Participation of Catalase in Voluntary Ethanol Consumption in Perinatally Low-Level Lead-Exposed Rats

Mara S. Mattalloni, Laura N. De Giovanni, Juan C. Molina, Liliana M. Cancela, and Miriam B. Virgolini

Background: Environmental lead (Pb) exposure and alcohol abuse pose significant public health problems for our society. One of the proposed mechanisms of action of the developmental neurotoxicant Pb is related to its ability to affect antioxidant enzymes, including catalase (CAT). Ethanol's (EtOH) motivational effects are postulated to be mediated by the CAT-dependent acetaldehyde generated in the brain. The current study sought to investigate the role of this enzyme in the elevated EtOH intake previously reported in perinatally Pb-exposed rats.

Methods: Thirty-five-day-old male Wistar rats exposed to 220 ppm Pb during gestation and lactation were offered escalating EtOH solutions (2 to 10%) or water, 2 h/d for 28 days. Once baseline 10% EtOH intake was achieved, they were injected with (i) saline (SAL), (ii) 3-amino 1,2,4 triazole (aminotriazole: AT, a CAT inhibitor, 250 mg/kg i.p., 5 hour before the last 8 EtOH intake sessions), or (iii) 3-nitropropionic acid (3NPA, a CAT activator, 20 mg/kg s.c., 45 minute before the last 4 EtOH intake sessions). Rats were then sacrificed, blood collected, and brain regions harvested for CAT activity determination. Additional studies evaluated EtOH intake and CAT activity in response to 10 and 30 mg/kg 3NPA. Both 3NPA and AT were evaluated for striatal cytotoxicity.

Results: We observed that AT pretreatment blunted the increased EtOH intake, as well as the elevated CAT activity in blood, cerebellum and hippocampus evidenced in the developmentally Pb-exposed rats that have consumed EtOH. Conversely, 20 mg/kg 3NPA further increased voluntary EtOH intake in these animals as compared with controls, concomitantly with a slight elevation in CAT activity both in blood and in the striatum, associated with no changes in striatal cytotoxicity.

Conclusions: These results suggest a participation of CAT, and possibly acetaldehyde, in Pb-induced high EtOH intake, and open up new avenues to elucidate the mechanism that underlies the Pb and EtOH interaction.

Key Words: Lead Exposure, Ethanol Intake, Catalase, Acetaldehyde.

LEAD ~~(PB) IS~~ a neurotoxicant, extensively used in the past that persists in the environment. Oxidative stress has been consistently related to its toxicity: ~~E~~levated Pb exposure inactivates free radical scavenging enzymes, while lower levels enhance their activity probably as a compensatory detoxification mechanism against excessive oxidant radicals (Antonio-Garcia and Masso-Gonzalez, 2008). The most adverse health effects are observed in the developmental brain manifested as hyperactivity, cognitive impairments, and an altered response to drugs (Virgolini et al., 2004; White et al., 2007). In this regard, preclinical studies demonstrate that Pb exposure increases ethanol (EtOH) anxiolytic and sedative responses and promotes its consumption in rats (Nation et al., 1986; Virgolini et al., 1999). However, the few studies that assessed the relationship between blood Pb levels and alcoholism or drug addiction in adult humans found no evidence of a significant interaction (Fishbein et al., 2008; Junco-Muñoz and Arrieta-Alcalde, 1993~~, respectively~~).

Compelling evidence supports a role for the metabolite acetaldehyde in several effects of EtOH (Deng and Deitrich, 2008; Quertemont et al., 2005). Pharmacological (Aragon and Amit, 1992; Escarabajal et al., 2000) or more recently genetic (Karahanian et al., 2011) manipulations of the enzyme catalase (CAT) have provided indirect evidence that acetaldehyde contributes to EtOH's pharmacological effects, supporting pioneer studies in acatalasemic mice (Aragon and Amit, 1993). CAT is an antioxidant enzyme that represents the main metabolic pathway of EtOH oxidation to acetaldehyde in the central nervous system (Oshino et al., 1973; Zimatkin and Buben, 2007), given that alcohol dehydrogenase (ADH) is hardly present in the brain (Zimatkin et al., 2006).

Many reports indicate that CAT activity inhibition with specific antagonists such as 3-amino 1,2,4-triazole (AT;

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Aragon and Amit, 1992) or sodium azide (Sanchis-Segura et al., 1999) reduces EtOH-induced locomotor activity (Correa et al., 2001; Pastor et al., 2002), its anxiolytic effects (Correa et al., 2008), reinforcing properties (Nizhnikov et al., 2007), and voluntary EtOH consumption (Tampier et al., 1995). Conversely, 3-nitropropionic acid (3NPA), a ~~ROS~~ generator, has been related to an elevation in CAT activity along with EtOH-induced hyperlocomotion (Binienda et al., 1998; Manrique et al., 2006). Interestingly, Pb also increases CAT activity when administered either acutely in adult rats (Correa et al., 2000) and mice (Correa et al., 2004), or chronically in developmental rats (Valenzuela et al., 1989) or chickens (Somashekaraiah et al., 1992).

On the basis of these antecedents, the present study attempted to delineate a possible converging mechanism of action for the interaction between Pb and EtOH, by postulating that the enzyme CAT may be related to the elevated EtOH intake evidenced in perinatally Pb-exposed rats. We thus hypothesized that CAT overactivation would be a compensatory response to both an exaggerated production of Pb-induced ROS (particularly H_2O_2) and to significant amounts of EtOH in the brain after voluntary EtOH intake.

MATERIALS AND METHODS

Animals and Perinatal Treatment

Adult male and female Wistar rats born and bred in our vivarium (IFEC-CONICET, Córdoba, Argentina) were maintained with food and water ad libitum in a 12-hour dark/light cycle with lights on at 07:00 AM (except for the self-administration procedure) under constant conditions of temperature and humidity. From the day of pregnancy, dams were weighed and housed in pairs, with unlimited access to a standard diet (Purina chow, Batistella, Argentina). They were randomly allocated to 1 of 2 subsets according to the fluid source: control group (C), which received filtered tap water; or lead group (Pb), provided with a 220 ppm Pb solution (0.4 g/l lead acetate; J.T. Baker, Mallinckrodt Baker, ~~Argentina~~). Within 24 hour of delivery (designated as postnatal day 1: PND1), litters were culled to 8 pups and weighed once a week until weaning at PND25, when Pb exposure was interrupted. Tests started at PND35, selecting 1 male per litter for each experimental condition (except for EtOH and sucrose intake tests where 2 pups from the same litter were housed together and considered a single case)~~, as suggested by Maurissen (2010)~~. Animal manipulations were in accordance with the Guiding Principles in the Use of Animals in Toxicology adopted by the Society of Toxicology. The number of animals used for each experiment is indicated in the corresponding figure legend.

Drugs and Dosing

Ethanol solutions were prepared daily in filtered water from a stock of 96% redistilled EtOH (Porta Hnos., Córdoba, Argentina). