or L-cysteine (20 and 40 mg/kg) before each training of self-administration of tap water or EtOH (5–10%) in 30 min daily sessions on a fixed-ratio 1 schedule of reinforcement, where each response resulted in delivery of 0.1 ml of fluids as previously described by others with some modifications (Weiss et al., 1993; Heidbreder et al., 2007; Cippitelli et al., 2007). Briefly, for the first 3 days of training, tap water availability in the home cage was restricted to 5 min/day in order to facilitate acquisition of operant responding for a liquid reinforcer. During this time, rats were permitted to nose-poke explore for tap water or 5% EtOH (v/v) solution. Thereafter, tap water was made freely available in the home cage and tap water or EtOH oral self-administration training continued for another 3 days. Starting on day 7, the concentration of EtOH solution was gradually increased from 5 to 10%. For the first 3 days of saccharine training, tap water availability in the home cage was also restricted to 5 min/day and rats were permitted to nose-poke explore for a 0.2% saccharine (w/v) solution without increasing concentrations during the training. Following completion (11 days) of the tap water, EtOH and saccharine procedure, the rats were trained in a 30 min session/day to nose-poke until acquisition of a stable baseline of responding was reached (4 days). Inactive nose-poke responses were recorded during all testing phases as a measure of non-specific behavioral activation but they had no programmed consequences.

### 2.4. Effect of L-cysteine on maintenance of oral ethanol self-administration

Following the acquisition of a stable baseline of responding (15 days), after the last self-administration day with availability of EtOH (10%) solution, the same group of rats was i.p. pretreated with saline or L-cysteine (0, 40, 60, 80 and100 mg/kg) 30 min before each operant session for five day/sessions pro each dose. In particular, the same group of rats was tested with increasing doses of L-cysteine. Saline solution was administered between various L-cysteine doses for five days. These sessions lasted 30 min under conditions identical to those during the acquisition of self-administration session.

### 2.5. Extinction phase

Following the acquisition of a stable baseline of responding was reached, after the last self-administration day, rats were subjected to 30 min extinction session for 5 consecutive days in which only i.p. saline was administered, 30 min prior to each session. During this phase, the white light placed over the active hole was on and the exploration was without liquid delivery. Responses at the nose-poke activated the delivery mechanism (house light, tone pump, white light over the active hole) but did not result in the delivery of EtOH solution.

### 2.6. Reinstatement testing

Reinstatement sessions began the day after the last extinction session. These sessions lasted 30 min under conditions identical to those during the self-administration session with availability of EtOH (10%) solution. Reinstatement experiments were conducted for 5 days, in which saline or L-cysteine (80 mg/kg) was administered 30 min prior to each session. Responding for the inactive nose-poke was constantly recorded to monitor possible non-specific behavioral effects.

### 2.7. Measurements of blood and brain ethanol and acetaldehyde levels

The same groups of rats ($n = 6 + 6$) trained for reinstatement of EtOH-drinking behavior (Fig. 4) were used for measurements of blood

and brain EtOH and ACD levels. In particular, after the last reinstatement session, animals were anaesthetized with ethyl urethane in saline (200 mg/kg/ml) and afterwards (about 45 min) blood samples and brains *in toto* were taken. A 1000 µl aliquot of heart blood was diluted with 1000 µl of cold milliQ water in 10 ml HS-vials (Hewlett-Packard) for analysis. Thus, deproteinization which could give rise to ACD (Stowell et al., 1977) was not part of the procedure. The rat brain (medium weight 1.7833 g) was homogenized with 1000 µl of cold milliQ water in 10 ml HS-vials (Hewlett-Packard) for analysis. The vials to be analyzed for EtOH and ACD were placed in a heating block at 45 °C for 10 min. The samples were analyzed on a HS-GC-FID system with a Dani 86.50 HSS-autosampler, and a Hewlett-Packard gas chromatography HP 6890 Plus. The capillary column used was an Econo CAP EC-5 (Alltech, Italy) (30 m, 0.53 mm i.d., 1.2 µm d.f.). The injection port temperature was maintained at 250 °C. The GC oven temperature was maintained at 45 °C in isothermal for 8 min. The flow rate of the carrier gas (helium) was 6.1 ml/min. The FID temperature was maintained at 250 °C. The HS parameters were: 75 °C manifold temperature, 150 °C transferline temperature, 1.57 psi carrier gas pressure, 1 min vial pressurization time, 1000 µl injection volume of headspace gas.

