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Silencing brain catalase expression reduces ethanol intake in developmentally-lead-exposed rats

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ABSTRACT

Lead (Pb) is a developmental neurotoxicant. We have demonstrated that perinatally Pb-exposed rats consume more ethanol than their control counterparts, a response that seems to be mediated by catalase (CAT) and centrally-formed acetaldehyde, ethanol's first metabolite with attributed reinforcing effects in the brain. The present study sought to disrupt ethanol intake (2–10% ethanol v/v) in rats exposed to 220 ppm Pb or filtered water during gestation and lactation. Thus, to block brain CAT expression, a lentiviral vector coding for a shRNA against CAT (LV-antiCAT vector) was microinfused in the posterior ventral tegmental area (pVTA) either at the onset or towards the end of a chronic voluntary ethanol consumption test. At the end of the study, rats were euthanized and pVTA dissected to measure CAT expression by Western blot. The LV-antiCAT vector administration not only reversed, but also prevented the emergence of the elevated ethanol intake reported in the perinatally Pb-exposed animals, changes that were supported by a significant reduction in CAT expression in the pVTA. These results provide further evidence of the crucial role of this enzyme in the reinforcing properties of ethanol and in the impact of the perinatal Pb programming to challenging events later in life.

1. Introduction

Although most of its industrial applications have been banned or are strictly regulated, lead (Pb) is still considered a public health concern due to its persistence in the environment globally. Several evidences indicate that developmental Pb exposure induces cognitive impairment, behavioral disturbances, and an altered response to drugs, including ethanol, both in children and laboratory animals (Mason et al., 2014; Virgolini et al., 2017). The stimulant and/or motivational responses to ethanol have been attributed to acetaldehyde (ACD), a metabolite generated in the brain primarily by the action of catalase (CAT), an enzyme of minor importance in liver ethanol metabolism (Aragon et al., 1992; Gill et al., 1992). In addition to hydrogen peroxide (H_2O_2) metabolism, the CAT-H_2O_2 complex can also promote the reaction of H_2O_2 with hydrogen donors, such as low molecular weight alcohols through a peroxidation reaction, constituting a major metabolic step in brain ethanol oxidation (Vetrano et al., 2005). It is noteworthy that recent reports indicate that the fraction of ACD generated in the central nervous system is in fact reinforcing (Correa et al., 2012; Peana and Acquas, 2013). Indeed, ACD is self-administered in the ventral tegmental area (VTA), a limbic region related to the reward circuit (Rodd-Henricks et al., 2002), and the self-administration is accompanied by an increase in dopaminergic activity in that region (Foddai et al., 2004). Since centrally-formed ACD acts as a positive reinforcer, this affords new perspectives for interventions that focus on reduction of brain ACD by pharmacologic or genetic means to alter central ethanol metabolism.

Several reports indicate that the pharmacological inhibition of the enzyme CAT with 3-amino 1,2,4-triazole (AT), reduces ethanol-induced locomotor activity and brain CAT activity levels after systemically (Escarabajal et al., 2000) or intra hypothalamic (Pastor and Aragon, 2008) ethanol administration. A reduction in both parameters was also reported when ethanol consumption was evaluated in rats (Aragon and Amit, 1992) or mice (Koechling and Amit, 1994). Interestingly, increased CAT activity has been reported in acutely (Correa et al., 1999, 2000, 2001) or developmentally Pb-exposed animals (Mattalloni et al., 2013). The first report of an interaction among Pb, CAT, AT and ethanol

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showed that AT was able to revert the increase in ethanol-induced hyperlocomotion and brain CAT activity observed upon acute Pb administration (Correa et al., 2001). Similarly, we have reported that AT pretreatment prevented both the elevated ethanol intake and elevated blood CAT activity in developmentally Pb-exposed animals (Mattalloni et al., 2013). However, while the results are robust, a major limitation of these studies is the unspecific action of the drug. An excellent alternative for overcoming this limitation is the administration of viral vectors encoding for genes allowing to increase or silence the expression of specific proteins in discrete regions of the brain. The use of gene therapy was proposed as an alternative to traditional systemic drugs such as the ALDH blockers, disulfiram and cyanamide, which elicit aversive effects as a consequence of peripheral ACD accumulation (Lee et al., 2012). These adverse effects were also observed in Pb-exposed animals after systemic cyanamide administration. Oppositely, when brain ALDH2 was inhibited by i.c.v. cyanamide administration an increased ethanol intake was observed in the Pb-exposed animals compared to their control counterparts (Mattalloni et al., 2017). Overall, this study provided further evidence of the compartmentalized and contrary effects of central and peripheral ACD reported in different experimental models of excessive ethanol intake. In agreement, Karahanian et al. (2015) administered an ALDH-coding lentiviral vector in the posterior VTA (pVTA), a key region for the reinforcing effects of drugs of abuse and observed a diminished ethanol intake in naïve ethanol-preferring UChB rats. The same authors observed a similar reduction in ethanol intake after the administration of a lentiviral vector coding for a shRNA against CAT into the pVTA of high ethanol-consumingUChB rats (Karahanian et al., 2011).

