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# Effect of L-cysteine on acetaldehyde self-administration

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ABSTRACT

Acetaldehyde (ACD), the first metabolite of ethanol, has been implicated in several behavioural actions of alcohol, including its reinforcing effects. Recently, we reported that L-cysteine, a sequestrating agent of ACD, reduced oral ethanol self-administration and that ACD was orally self-administered. This study examined the effects of L-cysteine pre-treatment during the acquisition and maintenance phases of ACD (0.2%) self-administration as well as on the deprivation effect after ACD extinction and on a progressive ratio (PR) schedule of reinforcement. In a separate PR schedule of reinforcement, the effect of L-cysteine was assessed on the break-point produced by ethanol (10%). Furthermore, we tested the effect of L-cysteine on saccharin (0.2%) reinforcement. Wistar rats were trained to self-administer ACD by nose poking on a fixed ratio (FR1) schedule in 30-min daily sessions. Responses on an active nose-poke caused delivery of ACD solution, whereas responses on an inactive nose-poke had no consequences. L-cysteine reduced the acquisition (40 mg/kg), the maintenance and the deprivation effect (100 mg/kg) of ACD self-administration. Furthermore, at the same dose, L-cysteine (120 mg/kg) decreased both ACD and ethanol break point. In addition, L-cysteine was unable to suppress the different responses for saccharin, suggesting that its effect did not relate to an unspecific decrease in a general motivational state. Compared to saline, L-cysteine did not modify responses on inactive nose-pokes, suggesting an absence of a non-specific behavioural activation. Taken together, these results could support the hypotheses that ACD possesses reinforcing properties and L-cysteine reduces motivation to self-administer ACD.



## Introduction

Mostly known for its well-known parent compound ethanol, acetaldehyde (ACD) was initially studied, but then research interest in this compound waned. However, in the last two decades, research on ACD has seen a revitalized and uninterrupted interest. ACD, *per se*, and as a product of ethanol metabolism, could be responsible for many biological effects which are not clearly distinguishable from those of ethanol (Correa et al., 2012; Font, Aragon, & Miquel, 2006a; Font, Aragon, & Miquel, 2006b; Peana, Assaretti, Muggironi, Enrico, & Diana, 2009; Peana et al., 2008; Peana, Muggironi, & Diana, 2010; Quertemont, Tambour, & Tirelli, 2005). Several studies have demonstrated that ACD induces conditioned place preference in rats through intracerebroventricular (Smith, Amit, & Splawinsky, 1984) or intraperitoneal administration (Quertemont & De Witte, 2001). In addition, in 1979 Brown and colleagues found that rats self-administered ACD directly into the cerebral ventricles, and in 2002, McBride et al. reported that rats self-administered ACD directly into the ventral tegmental area (VTA). Other studies have shown that rats self-administered ACD intravenously (Myers, Ng, & Singer, 1984; Takayama & Uyeno, 1985), and Rodd-Henricks reported ACD VTA self-administration in alcohol-preferring rats (Rodd-Henricks et al., 2002). Furthermore, we previously found that intragastric administration of ACD caused conditioned place preference (Peana et al., 2008), was orally self-administered (Peana, Muggironi, & Diana, 2010) and that the opioid system could be implicated in its reinforcing properties suggesting also an involvement of extracellular signal regulated kinase (ERK) (Peana et al., 2011). In addition, our previous findings have shown that L-cysteine, a thiol non-essential amino acid, able to chemically neutralize ACD, prevented ethanol and ACD-induced conditioned place preference in the same strain of rats (Wistar) (Peana et al., 2009). Moreover, L-cysteine reduced the different phases of oral ethanol self-administration (Peana, Muggironi, Calvisi, et al., 2010). This amino acid binds ACD covalently (*in vitro*) with the high-reactivity carbonyl carbon atom of ACD to forming a stable nontoxic compound, 2-methylthiazolidine-4-carboxylic acid (Kera, Kiriyama, & Komura, 1985; Nagasawa, Goon, Muldoon, & Zera, 1984; Sprince, Parker, Smith, & Gonzales, 1974). To better characterise the ACD reinforcing effect and its probable

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involvement in animal models of alcohol addiction, the aim of the present study was to examine if L-cysteine would decrease oral ACD self-administration. In particular, L-cysteine was tested first on acquisition and then on maintenance of oral ACD self-administration. Subsequently, the effect of this sequestrating agent was assessed on the reinstatement for nose poking induced by oral ACD extinction. In addition, this study examined ACD self-administration under a progressive ratio (PR) schedule of reinforcement. In our experiments we included ethanol PR performance, to directly evaluate with ACD in identical experimental conditions and to test efficacy of L-cysteine against the two reinforcers. Under PR schedules, the response requirement for reinforcer delivery increases systematically. The point at which the animal ceases to respond within a determined period is defined as the "break point". Thus, the break point serves as an index of an animal's motivation to work for the reinforcers (e.g., 0.2% ACD or 10% ethanol) and the reinforcing efficacy of the drug. To determine if L-cysteine could alter motivation to self-administer a nondrug rewarding substance, we tested the equivalent dose range of L-cysteine on saccharin (0.2%) self-administration. We recorded inactive nose-poke responses through all testing phases as a measure of non-specific behavioural activation. In parallel, we measured blood ACD levels in rats that self-administered oral ACD.

## Method

The study was carried out in accordance with current Italian legislation [D.L. 116, 1992] that allows experimentation on laboratory animals only after submission and approval of a research project to the Ministry of Health (Rome-Italy) and was in strict accordance with European Council directives on the matter [n. 2007/526/CE]. All possible efforts were made to minimise animal pain and discomfort and to reduce the number of experimental subjects.

### *Animals*

Male Wistar rats (Harlan, Udine, Italy), weighing 175–225 g, were housed in pairs in Plexiglas cages with two bottles of tap water and food continuously available unless otherwise stated. The colony room was maintained under controlled environmental conditions (temperature 22 ± 2 °C; humidity 60–65%) on a reverse 12-h light/dark cycle (light on at 18:00 h; off at 06:00 h). To limit animal stress, the operant chambers were located in the same colony room where the rats were housed. All training and experimental sessions were conducted during the dark phase of the cycle every day.

