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ROLE OF ETHANOL-DERIVED ACETALDEHYDE IN OPERANT ORAL SELF-ADMINISTRATION OF ETHANOL IN RATS

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Running title: *Effects of D-penicillamine and 3-amino-1,2,4-triazole on ethanol self-administration.*

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This study was carried out in accordance with the Italian legislation (D.L. 116, 1992), which allowed experimentation on laboratory animals only after submission and approval of a research project to welfare and health organization on animal's experimentation of the University of Sassari (Sassari, Italy) and to the Ministry of Health (Rome, Italy), and in accordance with European Council directives on the matter (n. 2007/526/CE) and the "Guide for the care and use of laboratory animals" as approved by the Society for Neuroscience (National Research Council, 1996).

Keywords: Ethanol; Acetaldehyde; Oral operant self-administration; D-Penicillamine; 3-Amino-1,2,4-Triazole; Wistar rats.

Abstract

Rationale: The role of ethanol-derived acetaldehyde has not been examined on performance in a model of operant oral self-administration. However, previous studies reported that an acetaldehyde-sequestering agent, D-penicillamine (DP) and an inhibitor of catalase-mediated acetaldehyde production, 3-amino-1,2,4-triazole (3-AT) reduce voluntary ethanol consumption. **Objectives:** The aim of our investigation was to evaluate the effects of DP and 3-AT on acquisition (in rats without previous experience of ethanol consumption) and on maintenance (in habitual drinker rats) of oral operant ethanol self-administration. **Methods:** Using operant chambers, rats learned to nose poke in order to receive ethanol solution (5-10% v/v) under a FR1 schedule of reinforcement in which discrete light and tone cues were presented during ethanol delivery. **Results:** DP and 3-AT impair the acquisition of ethanol self-administration, whereas its maintenance is not affected by neither drug given alone, for both 5 or 10% ethanol, nor by drugs association for 5% ethanol. **Conclusions:** These findings suggest that brain acetaldehyde plays a critical role in the formation of Pavlovian learning elicited by ethanol during acquisition of operant self-administration in ethanol-naïve rats. In contrast, it is postulated that during the maintenance phase of operant self-administration, acetaldehyde could contribute to ethanol self-administration by a combined mechanism: on one hand its lack might result in further ethanol-seeking and taking and, on the other, inhibition of ethanol metabolism might release the action of un-metabolized fraction of ethanol onto $GABA_A$ receptors.

Introduction

The operant oral ethanol self-administration paradigm is a commonly used model in which animals are trained to emit a specific response (lever press or nose poke) for gaining the ethanol reinforcement (Grant and Samson 1986; Samson et al, 1988). It is based on Pavlovian conditioning, in which rats acquire conditioned reinforcing properties able to generate and maintain ethanol self-administration behaviour (Tomie et al, 2008; Samson et al, 1988). In this paradigm the schedule of drug reinforcement is critical when modelling distinct phases of addictive behaviour: thus, after animals have learned to perform a fixed number of specific responses to receive the delivery of the drug, lowering or rising the amount of drug received per operant response, results in an increase or decrease of the rate of self-administration, respectively. Accordingly, self-administration rates on fixed ratio (FR) schedules of reinforcement are dose-dependent and are inversely related to the dose received following each operant response (Graham and Self, 2010). Indeed, this model has been invaluable in the alcohol research field, as it has enabled researchers to explore the reinforcing aspects of ethanol taking and seeking, with the use of fixed and progressive ratio schedules, as well as with the use of reinstatement paradigms. Furthermore, it also had a critical role in the preclinical characterization and validation of two medications, presently approved by the US Food and Drug Administration, for the treatment of alcohol use disorders, naltrexone (Bienkowski et al, 1999) and acamprosate (Czachowski et al, 2001).

