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Brain ethanol-metabolizing enzymes are differentially expressed in lead-exposed animals after voluntary ethanol consumption: Pharmacological approaches

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ABSTRACT

Developmentally-lead (Pb)-exposed rats showed an enhanced vulnerability to the stimulating and motivational effects of ethanol (EtOH). This is accompanied by differential activity of the brain EtOH-metabolizing enzymes catalase (CAT) and mitochondrial aldehyde dehydrogenase (ALDH2). Based on the theory that brain acetaldehyde accumulation is associated with the reinforcing properties of EtOH, this study sought to determine brain CAT and ALDH2 expression in limbic areas of control and Pb-exposed animals after voluntary EtOH intake. Thirty-five-day-old rats perinatally exposed to 220 ppm Pb were offered with water or increasing EtOH solutions (2–10% v/v) during 28 days until postnatal day (PND) 63. Once intake was stable, the animals were administered: 1) saline (SAL; test days 21–24 or 21–28, as corresponds), or 2) a CAT inhibitor: 3-amine 1, 2, 4-triazole (AT; 250 mg/kg intraperitoneally [i.p.], 5 h before the last eight EtOH intake sessions -test days 21–24 and 25–28), or 3) a CAT booster: 3-nitropropionic acid (3NPA; 20 mg/kg subcutaneously [s.c.], 45 min before the last four EtOH intake sessions -test days 25–28). Two additional groups were centrally-administered cyanamide (CY, an ALDH2 inhibitor, 0.3 mg i.c.v. immediately before the last four EtOH sessions, test days 25–28) or its corresponding vehicle (VEH). Lead exposure increased EtOH intake, an effect potentiated in both groups by 3NPA or CY pretreatments and reduced by AT, albeit selectivity in the Pb group. Catalase abundance in limbic areas parallels these observations in the Pb group, showing higher CAT expression in all areas after EtOH consumption respect to the controls, an effect prevented by AT administration. In contrast, ALDH2 expression was reduced in the Pb animals after EtOH intake, with CY potentiating this effect in all brain areas under study. Based on these results and on previous evidences, we suggest that Pb exposure promotes acetaldehyde accumulation in limbic regions, providing some insights into the mechanism of action that underlies the vulnerability to the excessive EtOH consumption reported in these animals.

1. Introduction

The developmental neurotoxicant lead (Pb) is a non-essential metal that has been extensively used in the past. Although several industrial applications have been banned or restricted, neurobehavioral alterations are still reported in environmentally-Pb-exposed children. Experimental evidences in rodents indicate an enhanced vulnerability to the stimulating and motivational effects of ethanol (EtOH) as result of Pb exposure (Nation et al., 1986; Correa et al., 1999a; Virgolini et al., 2017). Since brain acetaldehyde accumulation is considered a key component of these responses (Aragon and Amit, 1985; Quertemont et al., 2005; Israel et al., 2015), EtOH metabolism emerged as an important aspect to consider when evaluating the mechanism that underlies the Pb-EtOH interaction.

Catalase (CAT) is an antioxidant enzyme that represents the major metabolic pathway of brain EtOH oxidation to acetaldehyde. This process requires H_2O_2 as a co-factor (Cohen et al., 1980; Aragon et al., 1992; Zimatkin and Buben, 2007), and plays a key role in EtOH pharmacological effects (Quertemont et al., 2005; Correa et al., 2012; Peana et al., 2017). Pb itself increases both, CAT activity and several EtOH responses when administered either acutely to adult animals (Correa et al., 1999b), or chronically to developmental rats (Valenzuela et al.,

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1989; Mattalloni et al., 2013) or chickens (Somashekaraiah et al., 1992). Interestingly, we have demonstrated that pharmacological (Mattalloni et al., 2013) or molecular (Mattalloni et al., 2019) manipulations of this enzyme have a direct impact on CAT activity and voluntary EtOH intake in developmentally Pb-exposed rats (reviewed in Virgolini et al., 2017). Similarly, CAT inhibition with specific antagonists such as 3-amino 1,2,4-triazole (AT) or sodium azide reduced both EtOH-induced locomotor activity (Sanchis-Segura et al., 1999; Correa et al., 2001) and the drug's anxiolytic responses (Correa et al., 2008). A comparable effect emerged when EtOH consumption was evaluated after pharmacological CAT blockade (Aragon and Amit, 1992; Tampier et al., 1995; Mattalloni et al., 2013). Moreover, an shRNA antiCAT vector abolished EtOH intake in the UChB rats, a strain which prefers EtOH (Karahanian et al., 2011). Conversely, CAT activation with 3-nitropropionic acid (3NPA) enhanced EtOH-induced locomotion (Manrique et al., 2006) and increased EtOH consumption (Mattalloni et al., 2013) in rodents.. Regarding its localization, CAT is an enzyme present in the brain, liver, and kidney, intracellularly in the peroxisomes of rats (Moreno et al., 1995; Zimatkin and Lindros, 1996) and humans (Houdou et al., 1991). Although low compared to liver, brain CAT expression has been reported in both neurons and glia, with preferential immunoreactivity in the aminergic system, providing a functional relevance to the presence of the enzyme in the brain (Moreno et al., 1995; Zimatkin and Lindros, 1996). These evidences contrast with the low expression of the other two enzymes that participate in minor pathways of brain EtOH metabolism. They are CYP2E1, which is localized preferably in cortical, striatal and hippocampal regions (Hansson et al., 1990) and ADH, present in neurons of the mammillary bodies, periaqueductal gray, and the cerebral and cerebellar cortices of normal adult rats (Kerr et al., 1989).

On the other hand, the next step of EtOH metabolism involves brain (and liver) acetaldehyde oxidation to acetic acid by the ALDH superfamily of enzymes. Of particular interest is the cytosolic ALDH1A1 and the mitochondrial ALDH2, with the latter having the highest affinity for acetaldehyde (Km < 1 μM) (Zimatkin et al., 2006; Marchitti et al., 2008). They are ubiquitously distributed in the rat brain, showing robust immunoreactivity in the extranuclear areas of the neuronal and glial cell bodies, accompanied by lower staining in the terminals and in aminergic neurons (Zimatkin et al., 1992). Interestingly, it has been reported that brain (rather than liver) ALDH2 activity closely correlates with EtOH preference (Amir, 1977). Moreover, low-doses of centrally-administered cyanamide (CY), an ALDH antagonist, enhanced subsequent EtOH intake (Critcher and Myers, 1987), and adding further support to the postulate that brain acetaldehyde is a metabolite involved in the positive reinforcing properties of EtOH (Aragon and Amit, 1985; Quertemont et al., 2005; Israel et al., 2015). In this regard, we have recently demonstrated that CY increased EtOH intake by reducing brain mitochondrial ALDH2 activity in rodents when the drug was administered directly in the brain. Interestingly, basal brain ALDH2 activity was lower in the Pb-exposed animals which had consumed EtOH compared to their control counterparts (Mattalloni et al., 2017), suggesting that central acetaldehyde accumulation make these animals more vulnerable to the development of excessive EtOH intake (reviewed in Virgolini et al., 2017).

Thus, on the basis of the critical role ascribed to CAT and ALDH2 in brain acetaldehyde accumulation, and their differential activity in the Pb-exposed rats, we aimed here to evaluate brain CAT and ALDH2 expression resulting of EtOH consumption in basal conditions and after pharmacological modulation of these two enzymes.

2. Material and methods

2.1. Animals and perinatal treatment

Adult male and female Wistar rats born and bred in our vivarium (IFEC − CONICET, Córdoba, Argentina) housed in a ratio of 1 male/2 females per cage were maintained with a standard diet (Purina chow, Batistella, Argentina) *ad libitum* in a 12 h dark/light cycle (lights on at 07:00 a.m.) under constant conditions of temperature and humidity. They were randomly allocated to one of two subsets according to the fluid source: control group (designated as C) which received filtered tap water; or lead group (designated as Pb), offered with a 220 ppm Pb solution (0.4 g/l lead acetate; Mallinckrodt, J.T. Baker; Argentina). It should be noted that the tap water contains less than 5.0 μg/l Pb as indicated by the filter company (Water Systems, Pure Air Technology, Argentina). Daily fluid intake measurement demonstrates the absence of differences in liquid consumption as a result of the presence of Pb acetate in the solution. The dam's weight was registered once a week to determine weight gain during pregnancy, and discard any treatment-related effects in the nutritional status. Within 24 h of delivery (designated as postnatal day 1: PND1), the number of male and female pups was counted and litters culled to eight pups, keeping all males whenever possible, though one female was always present in each litter. Pups were weighed once a week until weaning at PND25 when Pb exposure was interrupted by replacing the Pb solution for filtered tap water as the only source of beverage. Tests started at PND35, a time considered periadolescence in rats (Sengupta, 2013), and a highly risky period for the initiation of drug addiction (Spear, 2000), and continued until early adulthood at PND63. One male per litter was allocated to each experimental condition as suggested by Maurissen (2010), except for EtOH intake tests where 2 pups from the same litter were housed together and considered a single case. Efforts were made to minimize pain and suffering and to reduce the number of animals used. 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).