### *Drugs*

ACD (Sigma–Aldrich, Milano, Italy) was dissolved in tap water as w/v (0.1–0.2%). To avoid ACD evaporation, we prepared the solutions while keeping the preparation beaker on ice. In addition, ACD was always used in this diluted form, which reduced surface tension (solvation). Saccharin (Sigma–Aldrich, Milano, Italy) was dissolved in tap water as w/v (0.2%). L-cysteine, (R)-2 amino-3-mercaptopropionic acid as hydrochloride (Sigma–Aldrich, Milano, Italy) was dissolved in a Tris (Sigma–Aldrich, Milano, Italy) solution (0.3 M in demineralised water) to a final pH of 7.4. Intraperitoneal administration of L-cysteine (20–140 mg/kg/ml) or saline was performed 30 min before each oral operant session. All drug dilutions were freshly prepared before every experimental session.

### *Apparatus*

Training and testing were conducted in modular operant chambers located in ventilated sound-attenuating environmental cubicles (Med Associates Inc., USA). Each chamber was equipped with a non-retractable drinking reservoir (capacity 0.50 ml) and two nose-poke holes located at 3 cm to the left and right of the reservoir. As a discriminative stimulus, a white light was placed over the active hole and a red light over the inactive one. Only the active nose-poke, which was recorded, set off the dipper delivering the solution (0.1 ml) into the receptacle in a 3.05-s period. Explorations of the inactive nose-poke were also recorded. This served to control for specificity of the response in the operant chamber. The availability of liquid was signalled by a white house light placed on the wall in front of the liquid delivery system that would light up for the duration of liquid delivery. Following each delivery, there were 2-s time-out period during which responses had no consequences, and the white light placed over the active hole would go off. An infrared head detector was located in the reservoir and recorded all signals during the entire session, which lasted 30 min. The chambers were interfaced to a computer equipped with software that programmed the sessions and recorded the data.

### *ACD reinforcement*

#### *Acquisition of oral ACD self-administration behaviour and L-cysteine pre-treatment*

Rats learnt how to obtain ACD solution by nose-poke. During the self-administration period, we repeatedly evaluated the active and inactive nose-pokes for each daily 30-min session. For operant ACD self-administration (full range: 0.1–0.2%), rats were trained to nose-poke on a fixed-ratio 1 (FR1) schedule of reinforcement, in which each response resulted in 0.1 ml of solution delivery. For days 1–3 of training, tap water availability in the home cage was restricted to 5 min/day after each session to facilitate acquisition of the operant response. During these 3 days/sessions, tap water self-administration parallelled oral ACD (see Fig. 1 from Peana, Muggironi, & Diana, 2010). We showed a similar trend with oral ethanol self-administration during the fluid restriction for the first 3 days (Peana, Muggironi, Calvisi, et al., 2010). From the 4th day, tap water was made freely available in the home cage. During this time (days 1–6), rats were permitted to nose-poke explore for 0.1% ACD solution. Starting on day 7, the ACD solution concentration was gradually increased in stages from 0.12, to 0.14, 0.16 and 0.18% up at the final concentration of 0.2%.

During all self-administration sessions, the rats were pretreated with saline intraperitoneally to habituate the animals to the injection protocol to be used for the L-cysteine assessments (from 1 to 15 days). Rats were administered L-cysteine (0, 20 and 40 mg/kg, i.p.) and were returned to the home cage. After 30 min, the rats were placed in the self-administration chambers for the acquisition of ACD self-administration.

Inactive nose-poke responses were recorded during all testing phases as a measure of non-specific behavioural activation, but they had no programmed consequences.

#### *Maintenance of ACD self-administration behaviour and L-cysteine pre-treatment*

After a stable baseline of responding (maintenance) was reached (15 days), that is, after the last self-administration session with availability of ACD (0.2%) solution, another group of rats were pretreated with L-cysteine (0, 80 and 100 mg/kg) for five sessions over five days. These sessions lasted 30 min under conditions identical to those during the self-administration session under FR1. The L-cysteine doses were tested using a Latin-square design using

$n = 6–8$ animals. There were at least two drug-free intervening ACD self-administration sessions between the L-cysteine doses test. We found that self-administration quickly recovered to maintenance levels by the self-administration session that followed a test day, but two intervening sessions were allowed to ensure stable responding.

*Deprivation effect in oral ACD self-administration behaviour and L-cysteine pretreatment*

Once reliable ACD self-administration was acquired, ACD-reinforced responding was extinguished by withholding ACD solution (0.2%) but not the presentation of environmental stimuli (i.e., the house light, infusion pump sound and white light over the active hole). The extinction period lasted five consecutive sessions (one session/days). Deprivation-effect sessions began the day following the last extinction session. These sessions were identical to those with availability of ACD (0.2%) under FR1 and continued for five consecutive days. During this period, rats were pretreated with L-cysteine (0, 100, 120 and 140 mg/kg) for five sessions (one session/day) and were assigned randomly to each dose. The experiments were conducted every tenth day using a Latin square counterbalanced design using $n = 6–8$ animals for each dose evaluated.