Recently published studies support the suggestion that the motivational properties of ethanol are mediated by its first metabolite, acetaldehyde, formed either in the periphery and in the brain (Correa et al, 2012) as demonstrated i) by inhibiting the production of acetaldehyde in the periphery (blockade of alcohol dehydrogenase: Peana et al, 2008), ii) by inhibiting the generation of brain acetaldehyde (blockade or interference of brain catalase: Font et al, 2008; Ledesma et al, 2013, 2014; Peana et al 2013b; Tarragon et al, 2014) and, finally, iii) by decreasing acetaldehyde bioavailability (by using acetaldehyde sequestering agents: Font et al, 2005, 2006a,b; Peana et al,

2008; Enrico et al, 2009; Ledesma et al, 2013; Marti-Prats et al, 2013). These observations overall support the hypothesis that central and peripheral acetaldehyde generation actively contributes to the reinforcing properties of ethanol and raise the possibility that acetaldehyde's role can be exploited to devise novel pharmacological approaches to target alcohol abuse-related problems.

D-penicillamine (DP), a thiol amino acid that interacts non-enzymatically with acetaldehyde to form stable adducts (Nagasawa et al, 1978), inactivates acetaldehyde and therefore reduces its bioavailability (Nagasawa et al, 1978). Accordingly, DP has been shown able to modify the voluntary ethanol consumption (Font et al, 2006a) as well as to prevent the alcohol relapse-like drinking in rats (Orrico et al, 2013). On the same vein, the catalase-H_2O_2 inhibitor, 3-amino-1,2,4-triazole (3-AT), has been shown to significantly reduce voluntary ethanol consumption (Aragon and Amit 1992; Koechling and Amit 1994) and likewise, decreasing the cerebral H_2O_2 availability by alpha lipoic acid, results in inhibition of voluntary ethanol intake (Ledesma et al, 2014) and in interference with maintenance, breaking point and reinstatement of seeking behaviour of oral operant ethanol self-administration (Peana et al, 2013b).

However, while DP and 3-AT have proven to be effective in modifying voluntary ethanol intake in rodents, a behavioural outcome that can be used as indirect evidence of the significance of acetaldehyde in the brain (Peana and Acquas 2013a), the role of ethanol-derived acetaldehyde has not been fully examined using the operant ethanol self-administration paradigm. Therefore, since under these circumstances the role of ethanol-derived acetaldehyde, strictly related to the efficacy of DP and 3-AT treatments, could be different than on voluntary ethanol intake (Israel et al, 2015), the aim of the present investigation was to further clarify the role that ethanol-derived acetaldehyde might have in the motivational effects of ethanol. To this end we evaluated if DP and 3-AT could affect oral operant ethanol self-administration both in naïve rats, during acquisition and, in experienced rats, during maintenance, a condition in which rats perform a stable oral operant ethanol self-administration at either 5 or 10% v/v ethanol concentration.

Methods

This study was carried out in accordance with the Italian legislation (D.L. 116, 1992), which allowed experimentation on laboratory animals only after submission and approval of a research project to welfare and health organization on animal's experimentation of the University of Sassari (Sassari, Italy) and to the Ministry of Health (Rome, Italy), and in accordance with European Council directives on the matter (n. 2007/526/CE) and the "Guide for the care and use of laboratory animals" as approved by the Society for Neuroscience (National Research Council, 1996). All possible efforts were made to minimise animal pain and discomfort and to reduce the number of experimental subjects.

Animals

Male Wistar rats (Harlan Italy Spa, Udine, Italy), weighing 125-150 g at the beginning of the experiment, were housed in pairs in Plexiglas cages with tap water (provided by two bottles/cage) and food (Mucedola, Milano, Italy) available *ad libitum*. The colony room was maintained under controlled environmental conditions (temperature: 22±2°C; humidity: 60-65%) on a 12/12-light/dark cycle (light on at 8:00 h; off at 20:00 h). For the experiments of acquisition of ethanol self-administration behaviour 18 rats were used; for the experiments of ethanol self-administration during the maintenance phase 12 rats were used.