2.2. Ethanol consumption and drug treatment

The day before the start of the EtOH/water free-choice test, a random subset of 35-day-old male pups (Group PND35) was weighed and later sacrificed to obtain brain tissue as detailed below. Other groups of rats of the same age and gender were weighed, housed in pairs, and water-restricted for the 24 h prior to the initiation of the EtOH intake protocol. Access to fluids was scheduled to be limited to 2 h per day between 10:00 a.m. and 4:00 p.m. to model "binge drinking", which is a more realistic approach of alcohol consumption patterns in adolescents. Thus, starting on PND35, these animals had access for 28 days to four graduated tubes containing either water (two tubes) or escalating concentrations of EtOH (two tubes) according to the following scheme (expressed in v/v): days 1-4: 2%; days 5-8: 4%; days 9-12: 6%; days 13-16: 8%; days 17-28: 10% EtOH. This strategy consisted in the presentation of EtOH at a relatively low concentration followed by the gradual increase of its content over days to acclimatize the animals to the naturally aversive taste of the drug. During the whole test, rats were weighed and both EtOH and water intake registered daily to be expressed as grams of EtOH per kilogram of body weight.

Once liquid intake was stabilized at the 10% EtOH concentration, a group of rats were daily injected with 1) saline (SAL; test days 21–24 or 21–28, as corresponds), or 2) a CAT inhibitor: 3-amine 1, 2, 4-triazole (AT; 250 mg/kg dissolved in 0.9% SAL and administered intraperitoneally [i.p.], 5 h before the last eight EtOH intake sessions -test days 21–24 and 25–28), or 3) a CAT booster: 3 nitropropionic acid (3NPA; 20 mg/kg dissolved in 0.9% SAL and administered subcutaneously [s.c.], 45 min before the last four EtOH intake sessions -test days 25–28). The timing, route of administration and dosing of these drugs were based on available literature (Aragon et al., 1991; Binienda et al., 1998) and on previous data published by us (Mattalloni et al., 2013). At the same time, in a fourth group cyanamide (CY, a long-lasting ALDH2 inhibitor that does not cross the blood-brain-barrier) was microinfused intracerebroventricularly (i.c.v.) in a dose of 0.3 mg

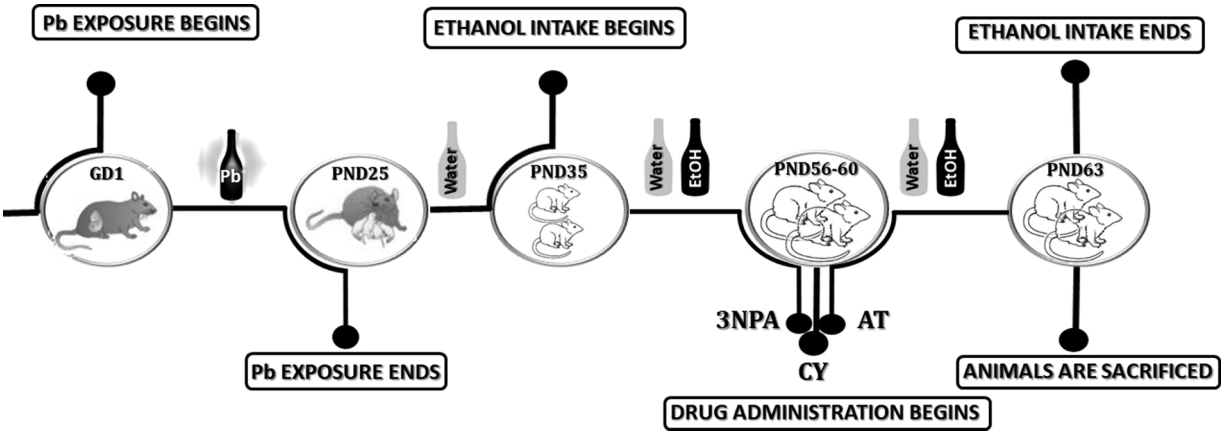

Fig. 1. Experimental timeline. The figure is a representative sequence of the protocol applied to the group of animals perinatally exposed to Pb (Pb groups) which underwent the EtOH/water free choice test (EtOH groups) and were later administered one of the drugs under study (AT, 3NPA or CY) to be finally sacrificed. Other groups not depicted here include the animals not exposed to Pb (C), those which were sacrificed at PND35, those injected with SAL or VEH, and the rats which did not consume EtOH (non-EtOH groups). GD = gestational day; PND = postnatal day; EtOH = ethanol; C = control; Pb = lead-exposed; SAL = saline; AT = 3-amino 1,2,4-triazole; 3NPA = 3-nitropropionic acid; VEH = vehicle; CY = cyanamide. Further details are provided in the main text of the manuscript.

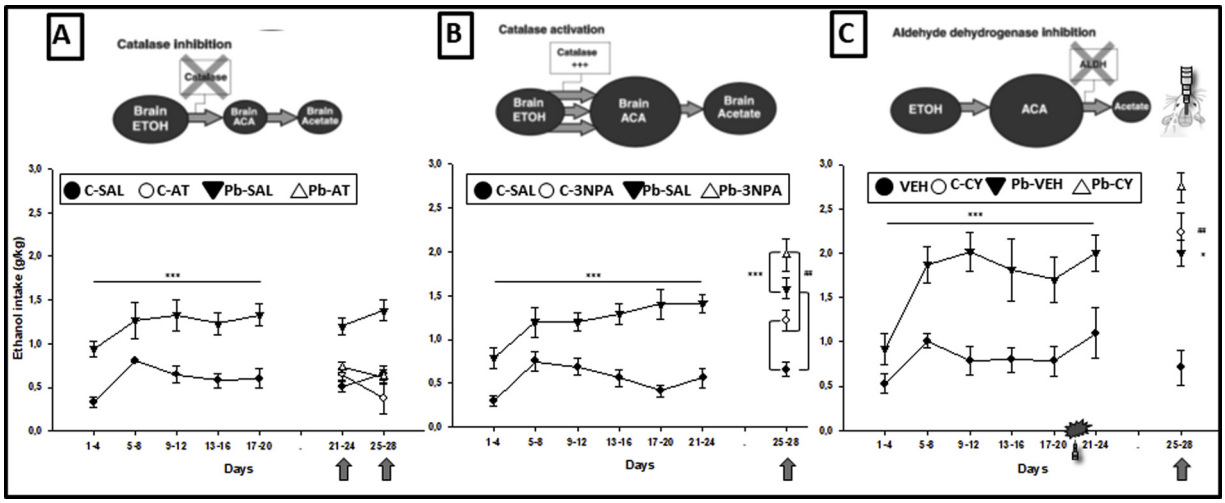

Fig. 2. Voluntary ethanol (EtOH) intake expressed as grams of EtOH per kilogram of body weight in response to AT (panel A), 3NPA (panel B) or CY (panel C) administration. At the top of each graph is depicted the EtOH metabolism after pharmacological manipulations (taken from Quertemont and Didone, 2006). Data (mean ± SE) grouped in 4-day blocks along the horizontal axis correspond to EtOH intake in response to increasing EtOH concentrations (days 1 to 4: 2%; days 5 to 8: 4%; days 9 to 12: 6%; days 13 to 16: 8%; and days 17 to 28: 10% v/v). C = control; Pb = lead-exposed; SAL = saline; AT = 3-amino 1,2,4-triazole; 3NPA = 3-nitropropionic acid; VEH = vehicle; CY = cyanamide. n = 5 animals for each group. *Panel A:* *indicates differences respect to the C animals at $^{***}p < 0.001$. *Panel B:* *indicates differences respect to the C animals at $^{***}p < 0.001$. *Panel C:* The symbol indicates the time of surgery. *indicates differences respect to the C animals at $^{***}p < 0.001$. Once VEH or CY was administered: *indicates significant differences between the C-VEH and Pb-VEH animals at $^{*}p < 0.05$ and $^{\#}$indicates significant differences between the C-VEH and C-CY-treated animals at $^{\#\#}p < 0.01$.

immediately before the last four EtOH sessions (test days 25–28). This administration protocol was based in Deitrich et al., 1976 and used by us in Mattalloni et al., 2017. It should be noted that CY was dissolved in standard Krebs solution and saturated with 95% O_2 and 5% CO_2, which was the solution administered i.c.v. to the fifth group labeled as vehicle (VEH). To perform the surgery on days 20–21 of the free-choice test, rats were anesthetized with ketamine hydrochloride (55 mg/kg) and xylazine (11 mg/kg) to unilaterally implant a cannula (14 mm, 22-gauge stainless steel) in the lateral ventricle (AP -0.9, ML -1.5, DV -3.6) with coordinates taken from Paxinos and Watson (2009). After the animals recovered from the surgery and the EtOH consumption test resumed, unilateral infusions of the drug delivered by a pump (Harvard Apparatus model #22, Holliston, MA) were performed daily for the last four days of the experiment. A representative scheme of the perinatal treatment, EtOH consumption and drugs administered time-line is shown in Fig. 1.

Therefore, the experimental groups for each prenatal treatment (C or Pb) were conformed as follows: PND35, PND63 EtOH-SAL, PND63 EtOH-AT and PND63 EtOH-3NPA for the systemically-injected animals, and PND63 EtOH-VEH and PND63 EtOH-CY for those centrally-injected. In addition, adult C and Pb-exposed animals of the same age administered with identical drug schemes, but not subjected to the EtOH intake protocol were sacrificed concurrently. They conform five additional groups: PND63 non-EtOH-SAL, PND63 non-EtOH-AT, PND63 non-EtOH-3NPA, PND63 non-EtOH-VEH, and PND63 non-EtOH-CY.

2.3. Brain sample collection

At the end of the experiments, all rats were anesthetized with an i.p. injection of ketamine hydrochloride (55 mg/kg) and xylazine (11 mg/kg) and transcardially perfused exposing the thoracic cavity and obstructing the blood flux in the descendent aorta. They were first perfused with a SAL/heparin solution 0.9% NaCl/1000 units/l (approx.

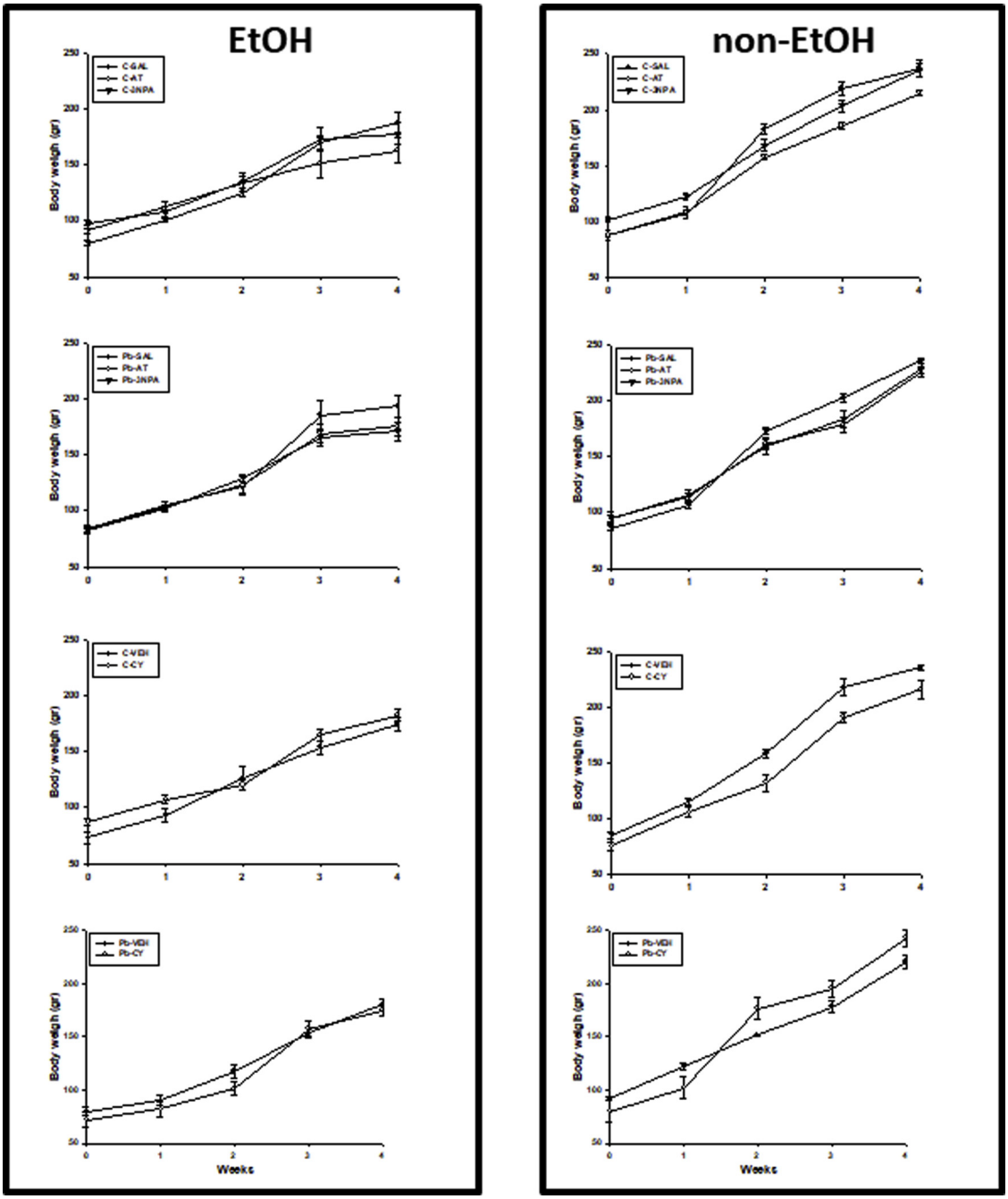

Fig. 3. Body weights of C and Pb-exposed animals which consumed EtOH (left panels) and their non-EtOH counterparts (right panels). All measurements were recorded weekly during 4 weeks (days 1–28 of the free-choice test). The number of animals corresponds to n = 10 per group for the animals which consumed EtOH and n = 3–5 for their non-EtOH counterparts. EtOH = ethanol; C = control; Pb = lead-exposed; SAL = saline; AT = 3-amino 1,2,4-triazole; 3NPA = 3-nitropropionic acid; VEH = vehicle; CY = cyanamide.

250 ml/rat) and later with 4% paraformaldehyde fixing solution in phosphate buffer (PB) 0.1 M pH = 7.4 (approximately 300–350 ml/rat). Brains were removed and kept on the 4% paraformaldehyde solution for 12 h at 4 °C to be later placed in a 30% sucrose in PB solution for 72 h at 4 °C until analysis.

The day of the experiment the brains were quickly frozen with an aerosol (Biofreeze, Biopack) and glued to the cryostat with a cryoprotector medium (Cryoplast, Biopack). To make the temperature uniform between the tissue and the mounting medium, the samples were left for 15–20 min before sectioned in 30 μm thick slices by the use of a cryostat (Leica CM15105). Sections from the nucleus accumbens (NAc), dorsal striatum (dStr), and prefrontal cortex (PFC) were obtained according to Paxinos and Watson (2009). The slices were washed with 0.01 PB three times for 10-min intervals and later incubated with 10% methanol, 30% H_2O_2 in PB for 1 h in a shaker to eliminate the endogenous peroxidase. Afterward, three 10-min washes were made with 0.01 PB and the sections incubated for 2 h under constant orbital agitation. This step included a blocking solution containing 1% bovine serum albumin (BSA Tethaedron), 0.1% Triton X-100, 10% goat serum or 10% normal horse serum (Sigma, Argentina) for CAT or ALDH2 respectively, in 0.01 M PBS in order to block unspecific antibody bindings. The sections were then incubated overnight at 4 °C in agitation with a rabbit polyclonal anti-CAT antibody (1:1500, Abcam) or a mouse monoclonal antiALDH2 antibody (1:1000, Abcam). The sections were then washed three times