*Acetaldehyde progressive ratio performance and L-cysteine pre-treatment*

To establish baseline PR responding and to test the feasibility of repeated testing, rats underwent 2 sessions under the PR schedule of reinforcement. Each session was separated by 2 days, with the regular training sessions under FR1 (Arnold & Roberts, 1997; Besheer, Faccidomo, Grondin, & Hodge, 2008; Richardson & Roberts, 1996). Under the PR schedule, the response requirement for ACD delivery increased by 1 each time a reinforcer was delivered (PR1, i.e., first reinforcer delivered after 1 response, second reinforcer delivered after 2 responses, third reinforcer delivered after 3 responses, etc.) (Besheer et al., 2008). Each ACD-reinforced response resulted in a 3.05-s illumination of the house light. Thus, the number of reinforcers received also reflects the highest response ratio completed (i.e., the break point) where no further reinforcers are emitted for a period of 30 min or more. L-cysteine was tested on responding under the PR schedule of reinforcement (PR1). Rats were administered L-cysteine (0, 120 and 140 mg/kg) and were returned to the home cage. After 30 min, the rats were placed in the self-administration chambers for the PR1 test session. For L-cysteine evaluation, the doses were tested using a Latin square counterbalanced design. The experiments were conducted every ninth day using $n = 12$ animals for each dose performed. There were at least two drug-free intervening ACD self-administration sessions between the PR1 test sessions. We found that self-administration quickly recovered to baseline levels by the self-administration session that followed a test day, but two intervening sessions were allowed to ensure stable responding. Responding under the PR1 was assessed again to determine if break points had changed after L-cysteine testing.

*Measurements of blood ACD concentrations*

Immediately after the final session of oral ACD self-administration, animals were anaesthetised with ketamine and medetomidine (75.0 + 0.5 mg/kg/ml, intraperitoneally), and approximately 10 min afterwards, blood samples were taken. For analysis, a 1-ml aliquot of rat blood was diluted with 1 ml of cold milliQ water in 10-ml HS-vials (Hewlett-Packard). The vials were placed in a heating block at 45 °C for 10 min. The samples were analysed on a HS-GC-FID system with a Dani 86.50 HSS-autosampler, and a Hewlett-Packard gas chromatography HP 6890 Plus. An Econo CAP EC-5 (Alltech, Italy) capillary column was used (30 m, 0.53 mm i.d., 1.2 μm d.f.). The injection port temperature was maintained at 250 °C. GC oven temperature was maintained at 45 °C in isothermal conditions for 8 min. The flow rate of the helium carrier gas was 6.1 ml/min, and the FID temperature was maintained at 250 °C. HS parameters were as follows: 75 °C manifold temperature, 150 °C transfer line temperature, 1.57 PSI carrier gas pressure, 1 min vial pressurisation time, and 1 ml injection volume of headspace gas.

*Ethanol reinforcement*

*Progressive ratio performance and L-cysteine pre-treatment*

Briefly, for oral ethanol self-administration (full concentration range: 5–10%), Wistar rats were trained to nose-poke on a fixed-ratio 1 (FR1) schedule of reinforcement, where each response resulted in 0.1 ml solution delivery. For days 1–3 of training, tap water availability in the home cage was restricted to 5 min/day (after each session) in order to facilitate acquisition of operant response. For days 4–6, tap water was made freely available in the home cage. During these days (1–6), rats were permitted to nose-poke explore for 5% ethanol solution. Starting on day 7, ethanol concentration was gradually increased in stages from 5, to 6, 7 and 9 ending up at the final concentration of 10%. Following the acquisition of a stable baseline of responding for 10% ethanol, rats pretreated with L-cysteine (0, 100, 120 mg/kg) were tested under the PR performance (see above: ACD progressive ratio performance).

*Saccharin reinforcement*

*L-cysteine on acquisition of oral saccharin self-administration*

For the first 3 days of saccharin training, tap water availability in the home cage was also restricted to 5 min/day after each session to facilitate acquisition of operant responding for a liquid reinforcer. During this time, rats were permitted to nose-poke explore for 0.2% (w/v) saccharin solution. At this point, tap water was made freely available in the home cage, and saccharin oral self-administration training continued for another 3 days. Six sessions over six days were allowed to ensure stable responding for oral saccharin self-administration. A group of rats were pretreated with L-cysteine (0 and 40 mg/kg, i.p.) throughout the acquisition of saccharin self-administration. The saccharin concentration was based upon previous work in our laboratory (Peana, Muggironi, Calvisi, et al., 2010) and other's (Ciccocioppo, Martin-Fardon, & Weiss, 2002; Cippitelli et al., 2007).

Responding at the inactive nose-poke was recorded throughout the experiment (acquisition, maintenance and deprivation effect) to monitor non-specific behavioural effects.

*L-cysteine on maintenance of saccharin self-administration behaviour*

After the acquisition of a stable baseline of responding (maintenance) was reached, another group of rats were pretreated with L-cysteine (0, 100 and 120 mg/kg) five sessions over five days. These sessions were identical to the self-administration sessions. The L-cysteine doses were tested using a Latin-square design using $n = 6–8$ animals with two drug-free intervening saccharin self-administration sessions between the L-cysteine doses tests.

*Deprivation effect in oral saccharin self-administration behaviour and L-cysteine pre-treatment*

The extinction period lasted five consecutive days, and during this time, responses at the active nose-poke set off the delivery mechanism under FR1 but resulted in no delivery of saccharin. Deprivation-effect sessions began the day following the last extinction session. These sessions were identical to those during

the self-administration session with availability of saccharin solution at 0.2% and proceeded for an additional five days. For L-cysteine (0, 100 and 120 mg/kg) testing, rats were pretreated five sessions over five days for each dose. The experiments were conducted every tenth day using a Latin square counterbalanced design using $n = 6–8$ animals for each different dose tested.

*Statistics*

For the analysis of ACD and saccharin self-administration (acquisition), two-way analysis of variance (ANOVA) with a repeated measure of "session" was used to analyse responses on the active nose-poke across the sessions. Nose-poke discrimination is a key factor to help distinguish reinforcement-contingent behaviour from a general increase or decrease in locomotor activity. For each L-cysteine dose, nose-poke discrimination was analysed by type (active or inactive) for an individual session, comparing the active and inactive nose-pokes. In the presence of significant main effects ($p$-values $< 0.05$), planned comparisons post-hoc tests were performed.