Drugs

Ethanol solutions (v/v) were obtained by dilution (U.S. Pharmacopeia National Formulary 1995) of ethanol (95%; Silvio Carta, Italy) with tap water (10% v/v ethanol solution contains 8.7 g of ethanol in 100 ml). D-penicillamine (DP) and 3-amino-1,2,4-triazole (3-AT) were purchased from Sigma-Aldrich (Milan, Italy). The doses and times of DP and 3-AT administration were selected based on previously published reports (Font et al, 2006a, 2008; Peana et al, 2008; Ledesma et al, 2013).

Apparatus

Training and testing was conducted in modular operant chambers located in ventilated soundproof environmental cubicles (Med Associates Inc., St. Albans, VT, USA). Each chamber was equipped with a non-retractable drinking cup (capacity 0.50 ml) and two nose poke holes located 3 cm to the left and right of the cup. A white light placed above the active hole and a weaker white lights placed above the inactive hole were used as environmental stimuli. Only the active nose poke hole set off the dipper-delivering solution (0.1 ml) into the drinking cup in 3.05-second period. The availability of liquid was signalled by a house light placed on the wall in front of the drinking cup that would light up for the duration of liquid delivery. Following each delivery, there was a 2-second time-out period during which responses had no consequences and the white light placed above the active hole went off. An infrared head detector was located in the reservoir and recorded all movements during the entire session. The chambers were interfaced to a computer equipped with software that ran the programmed sessions and recorded the data.

DP or 3-AT administration on acquisition of oral ethanol self-administration behaviour

Acquisition of self-administration was preceded by a shaping phase: briefly, in order to facilitate acquisition of operant responding for liquid delivery, during the first 3 days of training, tap water availability in the home cage was restricted to 5 min/day. During this period, naïve rats were allowed to nose poke explore for tap water in a 30 min session/day. On the 4th day tap water was made freely available in the home cage and tap water was replaced by 5% ethanol (v/v) solution (this switch in reinforcer occurred in the operant chambers). Treatments with saline, DP or 3-AT were started after shaping phase; in particular, rats (n=30) were administered intraperitoneally (ip) with saline (n=18; 1 ml/kg) or DP (n=6; 50-100 mg/kg, ip) 30 min before each of the 8 sessions of ethanol self-administration. 3-AT (n=6; 1 g/kg, ip) was administered 4 hours before each of the 8 sessions of ethanol self-administration. From day 4 to day 6, rats were permitted to nose poke explore, in a 30 min session/day, for 5% ethanol solution under a FR 1 schedule of reinforcement, in which each response resulted in 0.1 ml of solution delivery. Starting on day 7, the ethanol

percentage was gradually increased, with daily increases of 1% up to the final concentration of 10% (day 11).

Each ethanol-reinforced response was paired with a brief presentation of environmental stimuli such as the house light, the infusion pump sound, and the white light above the active hole. In this model, both ethanol administration and presentation of conditioned stimuli were contingent upon the rats' operant response. Responses at the inactive nose poke hole during self-administration throughout the experiments were recorded to monitor non-specific behavioural effects.

DP or 3-AT administration on maintenance of oral ethanol self-administration behaviour

For these experiments we used animals (n=18) that received daily ip injections of saline during acquisition of ethanol self-administration. However, after the acquisition phase, these rats were subjected to a daily self-administration session lasting 30 minutes (seven days a week, at least 10 days after acquisition). Following this experiment, given that DP and 3-AT had no effect under the experimental condition applied in the maintenance of ethanol self-administration we made available to the same rats a solution with a lower concentration of ethanol (5%) under a FR1 schedule of reinforcement.

Drugs' doses were tested under a random treatment order (n=6 to 12 for each dose studied). Three drug-free intervening sessions were allowed between pre-treatments in order to avoid possible confounding due to carry-over effects upon subsequent responding and ethanol intake. In particular, each animal was re-tested after reaching a stable baseline of self-administration.