Based on this evidence, we sought to silence the CAT gene in developmentally Pb-exposed rats by administering an anti-CAT lentiviral vector in an attempt to provide additional mechanistic evidence on the role of CAT (and central ACD) in the enhanced motivational effects of ethanol observed in animals developmentally exposed to an environmental neurotoxicant.

2. Methods

2.1. Generation of lentiviral vectors

Plasmid pLKO.1-shRNAantiCAT (The RNAi Consortium, Broad Institute of MIT and Harvard TRCN0000120679) which encodes a shRNA for silencing gene expression of mouse CAT, was used for generating the anti-CAT vector (LV-antiCAT). Its empty backbone, plasmid pLKO.1, was used for generating the control lentivector (LV-control). Either shuttle vector plus pDelta8.9 packaging vector and pVSV-G envelope vector were co-transfected into HEK 293 T cells at a 3:2:1 ratio (12 µg total DNA) with 1 µmol polyethyleneimine. Transfections were carried for 6 h in high-glucose DMEM medium (Gibco, Waltham, Massachusetts, USA) supplemented with 2 g/l sodium bicarbonate. Next, 10% fetal bovine serum was added, and cells were left undisturbed at 37 °C, 5% CO_2 for 48 h. The cell culture supernatant was then collected, filtered through a 0.45 µM polyethersulfone membrane and incubated overnight at 4 °C with 10% (v/v) polyethylene glycol 8000 (PEG-8000; Sigma-Aldrich, St Louis, MO, USA) and 0,3 M NaCl (Merck, KGaA, Darmstadt, Germany) on a rocking platform. The following morning the viral particles were precipitated by centrifugation 20 min at 3600 x g, 4 °C and resuspended in phosphate buffered saline (PBS) with the addition of 1% albumin serum (BSA). The viral titers of both lentivectors were determined with the qPCR Lentivirus Titration Kit (Applied Biological Materials (abm) Inc., Vancouver, Canada) according to the manufacturer's instructions.

2.2. Animals and perinatal treatment

Adult female Wistar rats (250–300 g) born and bred at the Facultad de Ciencias Químicas vivarium (IFEC-CONICET, Córdoba, Argentina) were separated in two a control group (C), which received filtered tap water; or a lead group (Pb) which received a 220 ppm Pb solution (0.4 g/l lead acetate in filtered tap water; Mallinckrodt, J.T. Baker; Argentina). On the same day they were mated following a 2 female/1 male index. The rats remained during the pregnancy and lactation periods in a 12 h dark/light cycle with lights on at 07:00 a.m. under constant temperature and humidity with unlimited access to a standard diet (Purina chow, Batistella, Argentina) and the respective fluid. Within 24 h from the birth of the pups, designed as postnatal day 1 (PND1), litters were culled to eight pups, maintaining all males whenever possible but at least one female in each litter. Pb exposure continued until weaning (PND25) and tests started ten days later during the peri adolescence, a period of high vulnerability to drug addiction (Smith, 2003). The Pb exposure protocol was selected based on previous results in which we reported blood Pb levels of 6.51 ± 0.26 µg/dl in the 35-day-old pups. Animal manipulations and all procedures were in accordance with the NIH Guide for the Care and Use of Laboratory Animals, as approved by the Animal Care and Use Committee of the Facultad de Ciencias Químicas (CICUAL), Universidad Nacional de Córdoba, Argentina (Protocol HCD FCQ-UNC 564/15).

2.3. Ethanol intake protocols

A total of 132 thirty-five-day old (PND35) male pups were housed in pairs with daily access during 2 h (anytime between 9.00 a.m. and 1.00 p.m.) to four tubes, two containing water and the other two increasing concentrations of ethanol (v/v) according to the following scheme: days 1-4: 2%; days 5-8: 4%; days 9-12: 6%; days 13-16: 8%; days 17-32: 10%. Daily intake was registered and expressed as grams of ethanol consumed per kg of body weight, and as percentage of ethanol consumption *versus* water. Intracerebral antiCAT shRNA vector administration took place in different groups at two time-points: before the beginning of the ethanol intake protocol (PND32) or once the differences in ethanol consumption between control and Pb-exposed animals were evident at the 10% concentration (PND55) (Fig. 1). In addition, a subset of each group was injected with a control lentiviral vector in identical conditions and time-points.