For the analysis of ACD and saccharin self-administration (maintenance), the data were averaged for two consecutive sessions (i.e., the last two sessions with saline and the first two sessions with L-cysteine doses). This was performed to avoid the complexity of a nested design. Data analyses consisted of treatment × session two-way ANOVA with a repeated measure of "session" on the number of nose-pokes. Additionally, for each treatment, nose-poke discrimination was determined by type × session. In the presence of overall significant main effects and interactions ($p$-values $< 0.05$), the Tukey's post-hoc test was performed.

Changes in nose-poke responding across the extinction-deprivation effect were analysed using a nose-poke type × session two-way ANOVA with a repeated measure of "session". All the mean values represent the average of the last two consecutive sessions of maintenance; sessions 1 and 2 together for extinction and sessions 1 and 2 together for the deprivation effect. Statistical significance was declared at $p$-values $< 0.05$; Tukey's post-hoc test was used.

The PR break point was determined to be the final response ratio completed before the end of the session. Break points to ACD and ethanol responses were analysed by one-way ANOVA. Inactive nose-pokes explored at the break point were analysed using one-way ANOVA. Tukey's post-hoc comparisons were performed to extract significant effects. Minimum statistical significance was set at $p < 0.05$.

Regarding measurements of blood ACD levels, one-way ANOVA we used to analyse responses on blood levels.

## Results

*Acetaldehyde reinforcement*

*Effect of L-cysteine on acquisition of ACD self-administration behaviour*

Fig. 1, panels A and B, shows the effect of L-cysteine (0, 20 and 40 mg/kg) throughout the acquisition of oral ACD self-administration. A two-way ANOVA conducted on these data revealed a significant main effect of pre-treatment [$F(2,18) = 7.17$, $p = 0.0051$], nose-poke-session [$F(29,522) = 13.66$, $p < 0.00001$] and a significant pre-treatment × nose-poke-session interaction [$F(58,522) = 3.01$, $p < 0.00001$]. During the acquisition sessions for oral ACD self-administration (Fig. 1, panel A and B), rats pretreated with saline responded significantly more on the active than inactive nose-pokes. In particular, from the ninth day of acquisition onward, rats discriminated between the active nose-poke that resulted in an ACD solution and the inactive nose-poke that had no effect. The lower dose of L-cysteine (20 mg/kg) did not reduce active nose-pokes for ACD self-administration (Fig. 1, panel A); instead, the higher dose (40 mg/kg) was more active, significantly decreasing the number of active nose-pokes for oral ACD (for most of the sessions; Fig. 1, panel B). This dose significantly reduced nose-poke discrimination throughout the ACD self-administration procedure compared to the saline group (Fig. 1, panel B). Pretreatment with L-cysteine did not alter responding for tap water [$F(3,44) = 0.92$, $p > 0.05$] (personal observation) and did not modify the number of inactive nose-pokes compared to the saline group, indicating an absence of a non-specific behavioural activation and an absence of motor depressant or stimulant effects (Fig. 1, panel A and B).

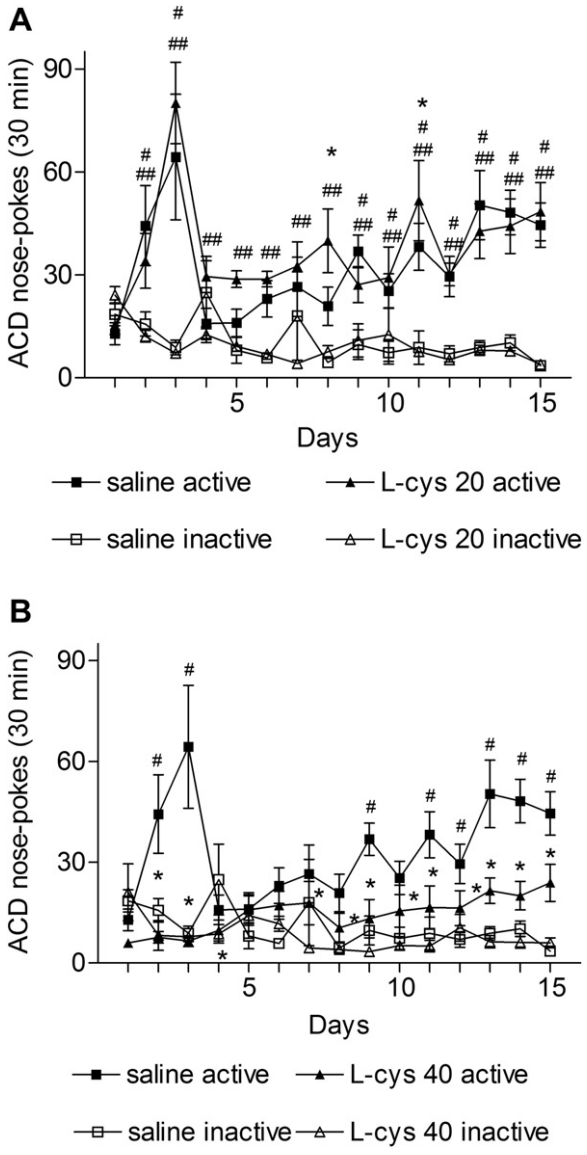


**Fig. 1.** Mean (±SEM) responses during the acquisition of oral ACD (0.1–0.2%) self-administration after L-cysteine pre-treatment at 20 mg/kg (panel A) or at 40 mg/kg (panel B). * indicates a significant difference in responding on the active nose-poke between an L-cysteine and saline group (two-way ANOVA performed on individual sessions contrasting active nose-pokes among groups). # (saline) or ## (L-cysteine) indicates significant nose-poke discrimination (two-way ANOVA for repeated measures and planned comparisons post-hoc test; $p < 0.05$).