Statistics

Results are reported as the mean ± SEM number of nose pokes/session. The analysis of ethanol self-administration (acquisition) was performed on data collected after the shaping phase i.e. from the fourth to eleventh session with saline, DP or 3-AT treatment. Data analyses consisted of a treatment x session two-way analysis of variance (ANOVA) with time as repeated measure. For the statistical

analysis of ethanol self-administration during maintenance, the last session with saline and the first session with DP, 3-AT or DP+3-AT administration were collected. Data analyses consisted of a treatment x session two-way ANOVA with session as repeated measure. In particular, for each treatment, nose poke discrimination (in the active and, separately, in the inactive hole) was determined by type x session (nose poke/session). In the presence of overall significant main effects and interactions (p-values <0.05) following two-way ANOVAs, the least significant difference (LSD) post-hoc tests (acquisition) and Tukey's honestly significant difference post hoc test (maintenance) were performed.

Results

Effect of DP and 3-AT on acquisition of ethanol self-administration behaviour

In these experiments, ethanol-naïve rats received daily ip injections of saline (0.1 ml/kg), DP (50 mg/kg) or 3-AT (1 g/kg) since the fourth session. Figure 1 shows the effect of DP and 3-AT administration on the acquisition of ethanol self-administration. Repeated-measures two-way ANOVA showed significant main effects of treatment [$F(2,9)=6.60$, $p<0.05$], nose pokes/session [$F(15,135)=16.16$, $p<0.0001$] and a significant treatment × nose pokes/session interaction [$F(30,135)=2.61$, $p<0.0001$]. During the acquisition sessions for ethanol self-administration, rats administered with saline responded significantly more on the active nose poke hole than on the inactive one ($p<0.05$, LSD test). Further analysis indicated that DP significantly decreased the average number of active nose pokes/session with respect to saline treated rats ($p<0.05$, LSD test), from the fifth to the eleventh day. Similarly, 3-AT significantly decreased the average number of active nose pokes/session with respect to the saline group (3-AT 0 mg/kg) from the sixth to the eleventh day except for the tenth day ($p<0.05$, LSD test). Notably, administration of DP and 3-AT did not modify the average number of inactive nose pokes/session with respect to the saline group, indicating the absence of nonspecific behavioural effects.

Effect of DP or 3-AT on maintenance of 10% ethanol self-administration behaviour

As shown in figure 2, DP (0, 50 and 100 mg/kg, ip) (panel a) and 3-AT (0 and 1 g/kg, ip) (panel b) failed to reduce nose poke responses during the maintenance phase for 10% ethanol self-administration. There was only a statistically significant discrimination between active and inactive nose poke holes in the group of saline and DP [$F(1,21)=139.45$, $p<0.0001$] (panel a) as well as in the group of saline and 3-AT [$F(1,9)=30.04$, $p=0.00039$] (panel b) pre-treatment.

Effect of DP or 3-AT or DP+3AT on maintenance of 5% ethanol self-administration behaviour

Self-administration rates on FR reinforcement schedules are dose-dependent and are inversely related to the dose received with each self-administration episode (Graham and Self, 2010). Due to the fact that neither DP (50 and 100 mg/kg) nor 3-AT (1 g/kg) could reduce operant ethanol self-administration behaviour for 10% ethanol, the present experiment assessed the effects of these drugs, either alone and in combination during the maintenance phase of self-administration for 5% ethanol concentration. For statistical purpose, data from the last session at 10% v/v were compared with data from the successive session at 5% v/v ethanol concentration. As can be seen in figure 3, rats exposed to the lower concentration of ethanol (5%) significantly increased nose poke responses with respect to 10% of ethanol (panel a) in order to increase ethanol intake (panel b) as demonstrated by repeated measures two-way ANOVA, which revealed a significant main effect of ethanol concentration [$F(1,10)=6.33$, $p=0.030$], of nose pokes/session [$F(1,10)=62.55$, $p<0.0001$] and a significant ethanol concentration x nose pokes/session interaction [$F(1,10)=6.28$, $p=0.031$], but not a significant main effect of ethanol intake (one way ANOVA).