2.4. Intracerebral lentiviral vectors administration

All rats were anesthetized with a mixture of 55 mg/kg ketamine chloridrate (Holliday Scott S.A. Argentina) and 11 mg/kg xilazine chloridrate (Richmond, S.A., Argentina) i.p. and mounted into a Stoelting stereotaxic instrument with the incisor bar at −3.3 cm above the interaural line. Using a microinjector, the active lentiviral vector (LV-antiCAT) or the empty vector (LV-control) were administered into the pVTA according to the following coordinates: AP-5.5; ML-0.8; DV 7.2 (Paxinos and Watson, 2009) The injectors were connected to a 10 µl Hamilton micro syringe (Reno, NV, USA) *via* a polyethylene catheter (P10, Becton Dickinson, MD, USA). Two minutes after injector implantations, rats were microinfused as described below. The injector was kept in place for an additional 5 min to allow diffusion through the tissue before its slow removal to prevent the reflux of the solution. The skin was then sutured and animals were transferred in pairs to individual cages with each box considered an experimental unit with free access to water and food.

2.4.1. Experiment 1: Intra pVTA lentiviral vector administration before the onset of the voluntary ethanol intake protocol

In this group of 21 animals (C group = 11; Pb group = 10), 0.5 µl of the corresponding solution (LV-antiCAT 2.34×10^6 viral particles) was infused at a rate of 0.329 µl/min with an infusion pump (MasterFlex, Model 77200-20). The empty lentiviral vector (1.18×10^6 particles in 0.5 µl of solution) was microinfused to 12 animals (C group = 6; Pb group = 6) under identical experimental conditions. All animals were allowed to recover during a 2-day-period before the onset of the

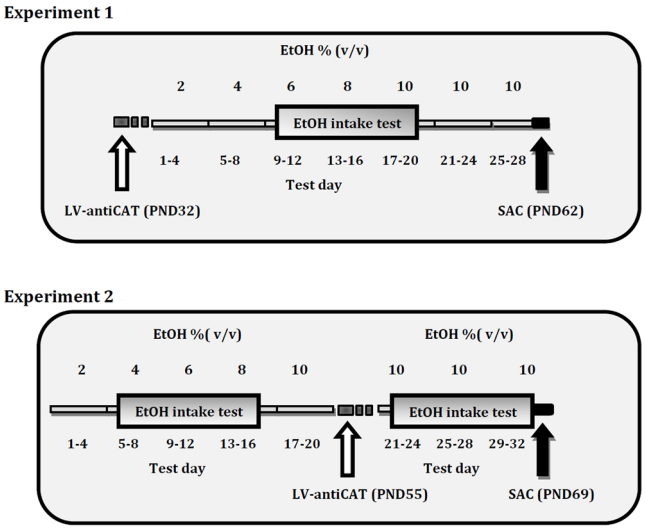

Fig. 1. Timeline for the ethanol intake protocol used in animals assigned to Experiment 1 (top) or Experiment 2 (bottom). The timing for the intra pVTA LV-antiCAT administration is denoted by a white arrow, whereas the time for brain dissection (sacrifice-SAC) is indicated with a black arrow.

voluntary ethanol scheme explained above.

2.4.2. Experiment 2: Intra pVTA lentiviral vector administration after the higher ethanol intake emerged in the Pb-exposed animals as compared to controls

On test day 21 of ethanol consumption (PND55), another group of 21 animals (C group = 11; Pb group = 10) was microinfused with 1 µl of LV-antiCAT (4.68×10^6 viral particles) in a time period of 3 min and 3 s. The empty lentiviral vector was microinfused (2.36×10^6 particles) to 12 animals (C group = 6; Pb group = 6) under identical experimental conditions. After a 2 day-period of recovery, ethanol consumption resumed for an additional 12 days to assess the impact of the silencing of the CAT gene on the already acquired behavior.

2.5. CAT expression

One hour after the last ethanol intake session (test day 28 -PND62- for Experiment 1 or test day 32-PND69- for Experiment 2), all animals were euthanized and their brains rapidly removed. Coronal brain slices (2 mm) containing pVTA as observed by visual inspection were obtained using an acrylic brain matrix (Stoelting CO, USA) on ice. The pVTA side injected with the lentiviral vector from two animals of the same group was pooled and homogenized in RIPA buffer (0.1% Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecylsulphate [SDS], 50 mM Tris, pH 7.4) with protease inhibitors (10 µg/ml aprotinin, 1 µg/ml leupeptin, 1 µg/ml pepstatin A and 100 µg/ml phenylmetylsulfonyl fluoride) and phosphatase inhibitor (1 mM sodium orthovanadate). The contralateral pVTAs were also pooled and analyzed as the non-injected controls. Homogenates were centrifuged at 1000 *g*and 4 °C for 10 min, and aliquots of the supernatant were used for protein quantification using the Bio-Rad Bradford Protein Assay Kit (Hercules, CA, USA). The remainder homogenate was mixed 3:1 with 4X Laemmli buffer (2% SDS, 20% glycerol, 10% mercaptoethanol, 0.01% bromophenol blue, 125 mM Tris, pH 6.8), boiled at 100 °C for 5 min and stored at −80 °C until use. The same procedure was followed to measure basal CAT expression in a 35-day-old and a 63-day-old group of rats, which are considered age-matched groups to the animals at onset of the ethanol intake protocol and at the end of the ethanol consumption, respectively.