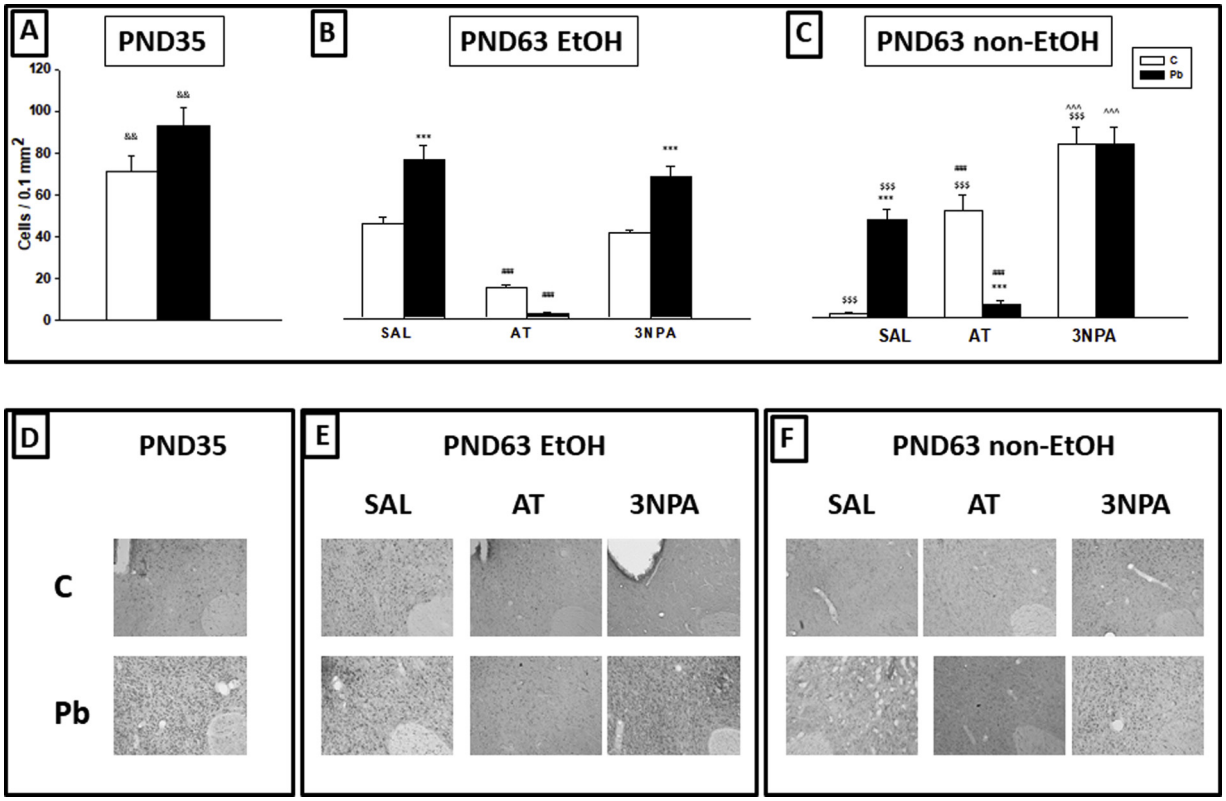

Fig. 4. CAT expression in the nucleus accumbens (NAc) in basal conditions and after drug administration. Panels A & D: Cell count (A) and representative pictures (D) of 35-day-old rats. Panels B & E: Cell count (B) and representative pictures (E) of 63-day-old rats which had consumed EtOH. Panels C & F: Cell count (C) and representative pictures (F) of 63-day-old rats which had not consumed EtOH. The data represent the results of 4 animals per group. PND = postnatal day; EtOH = ethanol; C = control; Pb = lead-exposed; SAL = saline; AT = 3-amino 1,2,4-triazole; 3NPA = 3-nitropropionic acid.
Panel A: $^{\&}$denotes significant differences respect to the PND63 non-EtOH animals from the same group (C or Pb) at $^{\&\&}p < 0.01$. *Panels B & C:* *indicates significant differences respect to the C animals from the same drug (SAL, AT or 3NPA) and EtOH (EtOH or non-EtOH) conditions at $^{***}p < 0.001$; $^{\#}$denotes significant differences between the SAL and AT-pretreated animals from the same group and EtOH conditions at $^{\#\#\#}p < 0.001$; ^indicates significant differences between the SAL and 3NPA-treated animals from the same group and EtOH conditions at $^{\hat{}\hat{}\hat{}}p < 0.001$; $^{\$}$denotes significant differences respect to their respective EtOH counterparts from the same group and drug conditions at $^{\$\$\$}p < 0.001$.

for 10 min and incubated for 2 h in a shaker with a biotinylated secondary antibody (anti-rabbit 1:1000, Vector Laboratories, Burlingame, CA, USA or anti-mouse, 1:2000, Jackson Immunoresearch, as appropriate). This was followed by the incubation with the avidin-biotin-peroxidase complex (Vector ABC kit, Burlingame, CA, USA) for 1 h at room temperature. Diamine benzidine (DAB, Sigma Aldrich, Argentina) as a chromogenic was used for 5–7 min in a solution containing 0.05% DAB and 0.006% H_2O_2 in 0.1 M PB. The final product developed a brownish coloration in the presence of the enzyme of interest, labeling with no coloration in the negative control. Once finished, three 10-min washes were made with 0.1 M PB. The slices were later mounted in glasses with a solution conformed by 1.5% gel and 80% alcohol and once dried, cover with mounting solution (DPX, Sigma, Argentina) and coverslip for visualization. In the case of CAT, to unmask the antigenic epitopes, the HIER (heat induced epitope retrieval) technique was employed before the immunohistochemistry protocol (Cholich et al., 2008).

Catalase and ALDH2 positive cells quantification: A light field microscope (Leica DM 4000B, Cambridge, UK) with a mounted camera (Leica DFC 300Fx) and the IM50 (Leica) software with a 20x magnification was used to count CAT positive cells, while 40x magnification was used for ALDH2 quantification. Two slices of each brain region (4 fields of each area) were selected and both hemispheres quantified to obtain a final count average for each experimental group with the aid of the software IMAGE *J* (National Institutes of Health, NIH, US).

2.4. Statistical analysis

To facilitate analysis, daily EtOH intake data were collapsed into the four days corresponding to the same EtOH concentration. Thus, the average for the 2%, 4%, 6%, 8% and the 10% EtOH concentrations (days 1–20) were analyzed by a two-way repeated measure analysis of variance (ANOVA), contrasting the group (C *vs.* Pb) against the time/EtOH concentration as the repeated variable. Once the AT administration protocol was implemented (test days 21–24 and 25–28), data were analyzed by a three-way-repeated-measures ANOVA by comparisons between group (C *vs.* Pb), drug (SAL *vs.* AT) and time/EtOH concentration as the repeated variable. In the case of 3NPA or CY (days 25–28), a two-way ANOVA was applied, with the group (C *vs.* Pb) and drug (SAL *vs.* 3NPA or VEH *vs.* CY, as appropriate) and time/EtOH concentration as the variables to contrast. In all cases, when significant interactions were found, a Tukey´s test was performed as a *post hoc* analysis, with resulting *p* values indicated in the corresponding figure legends.

To avoid large data comparison, body weights values were analyzed in a separate analysis for each EtOH condition by contrasting the group and the drug parameters and time as the repeated variable.

In relation to the immunohistochemical data, the analysis of each brain region was evaluated separately. On one hand, the effect of postnatal age on the enzymes' expression was analyzed by a two-way ANOVA contrasting group (C *vs.* Pb) and age/EtOH condition (groups PND35, PND63 EtOH, and PND63 non-EtOH). In addition, to evaluate the effects of the drugs on each enzyme expression a separated group of

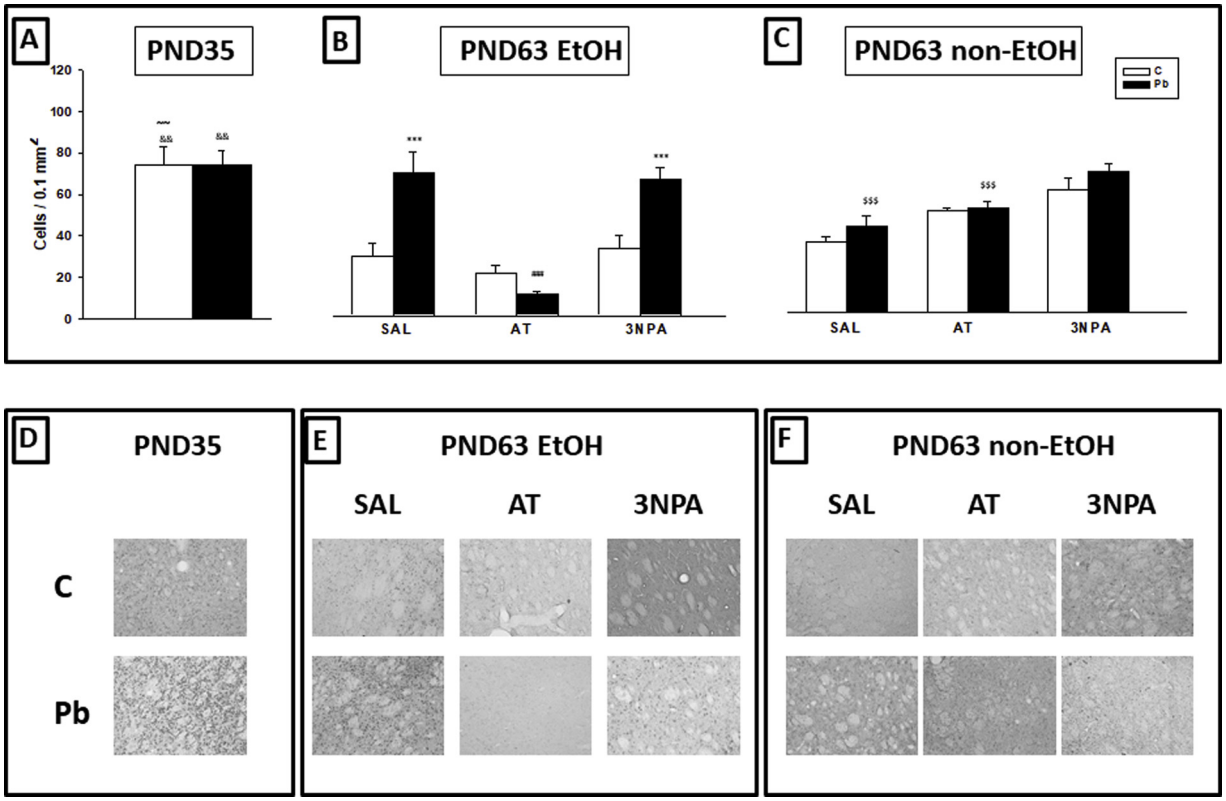

Fig. 5. CAT expression in the dorsal striatum (dStr) in basal conditions and after drug administration. Panels A & D: Cell count (A) and representative pictures (D) of 35-day-old rats. Panels B & E: Cell count (B) and representative pictures (E) of 63-day-old rats which had consumed EtOH. Panels C & F: Cell count (C) and representative pictures (F) of 63-day-old rats which had not consumed EtOH. The data represent the results of 4 animals per group. PND = postnatal day; EtOH = ethanol; C = control; Pb = lead-exposed; SAL = saline; AT = 3-amino 1,2,4-triazole; 3NPA = 3-nitropropionic acid.
Panel A: ¯indicates significant differences respect to the PND63 EtOH animals from the same group (C or Pb) at $^{\bar{}}p < 0.01$; $^{\&}$denotes significant differences respect to the PND63 non-EtOH animals from the same group at $^{\&\&}p < 0.01$. *Panels B & C:* *indicates significant differences respect to the C animals from the same drug (SAL, AT or 3NPA) and EtOH (EtOH or non-EtOH) conditions at $^{***}p < 0.001$; $^{\#}$denotes significant differences between the SAL and AT-pretreated animals from the same group and EtOH conditions at $^{\#\#\#}p < 0.001$; $^{\$}$denotes significant differences respect to their respective EtOH counterparts in the same group and drug conditions at $^{\$\$\$}p < 0.001$.