*Effect of L-cysteine on maintenance of ACD self-administration behaviour*

Maintenance indicates on-going oral ACD self-administration. Baseline data (mean ± SEM) for the 2 days preceding testing with

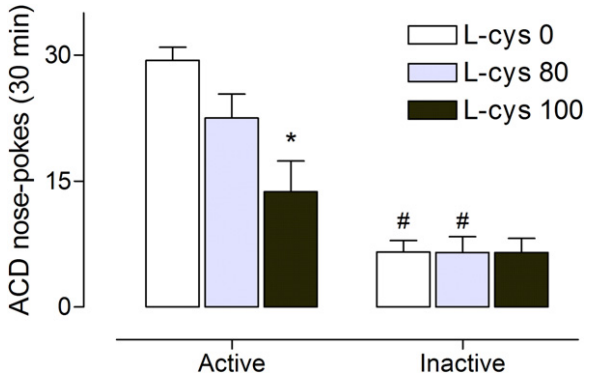


**Fig. 2.** Mean (±SEM) responses during the maintenance of oral ACD (0.2%) self-administration after L-cysteine pre-treatment (0, 80 and 100 mg/kg). * indicates a significant difference in responding on the active nose-poke between an L-cysteine and saline group. # indicates significant nose-poke discrimination (two-way ANOVA for repeated measures and Tukey post-hoc test; $p < 0.05$).

L-cysteine on ACD self-administration were averaged as follows: 28.69 ± 2.20 and 29.98 ± 2.17 (active nose-pokes). L-cysteine significantly reduced nose-poke responses for ACD reinforcement (Fig. 2). Two-way repeated measure ANOVA showed a significant main effect of pre-treatment [$F(2,95) = 4.76$, $p = 0.011$], nose-poke-session [$F(1,95) = 62.37$, $p < 0.00001$] and a significant pre-treatment × nose-poke-session interaction [$F(2,95) = 7.05$, $p = 0.0014$]. The higher dose of L-cysteine (100 mg/kg) significantly reduced both the active nose-poke responses for ACD and the ACD intake (6 mg/kg), inferred from the number of nose-pokes, compared to the saline pre-treatment (or L-cys 0: 20 mg/kg; Tukey post-hoc test; $p = 0.00014$). Furthermore, this dose of L-cysteine reduced nose-poke discrimination compared to the saline pre-treatment ($p = 0.00012$).

*Effect of L-cysteine on deprivation effect of ACD self-administration behaviour*

Rats that self-administered 0.2% ACD showed extinction behaviour with a loss of nose-poke discrimination and subsequently, after ACD restoration, rats showed a gradual reinstatement of active nose-poke responses. Fig. 3 shows the effect of L-cysteine (0, 100, 120 and 140 mg/kg) on the deprivation effect of oral ACD self-administration after ACD extinction. For L-cysteine pre-treatment, the two-way ANOVA revealed a significant main effect of pre-treatment [$F(5217) = 53.43$, $p = 0.00012$], nose-poke-session [$F(1217) = 1.65$, $p < 0.000001$] and a significant pre-treatment × nose-poke-session interaction [$F(5217) = 78.05$, $p = 0.000001$]. During the ACD maintenance session, responding was significantly more frequent on the active nose-pokes than the inactive nose-pokes (Fig. 3). L-cysteine (100, 120 and 140 mg/kg), administered 30 min before each deprivation session, significantly reduced active nose-pokes for oral ACD self-administration compared to the saline group (L-cys 0; Fig. 3). The effect of the higher dose of L-cysteine (140 mg/kg) was also present compared to the maintenance phase in which rats received saline alone. Furthermore, this dose of L-cysteine slowly reduced nose-poke discrimination ($p = 0.054$) compared to the maintenance and L-cysteine (0, 100 and 120 mg/kg) pre-treatment.

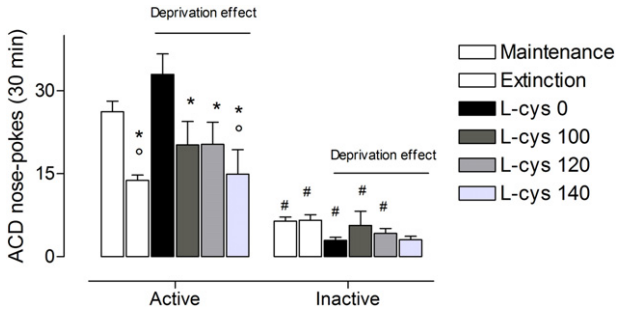


**Fig. 3.** Mean (±SEM) responses during the deprivation effect after L-cysteine pre-treatment (0, 100, 120 and 140 mg/kg) following ACD extinction. All the mean values represent the average of two consecutive self-administration sessions throughout the five sessions (i.e., the last two sessions together for maintenance, the first two sessions together for extinction and the first two sessions together for the deprivation effect). * indicates a significant difference in responding on the active nose-poke compared to the ACD deprivation effect. ° indicates a significant difference in responding on the active nose-poke compared to the maintenance phase. # indicates significant nose-poke discrimination (two-way ANOVA for repeated measures and Tukey post-hoc test; $p < 0.05$).

*Effect of L-cysteine on acetaldehyde progressive ratio performance*

L-cysteine pre-treatment induced a significant decrease in the ACD break point (Fig. 4, panel A) [$F(2,69) = 11.50$, $p = 0.000049$]. Both L-cysteine doses (120 and 140 mg/kg) reduced the ACD break point (6.42 ± 0.98, $p = 0.0026$ and 6.42 ± 1.42, $p = 0.0007$, respectively; Tukey post-hoc test) compared to the saline group (10.29 ± 0.52). Furthermore, the same L-cysteine doses (0, 120 and 140 mg/kg) did not change the number of inactive nose-pokes during ACD PR performance [$F(2,69) = 2.77$, $p = 0.07$], indicating an absence of a non-specific behavioural effects. The saline (L-cys 0) measurement is the average total break point for the ACD PR baseline preceding testing. A reliable ACD PR baseline was established because break point values did not differ with repeated testing or under the various conditions (i.e., saline injection and post-test).