### 2.8. Drugs

EtOH (Zedda-Piras, Alghero, Italy) and saccharine (Sigma-Aldrich, Milano, Italy) were dissolved in tap water. EtOH solutions (v/v) were obtained by dilution of ETOH (95%) (Medicamenta, 1991–1992; U.S. Pharmacopeia National Formulary, 1995) by considering each density of the EtOH solution; indeed is different the ETOH (95%) density from the ETOH (10%). For example a 10% EtOH v/v solution contains 8.7 g of EtOH in 100 ml. Saccharine was dissolved as w/v. L-cysteine, (R)-2amino-3-mercaptopropionic acid as hydrochloride was dissolved in a Tris (Sigma-Aldrich, Milano, Italy) solution (0.3 M in demineralized water) to a final pH of 7.4. I.p. administration of 20, 40, 60, 80 and 100 mg/kg/ml, or saline was performed 30 min before each oral operant session. L-cysteine doses are expressed as the bases. All drug dilutions where freshly prepared before every experiment session.

### 2.9. Statistical analysis

Data are expressed as mean ± S.E.M. Statistics were analyzed by repeated measures, two-way analysis of variance (ANOVA). Post hoc comparisons were undertaken if a significant effect of the interaction was found ($p<0.05$). The comparisons were carried out using Least Significant Differences (LSD) tests. In the study for which blood and brain EtOH and ACD levels were determined following the last reinstatement session, planned comparisons were undertaken using one way ANOVA between groups or two-way ANOVA between groups and within blood and brain levels.

## 3. Results

### 3.1. Acquisition of oral ethanol self-administration

Our experiments used oral EtOH self-administration, the most common procedure for the study of voluntary drug intake. Freely moving rats learned to obtain oral solution of EtOH, in tap water, by poking their noses into a hole. To allow the acquisition of addiction-like behavior to appear, we studied self-administration over a period of 15 days that is typical in self-administration experiments (Deroche-Gamonet et al., 2004). We used operant cages with nose-poke holes since nose-poking in rats is a more usual behavior than lever pressing (Samson et al., 1988, 2000). Likewise, assessment of the pattern of EtOH consummatory bouts and behaviors that precede them, is critical in understanding how EtOH functions as a reinforcer. For example, the rate of consumption of oral EtOH self-administration in a model of sipper-tube with licks is twice that of the dipper procedure with lever pressing (Samson et al., 2000). During the self-administration period, we repeatedly evaluated active and inactive nose-pokes as well as EtOH intake referred to the rats' weight (g/kg) for each daily session (30 min). The drinking cup is not retractable and has a drinking reservoir of 0.50 ml. Therefore each active nose-poke activated the dipper and delivered a solution in of 0.1 ml until 0.5 ml. Rats ($n=8$–10) pretreated with saline showed (Fig. 1, panel a) a higher number of responses for EtOH solution (5–10%) from the sixth day of the sessions than the tap water group. In fact, active nose-poke occurred during the six days of the sessions for EtOH solution [active nose-poke: $F(1,2)=8.73$, $p=0.05$]; session: $F(19,38)=3.12$, $p=0.0014$ and an effect active nose-poke × session interaction: $F(19,38)=2.07$, $p=0.027$]. For rats pretreated with saline on EtOH-self-administration (Fig. 1, panel a), nose-poke discrimination (active and inactive nose-pokes) was present for the major part of acquisition sessions [nose-poke: $F(1,4)=32.08$, $p=0.0047$]; session: $F(19,76)=3.09$, $p=0.00025$; nose-poke × session interaction: $F(19,76)=3.41$, $p=0.00007$]. The discrimination between active and inactive nose-poke for the saline group on oral tap water self-administration did not differ throughout the sessions (Fig. 1, panel a). Fig. 1, panel b shows the corresponding EtOH intake as a function of active nose-poke in the same group (saline/EtOH); after six days of oral EtOH self-administration rats consumed more EtOH (0.9–1.9 g/kg/30 min) with respect to the first five sessions of self-administration. EtOH intake after the tenth session (EtOH 10%) remained stable.