Protein samples (20 µg/lane) were separated in 10% SDS-PAGE gels, and subsequently blotted to polyvinylidene fluoride membrane (Merck KGaA, Darmstadt, Germany). Blots were blocked in TBS buffer (20 mM Tris–HCl, pH 7.5, 150 mM NaCl, and 0.1% Tween 20) with 5% powdered low-fat milk for 1 h at room temperature, and then incubated overnight at 4 °C with an antibody that recognized CAT (CAT-peroxisome marker 1:1000, AbCam, CA, USA). Membranes were then probed with a secondary anti-rabbit antibody conjugated to green fluorophore IRDye 800CW (1:25000, LI-COR Biosciences, NE, USA) for 2 h at room temperature and developed using an Odyssey CLx imager scanner. Thereafter, blots were washed 3 times for 15 min with TTBS (TBS + Tween 20), and re probed with anti-actin antibody (1:1000, Santa Cruz Biotechnology, Inc, TX, USA) at 4 °C overnight. The next day the blots were probed with an anti-goat secondary antibody conjugated to the red fluorophore IRDye 680 L T (1:10000, LI-COR Biosciences, NE, USA) for 2 h and developed as previously mentioned. The resulting film samples were scanned and analyzed with an image analysis program (Gel-Pro Analyzer Software). Data is presented as CAT/total actin expression and expressed as mean ± S.E.M.

2.6. Statistical analysis

For statistical comparisons, daily ethanol intake and preference data was averaged in blocks of four days and expressed as grams of ethanol consumed per kg body weight (g/kg) or the ethanol to water preference (%). In the experiment in which the lentiviral vectors were administered before to the voluntary consumption scheme (Experiment 1), data was analyzed by a three-way repeated measures ANOVA contrasting the variables group (control *vs.* Pb), treatment (LV-antiCAT *vs.* LV-control) and time/ethanol concentration as the repeated variable. When the lentiviral vectors were administered on test day 21 of the ethanol consumption scheme, a two-way-repeated-measures analysis of variance (ANOVA) was applied before the administration of the vector,

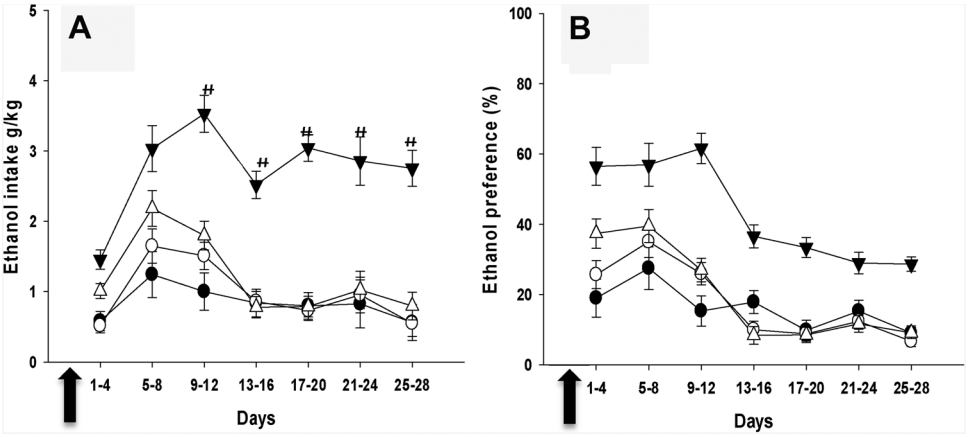

Fig. 2. The intra pVTA LV-antiCAT administration before the onset of the test prevented the emergence of the excesive ethanol intake evidenced in the Pb-LV group. C = control; Pb = Pb-exposed. Group symbols: ▼Pb-LV-control (n = 6); ∇ Pb-LV-antiCAT (n = 10); ● C-LV-control (n = 6); ○ C-LV-antiCAT (n = 11). A–B. Voluntary ethanol intake expressed as g ethanol/kg body weight (A) and percentage of preference ethanol/water (B). Data (mean ± SE) grouped in 4-day blocks along the horizontal axis correspond to ethanol intake in response to increasing ethanol concentrations (days 1-4: 2%; days 5-8: 4%; days 9-12: 6%; days 13-16: 8%, and days 17-32: 10%). The arrow indicates the lentiviral vector administration. *denotes significant differences between groups (C *vs.* Pb) at $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$. $^{\#}$indicates differences between treatments (LV-control *vs.* LV-antiCAT) at $^{\#}p < 0.05$, and $^{\#\#}p < 0.01$.

contrasting each group with the ethanol concentration/time. After the vector was administered, a three-way-repeated measures ANOVA was used to compare groups, treatment, and time/ethanol concentration as the repeated factor. In all cases, when a significant interaction was found ($p < 0.05$), a Tuckey test was performed as a *pos hoc* analysis.