data was analyzed by a three-way ANOVA (group x drug x EtOH condition) followed by a Tukey´s test as a *post hoc* analysis. It should be noted that although shown in separated figures, the EtOH and non-EtOH animals were analyzed together to contrast the effect of EtOH intake on each enzyme expression.

3. Results

3.1. Ethanol intake

Fig. 2 represents total EtOH consumption of escalating solutions (2–10% EtOH v/v) in basal conditions and in response to AT (panel A), 3NPA (panel B) or CY (panel C) administration. It should be noted that this data corresponds to a large group of animals that include the subset sacrificed for the immunohistochemistry assays described below. Thus, replicating previous results, (Mattalloni et al., 2013, 2017 & 2019) Pb-exposed rats increased their EtOH intake over time, which became relatively stable after the 6% EtOH concentration. In contrast, C animals demonstrated a steady low EtOH intake over the length of the experiment. The statistical analysis performed for each drug condition is detailed in the corresponding section.

3.1.1. Effect of AT administration

Starting on test day 21 (Fig. 2A), when EtOH intake was stabilized and showed to be significantly higher in the Pb-exposed rats (group effect: $F(172) = 34.71$, $p < 0.001$), CAT inhibition was in effect (as indicated by the two arrows on days 21–24 and 25–28). The results show that AT pretreatment abolished the elevated EtOH consumption observed in the Pb-exposed animals reaching similar values than their corresponding SAL-treated counterparts, while such an effect was not evident in the C animals. In support of this conclusion, a three-way-repeated-measures ANOVA indicated a significant effect in the group: $F(116) = 24.69$, $p < 0.001$ and in the drug: $F(116) = 4.62$, $p < 0.05$ variables, but no overall interaction was evident.

3.1.2. Effects of 3NPA administration

Baseline data plotted in Fig. 2 (panel B) shows a significant effect of the group: $F(190) = 63.25$, $p < 0.001$, and the time/EtOH concentration: $F(590) = 52.23$, $p < 0.001$ variables, as well as a significant interaction between both parameters: $F(590) = 2.64$, $p < 0.05$. The arrow indicates the start of 3NPA administration (test days 25–28) which promoted an increase in EtOH intake in both, the C and Pb-exposed animals compared to the respective SAL-injected rats. These differences were evidenced by the two-way ANOVA results revealing a significant effect in the group: $F(116) = 41.14$, $p < 0.001$, and drug: $F(116) = 13.04$, $p < 0.01$ variables, but not in their interaction.

3.1.3. Effect of CY administration

The data plotted in Fig. 2 (panel C) in basal conditions shows a significant effect of the group variable: $F(190) = 21.39$, $p < 0.001$, and of the time/EtOH concentration repeated variable: $F(590) = 15.25$, $p < 0.001$, but no interaction emerged between both parameters. Interestingly, CY administration (indicated by an arrow in test days

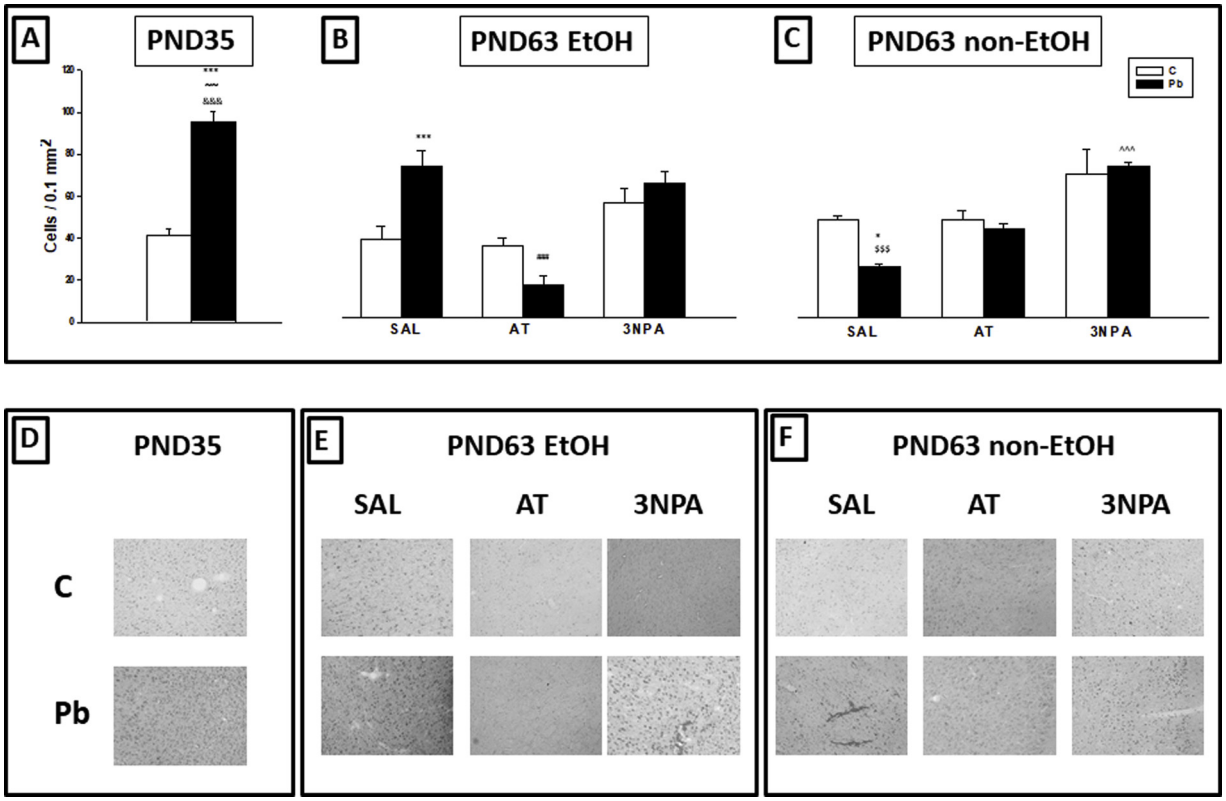

Fig. 6. CAT expression in the prefrontal cortex (PFC) in basal conditions and after drug administration. Panels A & D: Cell count (A) and representative pictures (D) of 35-day-old rats. Panels B & E: Cell count (B) and representative pictures (E) of 63-day-old rats which had consumed EtOH. Panels C & F: Cell count (C) and representative pictures (F) of 63-day-old rats which had not consumed EtOH. The data represent the results of 4 animals per group. PND = postnatal day; EtOH = ethanol; C = control; Pb = lead-exposed; SAL = saline; AT = 3-amino 1,2,4-triazole; 3NPA = 3-nitropropionic acid.
Panel A: *indicates significant differences respect to the C animals of the same age at $^{***}p < 0.001$; ~indicates significant differences respect to the PND63 EtOH animals from the same group (C or Pb) at $^{\sim}p < 0.01$; $^{\&}$denotes significant differences respect to PND63 non-EtOH from the same group (C or Pb) at $^{\&\&\&}p < 0.001$.
Panels B & C: *indicates significant differences respect to the C animals in the same drug (SAL, AT or 3NPA) and EtOH (EtOH or non-EtOH) conditions at $^{*}p < 0.05$ and $^{***}p < 0.001$; $^{\#}$denotes significant differences between the SAL and AT-pretreated animals from the same group and EtOH conditions at $^{\#\#\#}p < 0.001$; ^indicates significant differences between the SAL and 3NPA-treated animals from the same group and EtOH conditions at $^{\wedge\wedge}p < 0.001$; $^{\$}$denotes significant differences respect to their respective EtOH counterparts in the same group and drug conditions at $^{\$\$\$}p < 0.001$.