Afterward, figure 4 shows the effect of pre-treatment with DP (0 and 50 mg/kg, panel a), 3-AT (0 and 1 g/kg, panel b) and DP+3-AT co-administration (50 mg/kg +1 g/kg, panel c) on 5% ethanol self-administration. As indicated in the figure 4, neither DP or 3-AT nor DP+3-AT co-administration reduced nose poke responses during the maintenance phase for 5% ethanol self-administration. As confirmed by two ways ANOVA, there was a significant nose poke hole

discrimination between active and inactive nose pokes in the saline and DP groups [$F(1,10=89.94$, $p<0.0001$] (panel a), between active and inactive nose pokes in the saline and 3-AT groups [$F(1,10=84.97$, $p<0.0001$] (panel b) and between active and inactive nose pokes in the saline and DP+3-AT groups [$F(1,10=127.21$, $p<0.0001$] (panel c).

Discussion

In order to characterize the role that ethanol-derived acetaldehyde plays in distinct phases of oral operant ethanol self-administration behaviour, the present study investigated the effects of the acetaldehyde-sequestering agent, DP, and of the catalase inhibitor, 3-AT, on acquisition, in naïve rats, and on maintenance, in rats that had acquired this behaviour. In these experiments, during acquisition, rats learn to self-administer ethanol by nose-poking in order to receive the delivery of a solution whose ethanol content increases daily in a stepwise manner from 5% up to 10% v/v. Instead, the maintenance phase is characterized by on-going oral ethanol self-administration at 10% in which rats have reached a stable baseline rate of self-administration. Notably, during these phases discrete light and tone cues were presented upon ethanol delivery. These cues are particularly important as, through Pavlovian conditioning, rats acquire conditioned reinforcing and motivational properties able to generate and maintain drug self-administration behaviour.

The results of DP and 3-AT administration during acquisition appear overall in agreement with previous data reporting a critical role of acetaldehyde in ethanol-mediated behavioural responses (Correa et al, 2012). In fact, in agreement with a number of previous studies, we found that the administration of DP and 3-AT, at doses that have been previously shown to prevent ethanol-mediated behaviours (Aragon and Amit, 1992; Escarabajal et al, 2000; Font et al, 2005; 2006a; 2006b; 2008; Peana et al 2008; Ledesma et al, 2013; Orrico et al, 2013), could significantly hamper the rise of the number of nose pokes/session during acquisition. The observation that reduction of bioavailability of acetaldehyde as well as inhibition of catalase-mediated ethanol metabolism prevent the acquisition of ethanol oral self-administration is also in agreement with a previous study

reporting that 3-AT, administered along five consecutive days, significantly reduced voluntary ethanol consumption (Koechling and Amit 1994), and also with recent data showing the ability of DP and 3-AT to affect the acquisition of ethanol-elicited conditioned place preference (Font et al, 2006b; 2008; Peana et al, 2008; Ledesma et al, 2013) and to reduce ethanol-induced behavioural locomotion (Escarabajal et al, 2000; Font et al, 2005).

The most parsimonious explanation to interpret the effects of DP and 3-AT on acquisition, may be that this phase of ethanol self-administration behaviour depends on the availability of optimal levels of acetaldehyde during learning and trace memory formation as well as on the load of the task (i.e. the strength of the memory rats are called to engage with). In this regard it appears important to highlight that the effectiveness of DP to impair the acquisition of ethanol self-administration cannot be justified by taste alterations (Font et al, 2006a) since, during maintenance, neither DP (or 3-AT) did show any effect, even when rats were asked to nose poke for the lower concentration of ethanol.

In contrast, preclusion of acetaldehyde availability or inhibition of acetaldehyde formation by DP and 3-AT, respectively, given either alone on maintenance of 10 or 5% v/v ethanol, and in combination before the session in which ethanol was down-shifted to 5%, failed to affect oral ethanol self-administration in trained rats. In the experiments performed during the maintenance phase, DP and 3-AT were administered at the same doses at which were administered in the acquisition phase. The results of these experiments show that in none of these conditions DP and 3-AT did affect operant responses for ethanol delivery. In addition, in order to rule out the possibility that a single administration could not have been sufficient to affect maintenance of 10% ethanol self-administration behaviour, we administered animals DP (50 mg/kg) for up to five consecutive days, without observing any significant reduction in the number of nose pokes/session (data not shown).