Baseline pVTA CAT expression was analyzed separately for each age by a *t* test for independent variables. On the other hand, post lentiviral infusion pVTA CAT levels were analyzed in a separate analysis for each lentiviral administration time by a 2-way ANOVA with group and treatment as the contrasting variables. In all cases, the results are presented as the mean ± SE.

3. Results

3.1. Experiment 1: The intra pVTA LV-antiCAT administration prevented the emergence of the excessive ethanol intake previously reported in Pb-exposed animals

Fig. 2 A and B depict ethanol consumption and preference in control and Pb-exposed animals treated with either LV-antiCAT or LV-control vectors before the ethanol consumption protocol was initiated. The repeated-measured 3-way ANOVA analysis of ethanol intake (panel 2 A) revealed statistical significance for group ($F(1,174) = 96.50$, $p < 0.001$), treatment ($F(1,174) = 42.28$, $p < 0.001$), time/ethanol concentration ($F(6,174) = 15.14$, $p < 0.001$) and significant interactions between group x treatment ($F(1,174) = 59.39$, $p < 0.001$), treatment/ethanol concentration x drug ($F(1,174) = 2.36$, $p < 0.05$), and a significant overall interaction (group x treatment x time/ethanol concentration) ($F(6,174) = 2.363$, $p < 0.05$). A Tuckey *pos hoc* test demonstrated that starting at the 6% ethanol concentration (9–12 days period) the Pb-exposed animals administered with the control vector were different from the respective group injected with the lentiviral vector, revealing the effect of the LV-antiCAT administration in preventing the development of the elevated ethanol intake previously reported in these animals (Mattalloni et al., 2013, 2017). Additionally, the LV-antiCAT administration failed to reduce the ethanol consumption in the control group, probably as a consequence of the low overall ethanol consumption inherent to these animals.

With respect to the ethanol preference (panel 2B), the 3-way-repeated measures ANOVA showed a significant effect of group ($F(1,174) = 77.86$, $p < 0.001$), treatment ($F(1,174) = 41.55$, $p < 0.001$) and time/ethanol concentration ($F(6,174) = 38.86$, $p < 0.001$), a significant interaction of group x treatment ($F(1,174) = 54.28$, $p < 0.001$) and time/ethanol concentration x group ($F(6,174) = 4.31$, $p < 0.001$) as well as an almost significant overall interaction (group x treatment x time/ethanol concentration: $F(6,174) = 2.09$, $p = 0.06$). The Tuckey *pos hoc* test for the group x treatment interaction revealed a $p < 0.001$ significance among the ethanol preference of the Pb-treated animals injected with control vector and all other groups, demonstrating the selective effect of the LV-antiCAT in these animals.

3.2. Experiment 2: The intra pVTA LV-antiCAT administration reversed the installed excessive ethanol intake in the Pb-exposed animals

Fig. 3 depicts ethanol consumption (panel A) and preference (panel B) in control and Pb-exposed animals treated with either LV-antiCAT or LV-control vectors once the differences among groups were evident (test day 21, ethanol concentration = 10%). With respect to baseline ethanol intake (panel 3 A, test days 1–20), the 2-way-repeated measures ANOVA revealed a significant effect of group ($F(1,124) = 203.91$, $p < 0.001$), time/ethanol concentration ($F(4,124) = 16.88$, $p < 0.001$), and their interaction (group x time/ethanol concentration: $F(6,174) = 4.76$, $p < 0.01$). The Tuckey *pos hoc* test revealed differences between groups starting at the 4% ethanol concentration ($p < 0.01$ in all cases). Once the lentiviral vectors were administered, the 3-way-repeated measures ANOVA analysis for test days 21–32 revealed a significant effect of group ($F(1,58) = 37.09$, $p < 0.001$) and treatment ($F(1,58) = 97.20$, $p < 0.001$), a significant interaction of group x treatment ($F(1,58) = 24.70$, $p < 0.001$), time/ethanol concentration x drug ($F(2,58) = 4.46$, $p < 0.05$) and time/ethanol concentration x treatment ($F(2,58) = 6.42$, $p < 0.01$), although there was no overall significant interaction (group x treatment x time/ethanol concentration: $p = 0.43$). The 2-way-repeated measures ANOVA analysis of the preference for ethanol (panel 3B, test days 1–20) revealed a significant effect of group ($F(1,124) = 56.43$, $p < 0.001$) and time/ ethanol concentration ($F(4,124) = 19.53$, $p < 0.001$), although a non-significant interaction between both variables was present ($p = 0.58$). After lentiviral administration, the 3-way-repeated measures ANOVA for test days 21–32 evidenced a significant effect of group ($F(1,58) = 32.91$, $p < 0.001$), treatment ($F(1,58) = 91.07$, $p < 0.001$), a significant interaction of group x treatment ($F(1,58) = 39.22$, $p < 0.001$), and time/ ethanol concentration x group ($F(2,58) = 3.67$, $p < 0.05$), although there was no overall significant interaction (group x treatment x time/ ethanol concentration: $p = 0.32$). The Tuckey *pos hoc* test revealed that the Pb-exposed animals administered with vehicle showed higher preference for ethanol from all the other groups at all times ($p < 0.001$). In summary, the LV-antiCAT administration elicited a reduction in ethanol intake and preference selectively in the Pb-exposed animals.