25–28) elicited a significant increase in EtOH consumption, an effect evidenced in both groups as a result of brain ALDH inhibition. This was statistically supported by the significant difference that emerged both in the group: $F(116) = 8.29$, $p < 0.05$, in the drug: $F(116) = 13,32$, $p < 0.01$ variables, and in their interaction $F(116) = 4.62$, $p < 0.05$. Individual comparisons are denoted in the figure and detailed in the respective legend.

3.2. Body weights

Fig. 3 depicts body weights of the animals assessed in the EtOH free-choice test (lefts panels) and of their non-EtOH counterparts (rights panels). From the observation of the figure it is evident that all animals have similar weights at PND35 (the start of the free choice test) as noted in the points of the curves that intersect the y axis. The figure also shows that EtOH intake and most probably the 2-h-restricted access to fluids prevented the animals to reach the same weight than the non-EtOH group. In addition, the brain surgery performed on week 3 to implant the canula for i.c.v. administrations had minor impact on weight gain in both, C and Pb-exposed rats independently of the EtOH condition. The statistical analysis shows the absence of group or drug effects in the animals which consumed EtOH, albeit a significant effect in the time/EtOH concentration ($F(3162) = 216.32$, $p < 0.001$) was evident. Similar results were obtained when body weight of the non-EtOH animals was analyzed, with a significant difference present only in the repeated variable time ($F(387) = 1244.40$, $p < 0.001$). Overall, these results indicate normal curves of weight gain of the animals evaluated in this study, which suggest a good health status with and minor disturbances resulting of the treatments applied.

3.3. Immunohistochemical studies

3.3.1. CAT expression

CAT expression in the NAc, dStr and PFC brain regions of 35-day-old animals is shown in panel A of Figs. 4–6, with a representative picture of each brain area shown in Panel D of the corresponding figure. In addition, panels B and C depict CAT expression in the 63-day-old animals after EtOH intake (Panel B) or in their non-EtOH counterparts (Panel C). Representative pictures of the corresponding brain area and drug administered are shown in Panels E and F, respectively.

3.3.1.1. Effect of age and EtOH intake on brain CAT expression. The two-way ANOVA performed for each region separately shows that in the NAc (Fig. 4A and SAL-bars of panels B & C), a significant effect of group: $F(1,18) = 72.80$; $p < 0.001$, and age/EtOH intake: $F(2,18) = 120.75$; $p < 0.001$ was found, with no interaction between them. This analysis demonstrates a higher abundance of the enzyme in the Pb-exposed animals independently of the age and EtOH intake. A different effect was observed in the dStr (Fig. 5A and SAL-bars of panels B and C) where a significant effect of group: $F(1,18) = 22.03$; $p < 0.001$, age/EtOH intake: $F(1,18) = 7.38$; $p < 0.01$, and of the interaction between them: $F(2,18) = 5.08$; $p < 0.05$ was observed. These results indicate that the

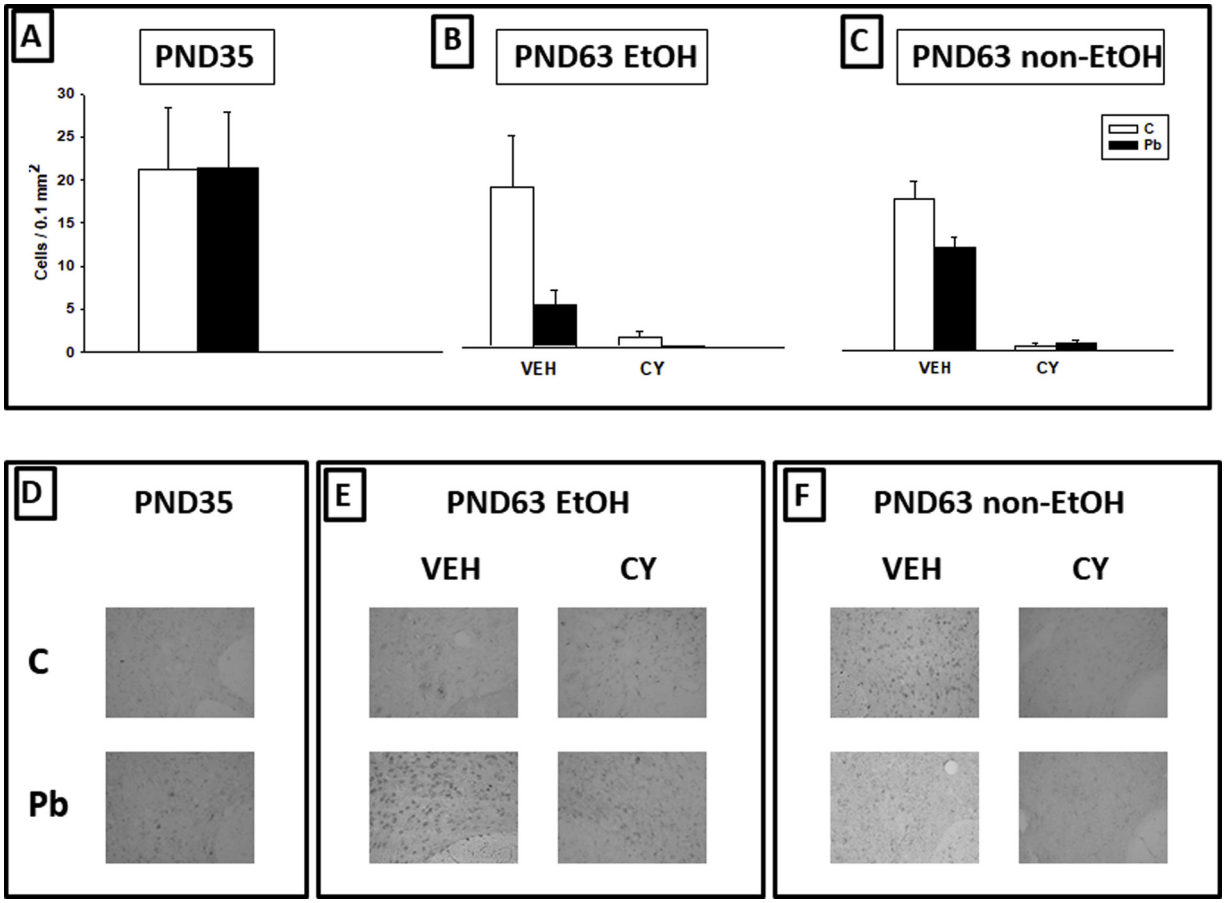

Fig. 7. ALDH2 expression in the Nucleus Accumbens (NAc) in basal conditions and after drug administration. Panels A & D: Cell count (A) and representative pictures (D) in 35-day-old rats. Panels B & E: Cell count (B) and representative pictures (E) in 63-day-old rats which had consumed EtOH. Panels C & F: Cell count (C) and representative pictures (F) in 63-day-old rats which had not consumed EtOH. The data represent the results of 3–6 animals per group. PND = postnatal day; EtOH = ethanol; C = control; Pb = lead-exposed; VEH = vehicle; CY = cyanamide.

enzyme show a higher expression selectively in the Pb-exposed rats only after voluntary EtOH consumption. On the other hand, in the PFC (Fig. 6A and SAL-bars of panels B and C) there was a significant effect of group: $F(1,18) = 34.75$; $p < 0.001$, and of age/EtOH intake $F(2,18) = 27,55$; $p < 0,001$, as well as in the interaction between them: $F(2,18) = 37.26$; $p < 0.001$. Thereby, a higher CAT expression was present in the Pb-exposed animals at young age, an effect that persisted only after EtOH intake.

3.3.1.2. Effect of drug (SAL, AT or 3NPA) pretreatment on brain CAT expression. The three-way ANOVA performed for each area shows that in the NAc (Fig. 4B and C), a significant difference was observed in the group: $F(1,36) = 9.70$; $p < 0.05$, EtOH intake: $F(1,36) = 5.98$; $p < 0.05$, and drug: $F(2,36) = 110.71$; $p < 0.001$ variables. This was also evident in the interactions between group x drug: $F(2,36) = 47.97$; $p < 0.001$, EtOH intake x drug: $F(2,36) = 56.21$; $p < 0.001$, group x EtOH intake: $F(1,36) = 8.85$; $p < 0.01$, and drug x group x EtOH intake: $F(2,36) = 4.61$; $p < 0.05$. These results demonstrate a reduction in CAT expression in this area in both, C and Pb-exposed rats after EtOH intake as a consequence of AT administration. However, in non-EtOH conditions this effect was only evident in the Pb-exposed group. On the other hand, 3NPA administered at the end of the EtOH intake protocol failed to change basal CAT expression in both C and Pb-exposed rats. This result contrast with the significant increase in CAT expression observed in both groups in a non-EtOH condition. In the dStr (Fig. 5B and C) the analysis shows a significant difference in the group: $F(1,36) = 21.43$; $p < 0,001$ and drug: $F(2,36) = 22.19$; $p < 0.001$ variables, and in their interaction: $F(2,36) = 10.11$; $p < 0.001$. In the same line, differences emerged in the group x EtOH intake: $F(1,36) = 9.05$; $p < 0.005$, EtOH intake x drug: $F(2,36) = 20.47$; $p < 0.001$ and in the group x drug x EtOH intake overall interaction: $F(2,36) = 6.67$; $p < 0.01$. These differences were evidenced as a significant reduction in CAT expression selectively in the Pb-exposed group after voluntary EtOH intake as a consequence of AT administration. Conversely, 3NPA reproduced the CAT expression observed in the SAL-injected animals in both groups and EtOH conditions. In the PFC (Fig. 6B and C) there was a significant effect in the drug variable: $F(2,36) = 29.45$; $p < 0.001$, as well as of the group x EtOH intake: $F(1,36) = 6.30$; $p < 0.05$, group x drug: $F(2,36) = 3.27$; $p < 0.05$, drug x EtOH intake: $F(2,36) = 12.85$; $p < 0.001$ interactions, and of the group x drug x EtOH intake: $F(2,36) = 10.71$; $p < 0.001$ overall interaction. These results demonstrated that AT reduced CAT expression selectively in the Pb-exposed rats after EtOH intake, whereas 3NPA blunted the differences observed between the SAL-injected C and Pb-exposed animals in both the EtOH and non-EtOH conditions.