### 3.2. Effect of L-cysteine on acquisition of oral ethanol self-administration

L-cysteine, administered 30 min before each procedure of EtOH-drinking behavior reduced acquisition of oral EtOH self-administration. Fig. 2, shows the effect of saline or L-cysteine (20 and 40 mg/kg) on

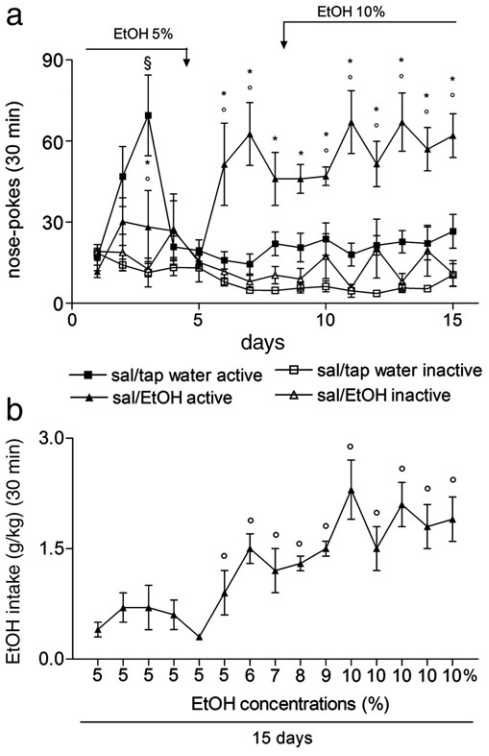


**Fig. 1.** Panel a. Acquisition of oral EtOH self-administration behavior. Responses per session (30 min) on the active and inactive nose-pokes by rats, pretreated with saline, for the acquisition of oral water or EtOH self-administration. *Indicates $p<0.05$ between tap water and EtOH, active nose-pokes; °indicates $p<0.05$ between active and inactive nose-pokes for EtOH; § indicates $p<0.05$ between active and inactive nose-poke for tap water. Panel b. EtOH intake expressed in g/kg during each operant session (30 min) as function of active nose-poke in the same saline group. +Indicates $p<0.05$ with respect to 1°day of EtOH session (two-way ANOVA for repeated measures and LSD post hoc test). Data are means ± SEM ($n=5$–8).