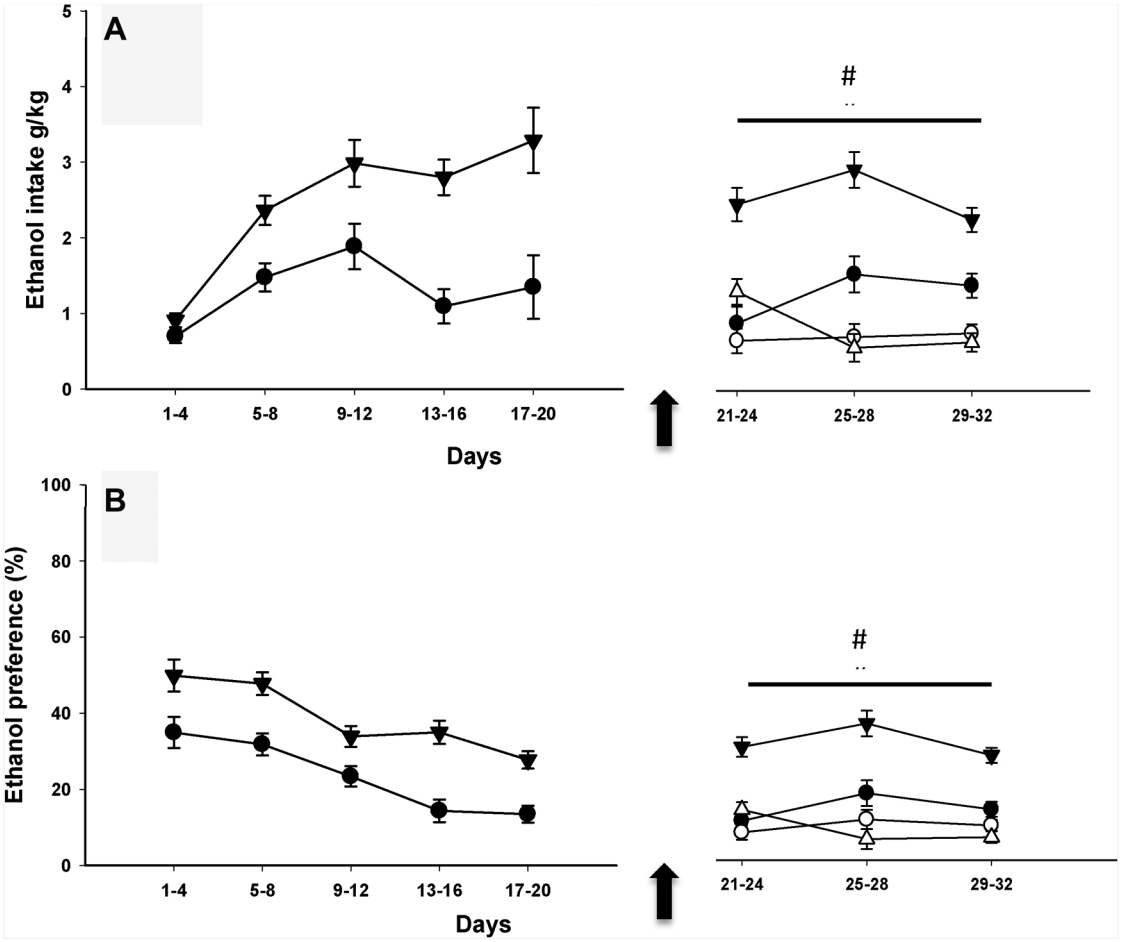

Fig. 3. The intra pVTA LV-antiCAT administration after stable ethanol intake was achieved reversed the excesive consumption of the drug evidenced in the Pb-exposed animals. C = control; Pb = Pb-exposed. Group symbols: ▼ Pb-LV-control n = 6); ∇ Pb-LV-antiCAT (n = 10); ● C-LV-control (n = 6); ○ C-LV-antiCAT (= 11). A–B. Voluntary ethanol intake expressed as g ethanol/kg body weight (A) and percentage of preference ethanol/water (B). Data (mean ± SE) grouped in 4-day blocks along the horizontal axis correspond to ethanol intake in response to increasing ethanol concentrations (days 1-4: 2%; days 5-8: 4%; days 9-12: 6%; days 13-16: 8%, and days 17-32: 10%). The arrow indicates the lentiviral vector administration. *denotes significant differences between groups (C *vs.* Pb) at $^{***}p < 0.001$; $^{\#}$indicates differences between treatments (LV-control *vs.* LV-antiCAT) at $^{\#\#\#}p < 0.001$.