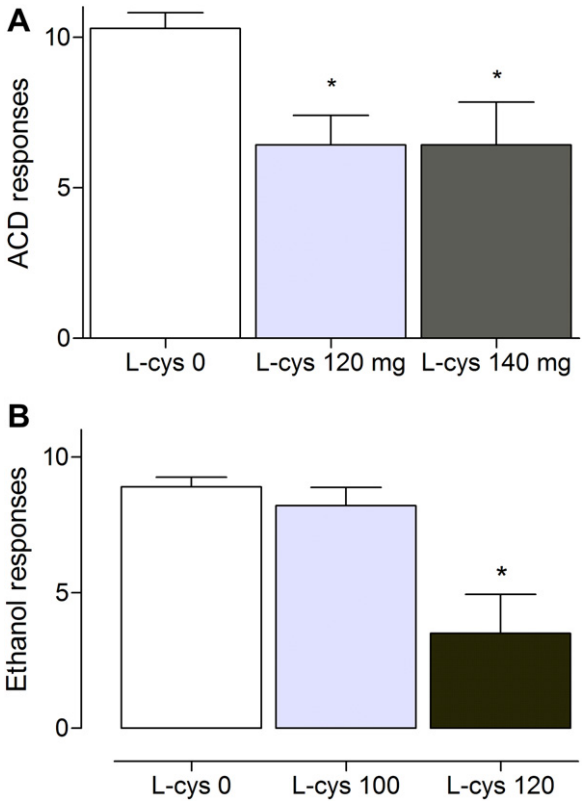


**Fig. 4.** Mean (±SEM) ACD (0.2%) (panel A) and ethanol (10%) (panel B) break point achieved after L-cysteine pre-treatment (0, 100, 120 and 140 mg/kg). * indicates a significant difference compared to the L-cysteine 0 (two-way ANOVA followed by Tukey's post-hoc test, $p < 0.05$).

*Blood acetaldehyde levels*

Ten minutes after anaesthesia, which immediately followed the final session of oral ACD self-administration, the ACD levels in blood were determined in a different group of rats ($n = 4–6$). Saline pre-treatment resulted in blood ACD concentrations of 0.0047 ± 0.00066 mg/ml while L-cysteine (100 mg/kg) pre-treatment resulted in blood ACD concentrations of 0.0051 ± 0.00082 mg/ml. The one-way ANOVA conducted on these data did not reveal a significant effect of pre-treatment [$F(1,8) = 1.47$, $p = 0.26$].

*Ethanol reinforcement*

*Effect of L-cysteine on ethanol progressive ratio performance*

L-cysteine-induced a significant decrease in the ethanol break point ([$F(2,76) = 14.48$, $p = 0.000005$]; Fig. 4, panel B). In particular, the higher dose tested (120 mg/kg) reduced the ethanol break point with respect to the rats pretreated with saline ($p = 0.00011$; Tukey post-hoc test). In addition, the mean number of inactive nose-poke explored, during the ethanol PR performance, relative to L-cysteine doses did not differ from the saline group [$F(2,76) = 0.72$, $p = 0.49$] indicating an absence of a non-specific behavioural effects. The saline (L-cys 0) bar was the average total break point for the ethanol PR baseline preceding testing.

*Saccharin reinforcement*

*Effect of L-cysteine on acquisition of oral saccharin self-administration*

Fig. 5, panel A shows the average number of nose-pokes in the active and inactive holes performed by rats during daily oral self-administration behaviour for saccharin solution (0.2%) for 15 consecutive days. L-cysteine (40 mg/kg) did not modify operant responding for saccharin (Fig. 5, panel A). In fact, the ratio of active to inactive nose-pokes was similar during all acquisition sessions, and the number of active nose-pokes for saccharin self-administration did not differ among the saline or L-cysteine tests throughout all sessions [pre-treatment: $F(1,6) = 0.10$, $p = 0.76$]; nose-poke-session: $F(29,174) = 3.49$, $p < 0.00001$; pre-treatment × nose-poke-session interaction: [$F(29,174) = 0.42$, $p = 0.99$].

*Effect of L-cysteine on maintenance of oral saccharin self-administration*

L-cysteine did not modify active nose-poke responses for saccharin reinforcement or nose-poke discrimination (Fig. 5, panel B). Two-way repeated measure ANOVA did not show a significant main effect of pre-treatment [$F(2,53) = 1.18$, $p = 0.31$] but did show a significant effect of nose-poke-session [$F(1,53) = 533.12$, $p < 0.00001$] and no significant pre-treatment × nose-poke-session interaction [$F(2,53) = 1.38$, $p = 0.26$]. No changes in inactive nose-pokes were significant among all pretreatments ([$F(2,53) = 0.67$, $p = 0.52$]; Fig. 5, panel B).

*Effect of L-cysteine on deprivation effect in saccharin self-administration*

During the saccharin maintenance phase, the rats responded significantly more on the active nose-pokes than inactive nose-pokes ($p = 0.00016$). In the same experimental conditions of the ACD deprivation phase, rats that self-administered 0.2% saccharin showed an extinction behaviour with a reduction of nose-poke discrimination ($p = 0.088$; Tukey post-hoc test) and then, after saccharin restoration, a gradual reinstatement of active nose-poke responses (deprivation effect) (Fig. 5, panel C). For L-cysteine (0 or