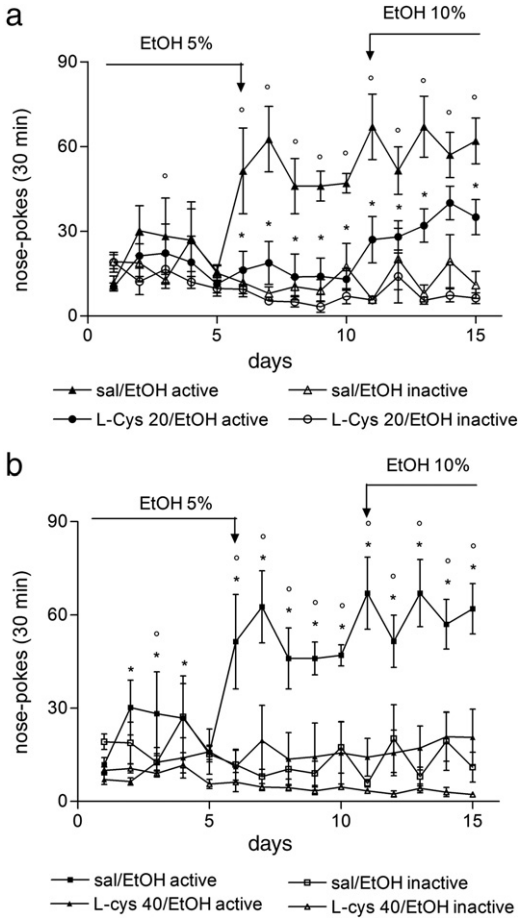


**Fig. 2.** Effect of L-cysteine at 20 mg/kg (panel a) or at 40 mg/kg (panel b) on acquisition of oral EtOH self-administration behavior. Number of active and inactive nose-pokes for oral self-administration during each operant session (30 min). °: indicates $p<0.05$ between active and inactive nose-poke for saline group, *: indicates $p<0.05$ between L-cysteine and saline groups, active nose-poke; (two-way ANOVA for repeated measures and LSD post hoc test). Data are means ± SEM ($n=6$).

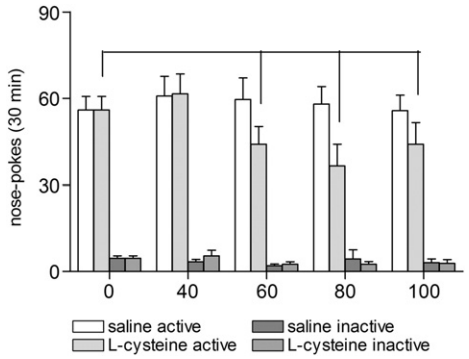


**Fig. 3.** Effect of L-cysteine (0, 40, 60, 80 and 100 mg/kg) on maintenance of oral EtOH self-administration behavior (10%). Data are shown as the number of active and inactive nose-pokes (means ± SEM; $n=6$) during each operant session (30 min). Significant differences between active nose-pokes are indicated ($p<0.05$); (two-way ANOVA for repeated measures and LSD post hoc test).

acquisition of oral EtOH self-administration. For two doses of L-cysteine the ANOVA conducted on these data revealed a significant main effect of dose [$F(2,9)=9.63$, $p=0.0058$], session [$F(29,261)=8.48$, $p<0.000001$] and a significant dose × session interaction [$F(58,261)=1.94$, $p=0.00025$]. During the acquisition sessions for oral EtOH self-administration (Fig 2), rats pretreated with saline, responded significantly more on the active nose-poke than the inactive. In particular, for the most part of self-administration sessions, rats discriminated the active nose-poke that resulted in a delivery of EtOH solution with respect to the inactive nose-pokes that had no effect. Further analyses indicated that 20 mg/kg of L-cysteine significantly reduced active nose-pokes for EtOH self-administration starting from the sixth day of acquisition (Fig. 2, panel a). The higher dose of L-cysteine (40 mg/kg) decreased significantly the active nose-poke numbers for oral EtOH self-administration from the second day of acquisition (Fig. 2, panel b). Pretreatment with L-cysteine did not alter responding for tap water [$F(3,44)=0.92$, $p>0.05$] (data not shown) and did not modify the number of the inactive nose-poke with respect to the saline group, indicating the absence of a non-specific behavioral activation/depression (Fig. 2).