3.3.2. ALDH expression

Figs. 7–9 (panel A) represent ALDH2 expression in the NAc, dStr, and PFC, respectively in 35-day-old animals with a representative picture shown in Panel D of each figure. Panels B and C depict ALDH2 expression of the 63-day-old animals after EtOH intake (Panel B) or of their non-EtOH counterparts (Panel C). A representative picture of the EtOH and non-EtOH groups in each drug condition is depicted in Panels E and F, respectively.

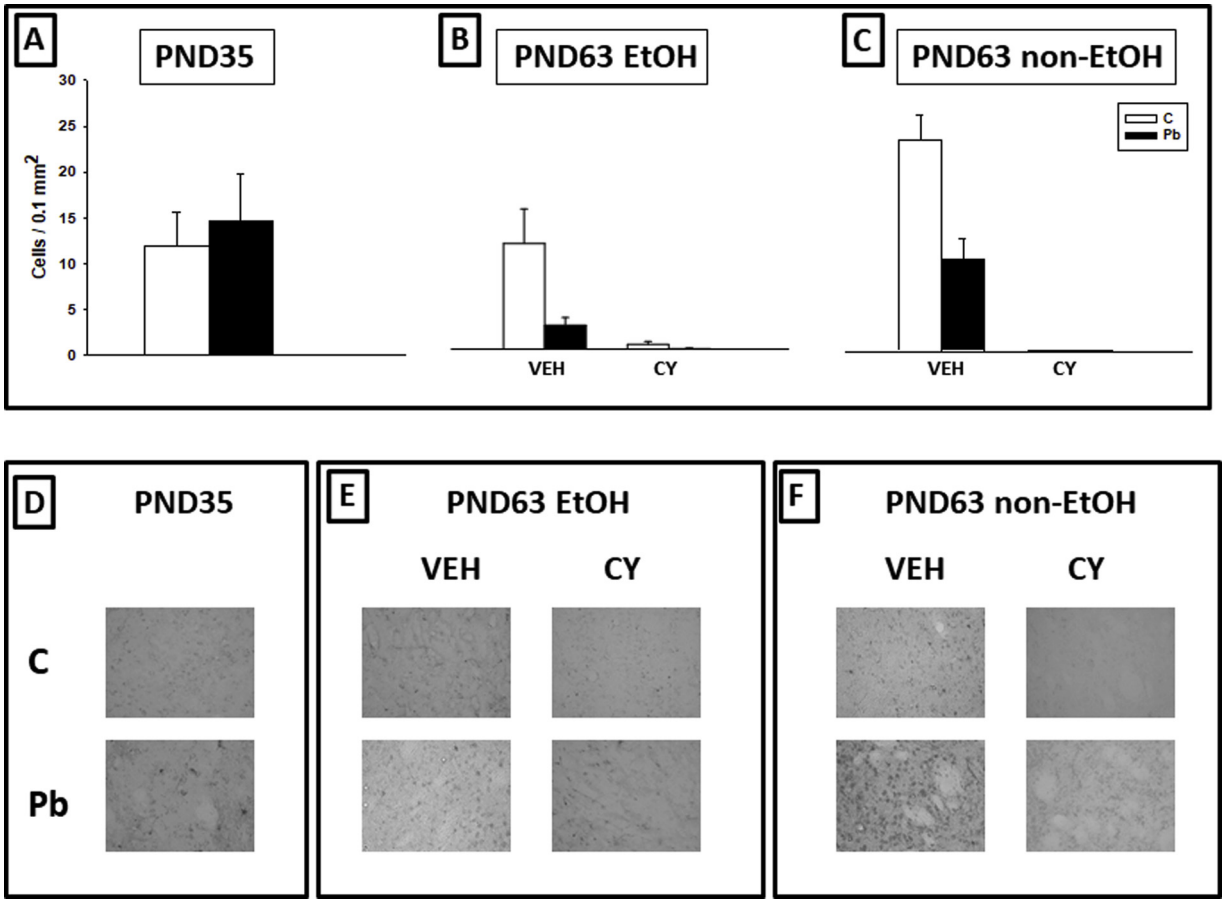

Fig. 8. ALDH2 expression in the dorsal striatum (dStr) in basal conditions and after drug administration. Panels A & D: Cell count (A) and representative pictures (D) in 35-day-old rats. Panels B & E: Cell count (B) and representative pictures (E) in 63-day-old rats which had consumed EtOH. Panels C & F: Cell count (C) and representative pictures (F) in 63-day-old rats which had not consumed EtOH. The data represent the results of 3–6 animals per group. PND = postnatal day; EtOH = ethanol; C = control; Pb = lead-exposed; VEH = vehicle; CY = cyanamide.

3.3.2.1. Effect of age and EtOH intake in brain ALDH2 expression. In the NAc (Fig. 7A and VEH-bars of panels B and C) the two-way ANOVA performed for each region separately failed to show a significant effect of the variables group, age/EtOH or their interaction. However, in the dStr (Fig. 8A and VEH-bars of panels B and C) a significant effect of the group variable was evident: $F(1,22) = 5.12$; $p < 0.05$; while in the PFC (Fig. 9A and VEH-bars of panels B and C) a significant difference in the interaction between group and age/EtOH condition: $F(2,22) = 5.08$; $p < 0.05$ emerged. Thus, the results shown here evidenced a non-significant reduction in ALDH2 expression in all areas of the adult Pb-exposed animals compared with the younger animals, independently of their EtOH consumption history.

3.3.2.2. Effect of drug (VEH or CY) pretreatment on brain ALDH2 expression. The data show that CY administration dramatically reduced ALDH2 expression in both groups independently of their EtOH intake, particularly in the NAc, dStr and to a lesser extent in the PFC. Thus, the three-way ANOVA performed for each area revealed that in the NAc (Fig. 7B and C), a significant difference was observed in the variables group: $F(1,31) = 6.12$; $p < 0.05$, drug: $F(1,31) = 39.70$; $p < 0.001$ and in the interaction between group x drug: $F(1,31) = 5.26$; $p < 0.05$. In the dStr (Fig. 8B and C) there were significant differences in the group variable: $F(1,31) = 16.09$; $p < 0.001$, EtOH intake: $F(1,31) = 16.60$; $p < 0.001$, and drug: $F(1,31) = 68.06$; $p < 0.001$ variables, and in the interactions group x drug: $F(1,31) = 14.76$; $p < 0.001$, and EtOH intake x drug: $F(1,31) = 10.82$; $p < 0.01$. In the PFC (Fig. 9B and C) a significant effect of the drug variable: $F(1,31) = 10.44$; $p < 0.01$, and of the group x EtOH intake interaction: $F(1,31) = 14.25$; $p < 0.001$ was evident. Hence, due to the absence of significant differences in the overall interaction *pos hoc* analysis was not performed.

4. Discussion

The results presented in this study provide further evidence of CAT and ALDH2 participation (and brain acetaldehyde accumulation) in the elevated voluntary EtOH consumption reported by us in male rats perinatally exposed to Pb.