3.3. Catalase expression

3.3.1. Basal CAT levels

One-way ANOVA analysis of the data presented in Table 1 revealed no significant differences in pVTA basal CAT expression between the naïve control and Pb-exposed groups neither for the 35-day-old ($p = 0.06$) nor for the 63-day-old rats ($p = 0.10$).

3.3.2. Experiment 1

The results of pVTA CAT expression in the animals that were treated with the LV-antiCAT vector before the onset of the voluntary ethanol intake protocol are plotted in Fig. 4A. The analysis of the data by a 2-way ANOVA revealed no significant differences for group effect ($p = 0.52$) or group x lentiviral vector interaction $p = 0.36$. As expected, a significant reduction on CAT expression resultant of the lentiviral vector administration was present ($F(1,15) = 14.10$, $p < 0.05$).

3.3.3. Experiment 2

Fig. 4B depicts the results of pVTA CAT expression in rats treated with the LV-antiCAT vector after the higher ethanol consumption was evident in the Pb-exposed animals. The analysis of the data by a 2-way ANOVA revealed no significant differences in the group variable $p = 0.61$ or in the group x lentiviral vector interaction $p = 0.83$. As expected, a significant effect of the lentiviral vector administration was evident ($F(1,16) = 6.51$, $p < 0.05$), corroborating a reduction in CAT expression after LV-antiCAT administration.

Table 1
Baseline catalase expression levels (pVTA CAT/actin) in peri adolescent and young adult rats. Between parenthesis is indicated the number of animals for each group.

Group	Control	Pb-exposed
35-day-old	1.05 ± 0.17 (5)	0.76 ± 0.14 (7)
63-day-old	1.19 ± 0.14 (8)	0.77 ± 0.14 (8)

4. Discussion

The present study establishes that an intra pVTA antiCAT lentiviral vector administration, not only reversed, but also prevented the increased ethanol intake previously reported in perinatally Pb-exposed animals (Mattalloni et al., 2013, 2017). Importantly, these results provide further evidence on the role of CAT (and presumably central ACD) in this behavior, with implications for the neurobiological basis of both, the reinforcing properties of ethanol and the impact of the cellular programming of early-life Pb exposure.

As mentioned before, most of ethanol -derived brain ACD formation is dependent upon the CAT-H_2O_2 peroxidase activity (Zimatkin and

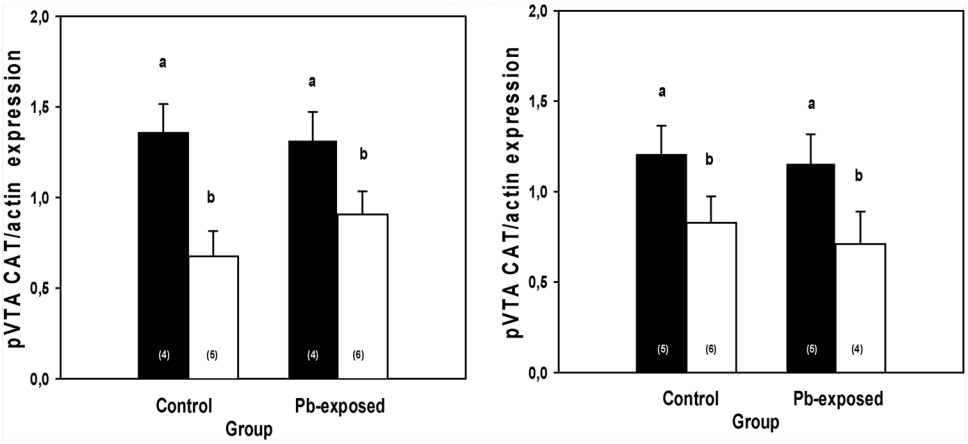

Fig. 4. Catalase expression (pVTA CAT/actin) in animals administered before the onset of the voluntary ethanol intake protocol (A) or once the higher ethanol intake emerged in the Pb-exposed animals (B). ≡LV-control; LV-antiCAT. Indicated between parentheses is the number of animals per group. Different letters indicate significance between the V and L-administered animals.

Buben, 2007), an enzyme that although present throughout the brain (Schad et al., 2003) is particularly abundant in monoaminergic cell body-rich areas (Moreno et al., 1995; Zimatkin and Lindros, 1996). Coincidentally, the VTA projects to the nucleus accumbens (NAc), conforming the mesolimbic dopaminergic circuit which is considered to be the main site of action for drugs of abuse. In particular, ethanol modulates the opioid system by β-endorphins release, resulting in inhibition of the GABAergic tone with consequential enhanced dopamine release in this system (Heilig et al., 2011). Given that VTA has also been considered a key area for brain ACD pharmacological effects (Orrico et al., 2013), and that dopamine and ACD can associate forming the tetrahydroisoquinoline salsolinol, it is possible that ethanol´s reinforcing effects may be mediated by salsolinol through its opioid agonistic properties (Deehan et al., 2013; Melis et al., 2015), an scenario that requires further discussion about the role of *in vivo* salsolinol formation (Collins et al., 1990; Lee et al., 2010). In addition, ACD neurotoxicity is another aspect that should not be neglected provided the capability of this metabolite to form adducts with brain proteins or nucleotides (Yu et al., 2010). Another consideration is related to ACD generation of an oxidative environment in the brain, a process mediated by the enzyme cytochrome P450 2E1 (CYP2E1), a secondary pathway for central ACD formation although a major source of reactive oxygen species resultant of ethanol metabolism (Koop, 2006). Although these scenarios are beyond the scope of this study, they reinforce the need for further consideration on the role played by ACD and bioproducts in ethanol´s motivational and neurotoxic effects.