### 3.3. Effect of L-cysteine on maintenance of oral ethanol self-administration

Fig. 3 shows the effect of L-cysteine (0, 40, 60, 80 and 100 mg/kg) on maintenance of oral EtOH self-administration. Pretreatment with L-cysteine, 30 min prior each oral EtOH self-administration session, significantly reduced the operant response for EtOH. For all doses tested of L-cysteine, ANOVA two-way for repeated measures conducted on these data revealed a significant main effect of dose [$F(4,10)=5.08$, $p=0.017$] , session [$F(3,30)=323.68$, $p<0.00001$] but not a dose × session interaction [$F(12,30)=1.94$, $p=0.069$]. During the sessions for oral EtOH self-administration (Fig. 3), rats pretreated with saline or L-cysteine at all doses, responded significantly more on the active nose-poke than the inactive nose-poke. During maintenance, the highest doses (60, 80 and 100 mg/kg) of L-cysteine appeared to produce similar results reducing active nose-pokes for EtOH self-administration. The lower dose of L-cysteine (40 mg/kg) did not alter responding for EtOH solution. L-cysteine did not modify the number of the inactive nose-poke at the doses tested with respect to the saline group (Fig. 3) indicating the absence of a non-specific behavioral activation/depression. For the analysis of the L-cysteine dose-response study, the number of active and inactive nose-pokes during the last 3 EtOH self-administration sessions prior to commencement and after L-cysteine treatment was averaged to obtain a mean value for EtOH self-administration.

### 3.4. Effect of L-cysteine on reinstatement of oral ethanol-drinking behavior

To study the propensity to relapse, we used the reinstatement procedure. After a 5 day period of withdrawal that followed the 15 days of self-administration, rats were exposed to conditions identical to those during the self-administration session with availability of EtOH (10%) solution. Fig. 4 shows the effect of saline or L-cysteine (80 mg/kg) on reinstatement of oral EtOH self-administration after EtOH extinction. For this dose of L-cysteine the ANOVA conducted on these data revealed a significant main effect of session [$F(29,232)=31.34$, $p<0.000001$] and a significant dose × session interaction [$F(29,232)=1.81$, $p<0.01$]. During the five sessions of self-administration before extinction (maintenance), responding was significantly more on the active nose-pokes than the inactive nose-pokes (Fig. 4). L-cysteine (80 mg/kg), administered 30 min before each reinstatement session significantly reduced active nose-pokes for the most part of EtOH self-administration with respect to the saline group (Fig. 4). We decided to use this dose of L-cysteine because, as suggested by previous observations, it was the mean dose that was able to reduce maintenance of oral EtOH self-administration. Also, this dose of L-cysteine did not modify the number of the inactive nose-pokes with respect to the saline group showing an absence of a non-specific behavioral activation.

### 3.5. Effect of L-cysteine on acquisition of oral saccharine self-administration

Fig. 5 shows the average number of cumulative nose-pokes in the active and inactive holes performed by rats during daily oral self-

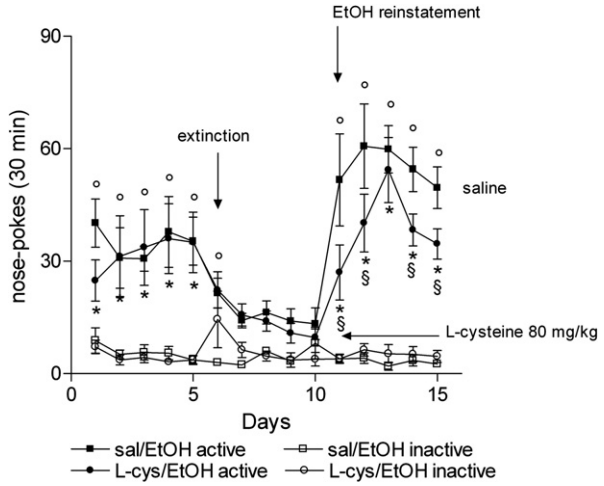


**Fig. 4.** Effect of L-cysteine(80 mg/kg) on EtOH reinstatement after EtOH extinction. Number of active and inactive nose-pokes for oral self-administration during each operant session (30 min). Significant differences between active and inactive nose-poke are indicated ($p<0.05$); °: saline group; *: L-cysteine group, before and after extinction). Significant differences between saline and L-cysteine group, active nose-poke, during the reinstatement session are indicated with §; (two-way ANOVA for repeated measures and LSD post hoc test). Data are means ± SEM ($n=5$–6).