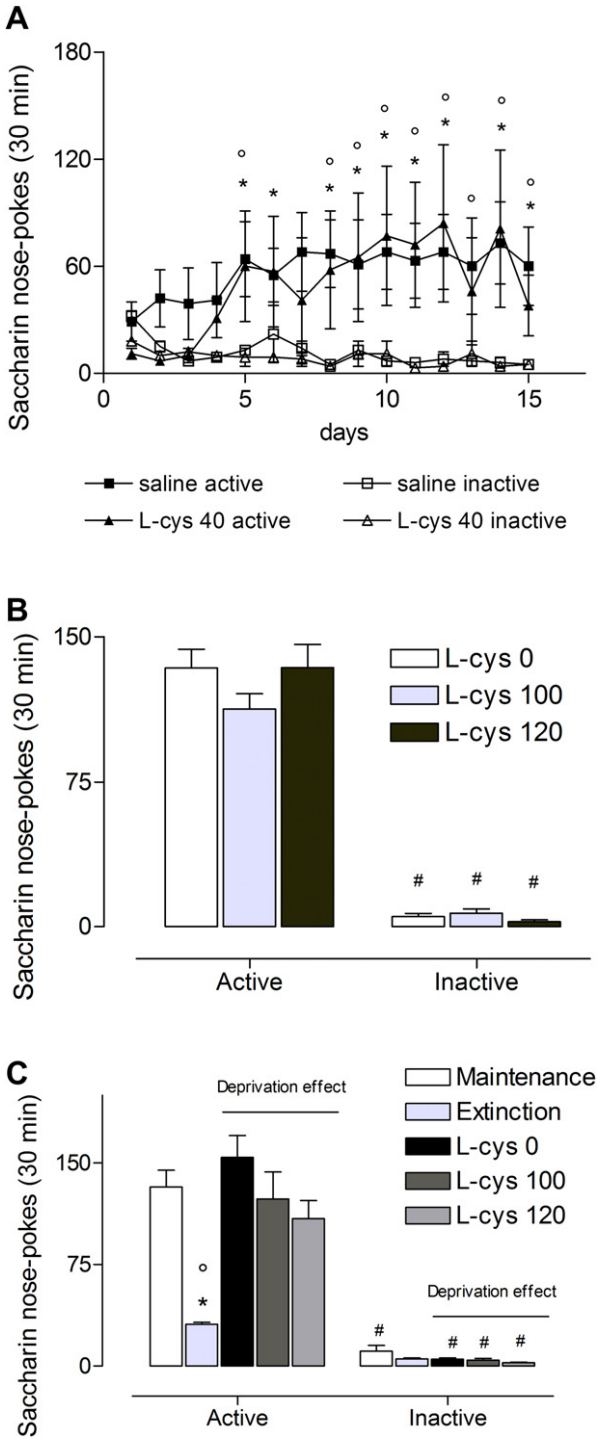


**Fig. 5.** Panel A. Mean (±SEM) responses during the acquisition of oral saccharin (0.2%) self-administration after L-cysteine pre-treatment at 40 mg/kg. * (saline) or ° (L-cysteine) indicates significant nose-poke discrimination (two-way ANOVA for repeated measures and planned comparisons post-hoc test; $p < 0.05$). Panel B. Mean (±SEM) during the maintenance of oral saccharin (0.2%) self-administration after L-cysteine pre-treatment (0, 100 and 120 mg/kg). # indicates significant nose-poke discrimination (two-way ANOVA for repeated measures and Tukey post-hoc test; $p < 0.05$). Panel C. Mean (±SEM) during the deprivation effect after L-cysteine pre-treatment (0, 100 and 120 mg/kg) following saccharin extinction. * indicates a significant difference in responding on the active nose-poke compared to the saccharin deprivation effect. ° indicates a significant difference in responding on the active nose-poke compared to the maintenance phase. # indicates significant nose-poke discrimination (two-way ANOVA for repeated measures and Tukey post-hoc test; $p < 0.05$).

saline, 100 and 120 mg/kg) pre-treatment, the two-way ANOVA conducted on the data revealed a significant main effect of pre-treatment [$F(4,63) = 260.18$, $p < 0.000001$], nose-poke-session [$F(1,63) = 1.65$, $p < 0.000001$] and a significant pre-treatment × nose-poke-session interaction [$F(4,63) = 227.65$, $p < 0.000001$]. L-cysteine (0, 100 and 120 mg/kg) pre-treatment did not modify the deprivation session after saccharin extinction (Fig. 5, panel C). Furthermore, these doses of L-cysteine did not alter nose-poke discrimination ($p < 0.05$) in rats during the maintenance phase and in rats pretreated with L-cysteine (0, 100 and 120 mg/kg).

## Discussion

The findings of the present study provide new insights on the involvement of ACD, first metabolite of ethanol, in the reinforcing effects of ethanol. We observed that rats orally self-administered ACD and that L-cysteine reduced this operant behaviour. In general, the results of the current study could suggest that ACD produces a reinforcing effect in Wistar rats. Evidence to support the interpretation that ACD could be reinforcing arises from the observation that rats acquire and maintain an oral ACD self-administration behaviour discriminating between active and inactive nose-pokes. This is in line with our previous study showed that ACD elicits a greater number of active nose-pokes for ACD solution (0.2%) with respect to a different group of rats self-administering tap water (Peana, Muggironi, & Diana, 2010). In addition, rats self-administering ACD demonstrated extinction behaviour when ACD was discontinued and gradually reinstated active nose-poke responses when ACD was reintroduced. L-cysteine pre-treatment, decreased acquisition, maintenance and the deprivation effect of oral ACD self-administration without interfering with inactive nose-pokes, suggesting the absence of a non-specific mechanism by which L-cysteine may reduce ACD self-administration. It is important to note that during acquisition of oral ACD self-administration behaviour, a lower L-cysteine dose (40 mg/kg for 15 days) was needed to reduce self-administration during the maintenance and deprivation effect after extinction phases (100 mg/kg for 5 days).

Furthermore, to study the possible reinforcing effects of ACD, we examined ACD self-administration under a PR schedule of reinforcement. In these experiments the ACD break point ($10.29 \pm 0.52$) was not statistically different from the ethanol break point ($8.9 \pm 0.35$) in which ACD concentration (0.2%) was 50 times lower than ethanol concentration (10%). Moreover the same doses of L-cysteine reduced both ACD and ethanol break point under identical experimental conditions. In addition, we observed consistent ACD and ethanol break point during repeated baseline tests and following L-cysteine evaluation. This could indicate that the PR performance in this study was stable over time and not influenced by repeated behavioural testing or factors associated with repeated drug administration. Furthermore, L-cysteine did not modify the exploration of inactive nose-pokes during ACD and ethanol PR performance compared to the saline group. This finding could suggest that the L-cysteine-induced reduction in the ACD and ethanol break point occurred because of a specific impact on ACD. It is important to consider that L-cysteine crosses the blood brain barrier through excitatory amino acid transporters (Chen & Swanson, 2003); leaving open the possibility, that L-cysteine could be acting centrally.