We observed a reduction in ethanol consumption in both naïve (Fig. 2) and habituated (Fig. 3) to chronic ethanol intake Pb-exposed groups, suggesting that CAT functionality is crucial for the higher ethanol intake reported in Pb-exposed rats. Thus, our data corroborate earlier reports in which the intra-VTA antiCAT vector administration markedly reduced ethanol intake in naïve UChB rats (Karahanian et al., 2011). In contrast, it has been shown that the administration of an antiCAT vector into the VTA of naïve animals blocked reward and alcohol self-administration, yet it was ineffective in inhibiting alcohol self-administration in rats that had been conditioned to ingest ethanol for over 2 months (Quintanilla et al., 2012). These differences may be related to the voluntary and shorter duration characteristics of our experimental protocol in comparison with those used in the above cited studies: Karahanian et al., 2011; Quintanilla et al., 2012.

Regarding the pVTA basal CAT expression, we observed similar enzymatic levels in the control and the Pb-exposed animals that have not consumed ethanol, both at young and adult ages (Table 1), as well as in both groups of animals exposed to voluntary ethanol intake and treated with the control lentiviral vector. As expected, LV-antiCAT administration reduced pVTA CAT levels in both the animals injected before the initiation of the experimental ethanol protocol, and in the group treated after stable ethanol intake (Fig. 4A and B). This reduction is in agreement with reports from Quintanilla et al. (2012), who demonstrated that intraVTA ACD levels were reduced for as long as 30 days after the lentiviral administration, a result also corroborated by Karahanian et al., 2011. Although a higher expression of the enzyme's mRNA or increased protein level does not necessarily result in increased activity, the similar expression in CAT levels in the control and Pb-exposed rats parallels the absence of differences in whole brain CAT activity levels reported in developmentally-Pb-exposed animals *versus* controls (Mattalloni et al., 2017; Valenzuela, 1989; Somashekaraiah et al., 1992), oppositely to adult Pb exposure that elicits brain CAT inhibition (Flora et al., 2012). However, when discrete brain areas were evaluated, we reported higher CAT activity accompanied by elevated blood CAT activity as a consequence of perinatally Pb-exposure (Mattalloni et al., 2013). Thus, it is possible the existence of area-related differences in the enzyme functionality, a scenario that deserves further studies in the Pb-exposed animals.

Finally, the results shown here recapitulate our previous findings that early-life exposure to Pb enhances ethanol intake in adolescent animals, an effect that we postulate is associated to changes in CAT activity. This assertion acquires relevance in the framework of several theories related to the enduring consequences of early-life environment, including the developmental origins of health and disease hypotheses (Gluckman et al., 2007), the Barker's hypothesis (Barker et al., 1989), and the recent "LEARn" model (Latent Early Associated Regulation; Lahiri et al., 2009), which combines genetic and environmental risk factors to explain the etiology of neurobiological disorders. Thus, and concerning Pb safety, the US EPA concluded that "the developing organism is of greatest risk, there is no evident threshold that has been found for lead's effects on the nervous system, and behavioral impairments of developmental exposures persist into childhood and adulthood" (Caito and Aschner, 2017). In this case, we assume that the initial hit (perinatal Pb exposure) is responsible for the increased vulnerability to the excessive ethanol consumption evidenced later in life. This assertion acquires relevance in the context of the low Pb blood levels we reported in the Pb-exposed animals at the onset of these experiment, levels that are slightly above the Pb-blood concentration advised for children by the CDC (US Centers for Disease Control and Prevention).

In summary, the present study establishes a key component in the Pb/ethanol interaction, ascribing a critical role to CAT in the heightened motivational properties of ethanol previously reported in developmentally Pb-exposed rats. In addition, and although these results provide mechanistic support for genetic approaches as an alternative therapy for the treatment alcoholism, further effort should continue to balance the peripheral *vs.* the central administration of these vectors (see Israel et al., 2013) in an attempt to implement these strategies to treat alcoholism in humans. Finally, efforts should continue to monitor the perinatal factors that may modify the environment of immature organisms that lead to adverse consequences later in life.

Conflicts of interest

The authors declare that there is no conflict of interest associated with this study.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.neuro.2018.10.010.

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