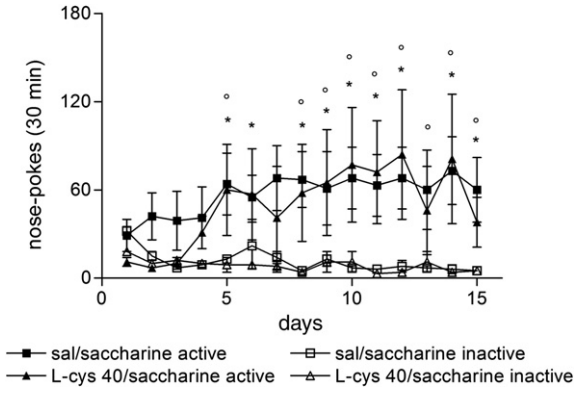


**Fig. 5.** Effect of L-cysteine (40 mg/kg) on oral saccharine self-administration behavior. Number of active and inactive nose-pokes for oral self-administration during each operant session (30 min). °: indicates $p<0.05$ between active and inactive nose-poke for sal/saccharine group, *: indicates $p<0.05$ between active and inactive nose-poke for L-cysteine/saccharine group; (two-way ANOVA for repeated measures and LSD post hoc test). Data are means ± SEM ($n=5$–6).

administration behavior for saccharine solution (0.2%) for 15 consecutive days. L-cysteine (40 mg/kg) did not modify operant responding for saccharine (Fig. 5). In fact, active vs inactive nose-pokes occurred approximately during all acquisition sessions and the number of active nose-pokes for saline or L-cysteine on saccharine self-administration did not differ throughout all sessions [group: $F(1,6)=0.10, p=0.76$]; session: $F(29,174)=3.49$, $p<0.00001$; group × session interaction: [$F(29,174)=0.42$, $p=0.99$].

### *3.6. Effect of L-cysteine on blood and brain ethanol and acetaldehyde levels*

L-cysteine is an ACD-sequestrating agent able to bind covalently ACD thereby forming a stable adducts (Nagasawa et al., 1984; Kera et al., 1985). With the aim to ascertain a possible effect of EtOH-derived ACD, we measured both blood and brain EtOH and ACD levels. The same groups of rats ($n=6+6$) trained for reinstatement of EtOH-drinking behavior (Fig. 4) were used. After the last reinstatement session, the animals were anaesthetized with ethyl urethane and afterwards (about 45 min) blood samples and brains *in toto* were taken. Means (±SEM) blood and brain EtOH and ACD levels at this time point are listed in Table 1. Means EtOH and ACD levels are presented as mg/ml in blood and mg/g in brain. Table 1 showed that blood EtOH concentrations ($0.11\pm0.05$) in saline group were significant higher than those in rats pretreated with L-cysteine ($0.06\pm0.04$), [$F(1,10)=6.38$, $p=0.03$]. Likewise the EtOH levels ($0.040\pm0.017$) in the brain were significantly higher than those in the L-cysteine group ($0.015\pm0.009$), [$F(1,10)=4.88$, $p=0.05$]. Two-way ANOVA conducted on these data revealed a significant effect between saline and L-cysteine group and within blood and brain levels [$F(1,10)=6.58$, $p=0.03$]. In contrast, no significant differences were observed in blood and brain ACD concentration between saline and L-cysteine group.