To determine if L-cysteine could produce a general reduction in motivation to self-administer we tested this agent on acquisition, maintenance and the deprivation effect with saccharin reinforcement. Although 40 mg of L-cysteine, during saccharin acquisition did not interfere with the rats' behaviour, it was tested in all subsequent phases in the same experimental conditions, either in chronic, during acquisition (40 mg/kg for 15 days at FR1); or in subacute, during maintenance and the deprivation effect (80–120 mg/kg for 5 days at FR1). No L-cysteine doses, effective on ACD reinforcement, reduced saccharin reinforcement or the number of inactive nose-poke responses during each self-administration phase. Thus, under the conditions of the present experiment, we could exclude a non-specific behavioural activation or reduction. Indeed, a motor impairment would probably produce a reduction in responding, which would be reflected as decreased self-administration phases.

In addition, given that L-cysteine is reported to be an ACD-sequestrating agent, able to bind covalently to ACD (Kera et al., 1985; Nagasawa et al., 1984) we measured blood ACD levels using a HS-GC-FID system, a gas chromatographer with headspace. From these analyses, we did not observe differences among samples. This result is in line with our previous report in which L-cysteine failed to reduce blood and brain ACD levels in rats self-administering ethanol (Peana, Muggironi, Calvisi, et al., 2010).

It is important to consider that ACD has a half-life of a few minutes (Correa et al., 2012; Novartis Foundation, 2007). In fact, ACD determination after peripheral ACD treatment could be difficult and compromised by its rapid conversion to acetate (Deng & Deitrich, 2008), which may markedly affect the recovery of ACD levels, thus it is very difficult to understand how the effects of L-cysteine are could be due to sequestering ACD. Therefore, we speculate that ACD could exert its potential via the condensation products (Correa et al., 2012; Talhout, Opperhuizen, & van Amsterdam, 2007) that could be implicated in ethanol and ACD reinforcement (Collins, 1988; Matsuzawa, Suzuki, & Misawa, 2000; Myers, Ng, Singer, Smythe, & Duncan, 1985; Quertemont & Tambour, 2004; Wodarz, Wiesbeck, Rommelspacher, Reiderer, & Boning, 1996). In this regard, salsolinol, the product of a direct cyclization between dopamine (DA) and ACD can be detected in the rat brain after contingently self-administered ACD (Myers et al., 1985). This possibility could be supported by our preliminary experiments in which rats were previously pretreated with an inhibitor of ACD dehydrogenase, disulfiram and then intragastrically treated with ACD, at doses comparable to the levels that occur during ACD self-administration (20 mg/kg). In this experiment the blood ACD levels were equally small/non-significant compared to the controls rats, pretreated with vehicle (personal observation).

We cannot exclude the possibility that the effectiveness of L-cysteine could be due to its properties as an ACD-sequestrating agent (Kera et al., 1985; Nagasawa et al., 1984). In fact, previous findings have reported that D-penicillamine, a structural analogue of L-cysteine, or L-cysteine decreased intragastric ethanol or ACD-induced conditioned place preference (Peana et al., 2008, 2009) and oral ethanol self-administration (Font et al., 2006a; Peana, Muggironi, Calvisi, et al., 2010). In addition, intracerebroventricular D-penicillamine reduced voluntary ethanol consumption in rats indicating that the central inactivation of ACD also blocks ethanol intake (Font et al., 2006b). On the other hand, D-penicillamine or L-cysteine prevented intragastric ethanol or ACD-induced stimulation of mesolimbic DA transmission (Enrico et al., 2009; Sirca, Enrico, Mereu, Peana, & Diana, 2011) at the same doses employed in behavioural experiments such as conditioned place preference (Peana et al., 2008, 2009) or ethanol self-administration (Peana, Muggironi, Calvisi, et al., 2010).

The effect of L-cysteine on ACD self-administration could reside in different mechanisms. Several reports show antioxidant properties of L-cysteine as precursor of the antioxidant glutathione (Soghier & Brion, 2006) and as direct scavenging of free radicals (Shackebaei, King, Shukla, Suleiman, 2005). 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 signalling 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). Evidence is also accumulating to suggest that sulphur-containing amino acids are analogues of glutamate (Thompson & Kilpatrick, 1996). 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 (Melendez, Hicks, Cagle, & Kalivas, 2005; Moran, McFarland, Melendez, Kalivas, & Seamans, 2005). Consistent with this mechanism of action, although not examined in the present study, it is possible that, like cocaine and heroin training, ACD 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 ACD-induced neuroadaptations in brain (Zhou & Kalivas, 2008).

Several studies have reported that ACD-induced robust effects in the VTA and increased the firing rate of dopaminergic neurons in the VTA (Diana et al., 2008; Enrico et al., 2009; Foddai, Dosia, Spiga, & Diana, 2004). In patch-clamp studies, ACD increased the inward current ($I_h$) in DA neurons, suggesting a direct effect for ACD on dopaminergic neural membranes (Melis, Enrico, Peana, & Diana, 2007). Furthermore, Vinci et al. (2010) reported that intragastric administration of ACD elicited the activation of ERK in the nucleus accumbens (Nacc) and in nuclei of the extended amygdala in a DA $D_1$ receptor-dependent manner (Vinci et al., 2010). The role of the $D_1$ receptors/pERK pathway has also been demonstrated in the motivational properties of ACD, as assessed by place conditioning (Spina et al., 2010). These findings could substantiate the probable reinforcing effect of ACD through activation of DA neurons in VTA directly through an interaction of ACD with receptors or ion channels and/or indirectly through an interaction of ACD with neurotransmitter systems regulating the activity of VTA DA neurons (Kalivas, 1993).

Irrespective of the mechanism, these results suggest that ACD could possess reinforcing properties and L-cysteine could reduce the motivation to self-administer ACD.

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