The lack of effect of DP and 3-AT on maintenance of operant ethanol self-administration observed in the present study could reside in different causes. One (paradoxical) explanation could be found

in the decrease of brain acetaldehyde, caused by DP or 3-AT pre-treatments. Notably, acetaldehyde has been shown able to induce and maintain the operant behaviour of its oral self-administration in rats (Peana et al, 2010) and this makes likely that also at this stage it plays a critical role. Indeed it might be suggested that when the concentration of ethanol was diminished from 10 to 5 %, rats felt the reduction of ethanol-derived acetaldehyde and, accordingly, increased the rate of self-administration behaviour. In other words, it might be possible that, either when ethanol-derived acetaldehyde's availability is pharmacologically prevented and when acetaldehyde's availability is reduced by down-shifting ethanol's concentration, animals do nose poke as they were seeking ethanol-derived acetaldehyde. This possibility would be in agreement with the observation that after the extinction of oral acetaldehyde operant self-administration, animals reinstate a drinking behaviour or engage in a deprivation effect (Peana et al, 2010; 2012). Likewise, the observation of an increased search for the reinforcing substance, following its reduction, is a remarkable finding, in line with that reported by Graham and Self (2010). In agreement with these observations, our data show that DP and 3-AT did not produce substantial differences in the rate of ethanol self-administration when ethanol concentration was reduced. Notably, although paradoxically the failure to affect maintenance could be attributed to the reduction of acetaldehyde availability (Peana et al, 2010), this condition might also unmask the concurrent effects of un-metabolized ethanol acting on γ-aminobutyric-acid A ($GABA_A$) receptors (Kumar et al, 2009; Tan et al, 2010; Kaminski et al, 2013). This possibility appears consistent with recent findings supporting the crucial role of the non-bio transformed fraction of ethanol in the depressant effects observed when acetaldehyde production is limited or inactivated (Martí-Prats et al, 2013). Consequently, it could be hypothesized that in rats treated with DP or 3-AT or DP+3-AT, the effects on ethanol self-administration could be the result of converging actions coming from the lack of optimal acetaldehyde concentrations, on one hand, and from the non-metabolized fraction of ethanol onto $GABA_A$ receptors on the other (Correa et al, 1999; Martí-Prats et al, 2013 Davies et al, 2003). Intriguingly, the stimulation of these receptors in the ventral tegmental area (VTA) has been

suggested to result in the activation of dopaminergic and non-dopaminergic reward pathways (Vashchinkina et al, 2014), as indicated by the fact that both the $GABA_A$ agonist, muscimol and the $GABA_A$ antagonist, bicuculline are reinforcing and rewarding when infused into the VTA (Ikemoto et al, 1997, 1998; Laviolette and van der Kooy, 2001). In particular, while muscimol, binding to VTA GABAergic neurons, disinhibits VTA dopaminergic neurons leading to dopamine antagonist-sensitive reward, bicuculline, binding to VTA GABAergic neurons, activates a yet poorly defined non-dopaminergic reward pathway insensitive to dopamine receptor antagonists (Laviolette and van der Kooy, 2001). In line with this possibility, when applied into the ventral pallidum (a convergent point for hedonic and motivational signalling), bicuculline also increases ethanol intake in voluntary ethanol consumption (Kemppainen et al, 2012) supporting the role of $GABA_A$ receptors on ethanol-mediated behavioural responses.

Although aware that the present data do not provide direct evidence in support of this interpretation, they indicate that maintenance of operant ethanol self-administration behaviour is not reduced when acetaldehyde is not available, in agreement with the observation that animals that had consumed ethanol chronically for 2-3 months do not modify their ethanol intake following VTA injections of lentivirus encoding anticatalase shRNA or aldehyde dehydrogenase-2 (Karahanian et al, 2011; 2015), indicating that factors other than (optimal) acetaldehyde concentrations in the VTA may be involved in the perpetuation of ethanol self-administration. Likewise, Israel and Colleagues (2015) suggest that in animals that have ingested ethanol chronically, the maintenance of ethanol intake is no longer influenced by ethanol metabolites (Israel et al, 2015). However, further alternative explanations should also be considered to interpret the present results; in fact, during perpetuation of ethanol self-administration, ethanol-related stimuli might elicit automatic responses that lead to ethanol seeking without recruiting conscious desire as reported by Ingjaldsson and Colleagues (2003). Moreover, the higher ethanol consumption, seen when ethanol concentration was decreased, may be associated to anxiety as recently suggested by Pelloux and Colleagues (2015).