## 4. Discussion

The present study shows that L-cysteine reduced both acquisition and maintenance of oral EtOH self-administration behavior. Moreover, L-cysteine reduced reinstatement of EtOH-drinking behavior after an oral EtOH extinction. The effect of pretreatment with L-cysteine that reduced reinstatement of EtOH-drinking behavior was paralleled by a significant reduction of EtOH intake and correlated with a reduction of blood and brain EtOH levels. In fact, L-cysteine (20–40 mg/kg, i.p.), administered 30 min before each acquisition session, significantly decreased active nose-pokes for oral EtOH solution with respect to the saline group with a reduction of discrimination between active and inactive nose-pokes from 5 to 10% concentrations of EtOH. In addition, rats pretreated with L-cysteine (40, 60, 80 and 100 mg/kg, i.p.) 30 min before each maintenance phase (after 15 days of acquisition) exhibited a reduction in oral EtOH self-administration. In particular, only the highest L-cysteine dose appeared to produce similar results. When the effect of L-cysteine was investigated on EtOH reinstatement paradigm, results also showed that 80 mg/kg dose of this agent significantly decreased the reinstatement of EtOH-drinking behavior for the main part of this session. It must be emphasized that pretreatment with L-cysteine, although only one dose was tested, was able to reduce the relapse response for EtOH. The effect of 80 mg/kg of L-cysteine on nose-pokes during reinstatement was similar to effect observed on maintenance. However, during the reinstatement session it is common to observe an increase of EtOH-drinking behavior, as can be seen in saline group (Fig. 4). This session induces high levels of responding (reinstatement) on the device previously associated with drug delivery. The rate of responding during the test for reinstatement is considered a measure of the propensity to relapse

**Table 1**
Effect of L-cysteine (80 mg/kg) on blood and brain EtOH and ACD levels.

| Pretreatment | EtOH intake g/kg | EtOH | | ACD | |
|---|---|---|---|---|---|
| | | Blood | Brain | Blood | Brain |
| Saline | 1.35 ± 0.16 | 0.11 ± 0.05 | 0.040 ± 0.017 | 0.0012 ± 0.0001 | 0.0004 ± 0.00001 |
| L-cysteine | 0.96 ± 0.14 | 0.06 ± 0.04 | 0.015 ± 0.009 | 0.0011 ± 0.0002 | 0.0003 ± 0.00001 |

Data are means ± SEM of EtOH and ACD levels, presented as mg/ml in blood and mg/g in brain. ETOH and ACD levels were measured as described in the "Materials and methods". (Planned comparisons were undertaken using one way ANOVA between group and two-way ANOVA between group and within blood and brain levels, $p<0.05$).

(Deroche-Gamonet et al., 2004). At the end of last reinstatement session, these two groups of rats were utilized to measure blood and brain EtOH levels. However, L-cysteine (80 mg/kg) induced a low decrease of nose-pokes discrimination. Our previous work reported that L-cysteine reduced, dose-dependently, the intragastric EtOH-induced motivational properties (Peana et al., 2009) and we have hypothesized a possible role of EtOH-derived ACD in the EtOH effect. In effect, L-cysteine is an ACD-sequestrating agent able to bind covalently ACD thus forming a stable adducts (Nagasawa et al., 1984; Kera et al., 1985). In view of this fact, with the aim to determine a possible effect due to EtOH-derived ACD we measured both blood and brain ACD levels. L-cysteine failed to reduce blood and brain ACD levels; therefore, at this time, the present results cannot support the role of EtOH-derived ACD in the oral EtOH self-administration and in the reinstatement of EtOH-drinking behavior.