In relation to CAT expression, we replicate here data reported by Zimatkin and Lindros (1996) and Moreno et al. (1995), evidencing the abundance of the enzyme in the brain. Furthermore, we demonstrated elevated CAT expression in the PFC in the 35-day-old developmentally Pb-exposed animals, differences that persisted (PFC) or emerged (NAc and dStr) after EtOH intake. Thus, a significantly higher number of CAT-positive cells in all areas was evident in the SAL-injected-Pb-exposed animals which had consumed EtOH. This effect was absent when the respective non-EtOH counterparts were analyzed (with the exception of the NAc). Interestingly, AT reduced the CAT-positive cells in the NAc (but neither in the dStr nor in the PFC) in the C animals after EtOH intake. In contrast, AT administration induced a drastic reduction of CAT abundance in all areas in the Pb-exposed animals which had consumed EtOH, although this effect was only present in the NAc of their non-EtOH counterparts. In contrast, no further increase in the enzyme expression was observed after 3NPA administration in most groups, with the exception of the NAc (of both, the C and Pb-exposed non-EtOH groups) and the PFC (of the Pb-exposed non-EtOH group). On

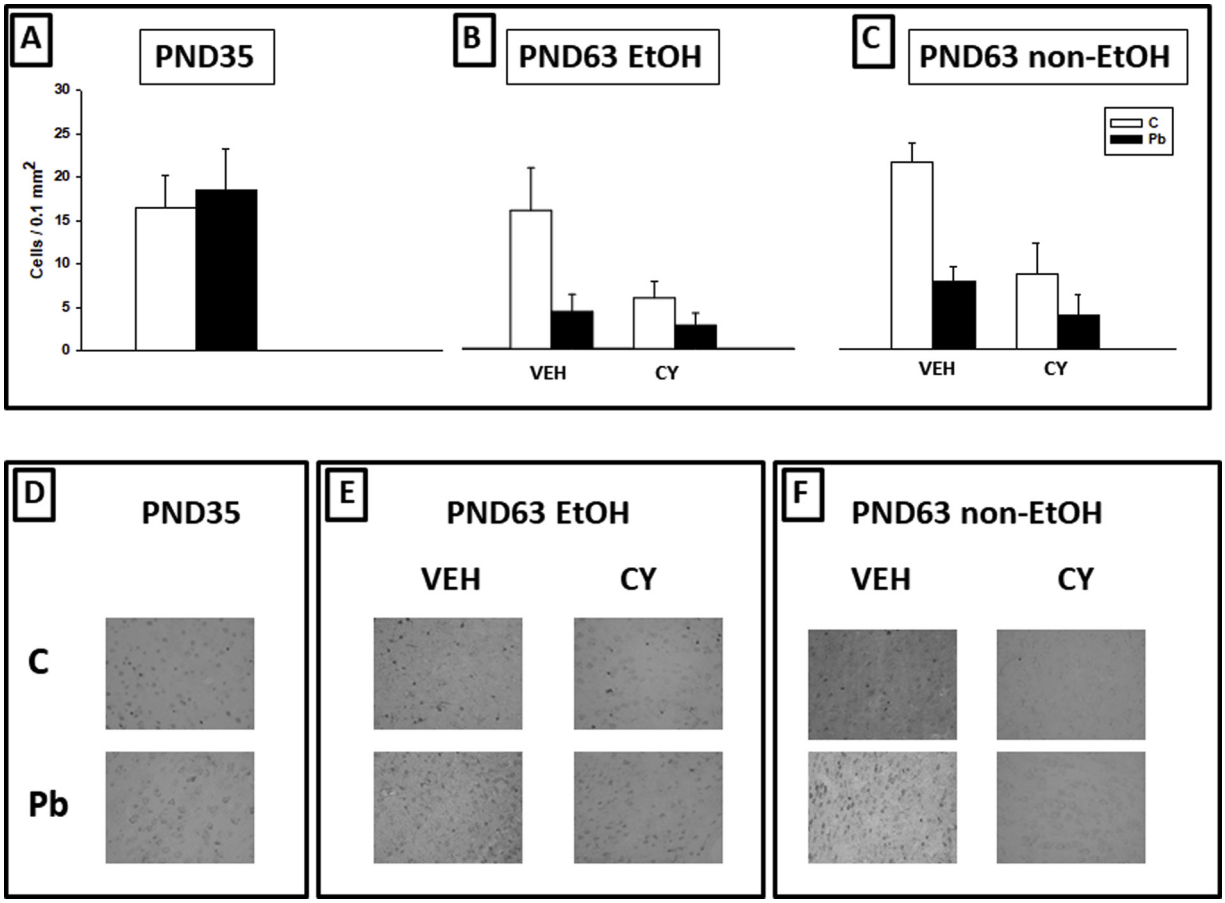

Fig. 9. ALDH expression in the prefrontal cortex (PFC) in basal conditions and after drug administration. Panels A & D: Cell count (A) and representative pictures (D) in 35-day-old rats. Panels B & E: Cell count (B) and representative pictures (E) in 63-day-old rats which had consumed EtOH. Panels C & F: Cell count (C) and representative pictures (F) in 63-day-old rats which had not consumed EtOH. The data represent the results of 3–6 animals per group. PND = postnatal day; EtOH = ethanol; C = control; Pb = lead-exposed; VEH = vehicle; CY = cyanamide.

the basis of these results, we propose that Pb induced a high CAT activity (Mattalloni et al., 2013) and expression (this data) that may have important consequences in the excessive EtOH intake we have reported in the Pb-exposed rats. Moreover, we have demonstrated that EtOH intake was reduced in these animals after pharmacological (Mattalloni et al., 2013 and this data) or molecular (Mattalloni et al., 2019) suppression of CAT activity or expression. In contrast, 3NPA effects were consistently less robust than those observed with AT. They show a modest restoration of CAT activity in blood and in the dStr (Mattalloni et al., 2013), with an increase in CAT expression restricted to the NAc (C and Pb-exposed rats) and PFC (Pb-exposed animals). These differences were evident despite the robust increase in EtOH intake observed in both C and Pb-exposed animals, an effect that may be related to the indirect mechanism that this drug exerts to increase CAT activity (Binienda et al., 1998). Thus, although a small portion of the neuronal and glial population is part of the CAT-positive cells (Zimatkin and Lindros, 1996; Moreno et al., 1995), it has been demonstrated that brain CAT activity determines EtOH-derived acetaldehyde production and the resulting pharmacological effects of this metabolite (Gill et al., 1992 & 1996, Aragon and Amit, 1985; Quertemont et al., 2005; Israel et al., 2015).

Regarding ALDH2, we observed low basal expression in the areas under study, replicating observations of Zimatkin (1991) and Zimatkin et al. (1992). Interestingly and opposing to CAT, ALDH2 expression failed to show differences between the 35-day-old C and Pb-exposed rats. However, Pb exposure selectively decreased ALDH2 expression in all the three areas under study after EtOH intake. This effect was potentiated by CY administration, a manipulation that further reduced ALDH2 activity (Mattalloni et al., 2017) and expression (present study) after EtOH intake in both C and Pb-exposed animals.

One limitation of this study that must be mentioned is the fact that no gender-related effects were assessed. At this respect, although differential toxicokinetic have been reported between male and females in humans (Ramchandani et al., 2001) and female rats voluntarily drink more EtOH than their male counterparts, the estrous-cycle had no effect on alcohol drinking evaluated in several strains and drinking protocols as reported by Priddy et al. (2017). Regarding the enzymes under study, neither ALDH nor CAT functionality have been shown to differ across genders, although some evidences in humans reported that ADH activity is higher in the males than in the females (Ramchandani et al., 2001; Kander et al., 2017).

Another aspect that deserves discussion is the possibility that the 2-h-limited-access to drinking fluids enhances EtOH toxicokinetic and elicits a rapid perception of the drug pharmacological effects (Pautassi et al., 2006). At this respect, we have previously shown that Pb exposure has no effect on EtOH toxicokinetic (Virgolini et al., 1999). Another consideration is related to the fact that the short period of fluid availability may promote deprivation stress in these animals. Interestingly, we and others have reported that adult (Haider et al., 2013) or early-life Pb exposure (Yu et al., 1996; Cory-Slechta et al., 2004; Virgolini et al., 2004) elevates basal corticosterone levels and triggers a high reactivity to stressful events compared to their non-exposed counterparts. Therefore, the possibility that the increased EtOH intake observed in the Pb-exposed animals was driven by the negative reinforcing or anxiolytic effects of the drug requires consideration. However, this scenario framed in the tension-reduction hypothesis

(Pohorecky, 1981) is more likely to occur in later stages of the addiction process. On the contrary, in the present conditions (initiation and maintenance of EtOH intake) the positive aspects of reinforcing that are mediated by the dopaminergic mesocorticolimbic pathway gain protagonism (Gilpin and Koob, 2008; Koob, 2006). In this line, accumulating evidence suggests that the motivational properties of EtOH are due not only to its first metabolite acetaldehyde but also to other derivates with known positive reinforcing properties. This includes the bioproduct salsolinol (1-methyl-1,2,3,4-tetrahydro-6,7-dihydroxy-isoquinoline), which results of the condensation of acetaldehyde and dopamine (Deng and Deitrich, 2008; Xie et al., 2013; Peana et al., 2016). Thereby, the high CAT and low ALDH2 activity and expression reported in the Pb-exposed animals after EtOH intake may have profound effects in terms of acetaldehyde accumulation. This, along with dopamine release would promote EtOH metabolites and resulting products accumulation in limbic areas, a scenario potentiated by the elevated EtOH consumption reported in these animals. In conclusion, these results provide further evidence that support the proposed critical role of acetaldehyde and related-bioproducts in the vulnerability to initiate an excessive EtOH intake pattern as a consequence of early-life exposure to Pb, a neurotoxicant still present ubiquitously in the environment.

Declaration of Competing Interest

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

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