In contrast with our results, previous data showed that DP and 3-AT were effective in reducing voluntary consumption of ethanol (Aragon and Amit 1992; Font et al, 2006a); however, this is quite a different paradigm based on the spontaneous intake of freely available ethanol that disregards the operant behaviour.

Overall, it could be assumed that, during the maintenance phase of the operant ethanol self-administration, acetaldehyde could play a critical role if we consider that its lack may also contribute to bring about an increase of seeking behaviour. However, the inhibition of catalase in the oral operant self-administration of ethanol does not result in a reduction of ethanol self-administration rates because of the effects of (the non-bio transformed fraction of) ethanol on $GABA_A$ receptor, sensible to induce an increase in behavioural responses in the paradigm of self-administration (Davies et al, 2003; Kumar et al, 2009; Kaminski et al, 2013).

In summary, we suggest that brain acetaldehyde could actively participate in the formation of Pavlovian learning elicited by ethanol during acquisition of operant self-administration in ethanol-naïve rats, an interpretation in agreement with the recently suggested "first hit" hypothesis (Israel et al, 2015). In contrast, during the maintenance phase of operant self-administration, it can be postulated that acetaldehyde could contribute to perpetuate ethanol self-administration by a combined mechanism: on one hand its lack might results in further ethanol-seeking and taking and, on the other, inhibition of ethanol metabolism releases the action of un-metabolized fraction of ethanol onto VTA $GABA_A$ receptors.

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Figures legends

Fig. 1 Mean (± SEM) responses during acquisition of oral ethanol (from 5 to 10% v/v) self-administration after saline (0.1 ml/kg), DP (50 mg/kg) or 3-AT (1 g/kg) injection (n=6/group). * Indicates significant difference in responding on the active nose poke between saline- and DP-injected rats. ° Indicates significant difference in responding on the active nose poke between saline- and 3-AT-injected rats. Data from the shaping phase were not included in the statistical analysis. Two-way analysis of variance (ANOVA) for repeated measures and LSD post-hoc test were performed ($p<0.05$).

Fig. 2 Nose poke responses during the maintenance of oral ethanol (10% v/v) self-administration after saline (0.1 ml/kg, DP 0 mg/kg or 3-AT 0 g/kg, panels a and b respectively), DP (50 and 100 mg/kg, panel a) or 3-AT (1 g/kg, panel b) injections. Values represent mean (± SEM) of data from 6-12 rats/group. ° Indicates significant nose poke hole discrimination between active and inactive nose poke holes in the saline and DP (panel a) or in the saline and 3-AT (panel b) pre-treated groups.

Fig. 3 Nose poke responses (panel a) and ethanol intake (panel b) during maintenance of oral ethanol (from 10% to 5%) self-administration after saline (0.1 ml/kg) injection. Values represent mean (± SEM) of data from 6-12 rats/group. * Indicates significant differences in active nose poke hole with respect to 10% ethanol concentration. ° Indicates significant differences in nose poke hole discrimination. Two-way ANOVA for repeated measures and Tukey's post-hoc test; $p<0.05$ (panel a) and one way ANOVA (panel b).

Fig. 4 Nose poke responses during the maintenance of oral ethanol (5%) self-administration after injection of DP 0 i.e. saline (0.1 ml/kg), DP (50 mg/kg, panel a), 3-AT (1 g/kg, panel b) or DP + 3-AT (50 mg/kg+1 g/kg, respectively, panel c). Values represent mean (± SEM) of data from 6-12 rats/group. ° Indicates significant differences in nose poke hole discrimination between active and inactive holes.