Furthermore, treatment with L-cysteine was unable to suppress responding for saccharine, suggesting that this effect is not related to a decrease in a general motivational state. This is also confirmed by the lack of action of L-cysteine on the motivational properties of morphine-induced conditioned place preference (Peana et al., 2009). Moreover, L-cysteine did not produce neither rewarding nor aversive effects since when paired with saline, it failed to affect place conditioning (Peana et al., 2009). The same response to L-cysteine in self-administration (presence of EtOH solutions) and relapse (after a period of extinction conditions) could reside in different mechanisms. L-cysteine could undergo redox reactions; cysteine has antioxidant properties; in fact, several reports show antioxidant properties of L-cysteine as precursor of the antioxidant glutathione (Soghier and Brion, 2006) and as direct scavenging of free radicals (Shackebaei et al., 2005). An alternative/additional explanation could be envisaged in an interaction with the presynaptic group I metabotropic glutamate (mGlu) autoreceptors that mediate a positive modulatory control on synaptic glutamate release both in vitro and in vivo. Evidence is also accumulating to suggest that sulphur-containing amino acids are analogues of glutamate (Thompson and Kilpatrick, 1996). Interestingly, low concentrations of L-cysteic acid (1 μM), at a similar concentration range to that of L-cysteine tested in our study, inhibited synaptic glutamate release by an interaction with presynaptic group mGlu autoreceptors (Croucher et al., 2001; Knackstedt and Kalivas, 2009). In line with these results, Blednov and Harris (2008) have shown that group I mGluR5 antagonists decrease EtOH self-administration. Furthermore, it is apparent that similar glutamatergic neuroadaptations arise after self-administration of EtOH. For example, reinstatement to EtOH can be prevented both by the stimulation of group II mGluR receptors (Zhao et al., 2006) and by the blockade of group I mGluR5 receptors (Backstrom and Hyytia, 2004). The similarities in the neurochemistry behind relapse could indicate that drugs that target the glutamate system could be effective at treating EtOH and relapse to multiple types of drugs. N-acetyl-L-cysteine is a derivative of cysteine where an acetyl group is attached to the nitrogen atom. N-acetyl-L-cysteine is known to increase exchanger activity, thereby promoting glutathione synthesis as well as an increased glutamatergic tone on group II mGluR autoreceptors (Moran et al., 2005; Melendez et al., 2005). Consistent with this mechanism of action, although not examined in the present study, it is possible that, like cocaine and heroin training, EtOH training is reducing cystine–glutamate exchange in the nucleus accumbens, and repeated L-cysteine treatment might cause an enduring restoration of exchanger function or other EtOH-induced neuroadaptations in brain (Zhou and Kalivas, 2008). Furthermore, L-cysteine acts as a natural substrate for the synthesis of hydrogen sulfide ($H_2S$). $H_2S$ is the newest member in a family of signaling molecules termed gasotransmitters; it is a small membrane-permeable gas molecule that is produced endogenously in a regulated manner to influence cellular function independently of membrane receptor interactions (Wang, 2003). In the mammalian brain, $H_2S$ enhances responses that are linked to activation of NMDA receptors (Abe and Kimura, 1996). In addition, a structural analog of L-cysteine, D-penicilamine prevents voluntary EtOH consumption in rats (Font et al., 2006b). The central mechanism, by which L-cysteine could act in oral EtOH self-administration could be envisaged in the fact that L-cysteine crossing into the brain by excitatory amino acid transporters that are rate limiting factors in neuronal cysteine uptake (Chen and Swanson, 2003) could mediate the reduction of motivational properties of EtOH.

In conclusion, our results show that L-cysteine reduced all phases of oral EtOH self-administration. The efficacy of L-cysteine on relapse to alcohol drinking, one of the major problems seen in alcoholism therapy, represents an interesting pharmacological approach and could open a new line of research for the development of pharmacological therapies to reduce EtOH intake in alcoholic patients. Although there are many mechanisms through which L-cysteine effect can occur, our data suggest that L-cysteine would be a potential therapeutic drug for alcoholics, since it is relatively inexpensive and safe to use (Dood et al., 2008).

## Acknowledgements

This work was supported, through grants from Fondazione Banco di Sardegna (2008.0729), Banca d'Italia, and Provincia di Sassari to A.T.P. and PRIN (MIUR, 2006057754) to M.D. The authors wish to thank the Basile (Italy) company for the kind supply of software program and to Engineer Sergio Sanna for his help on the software program.

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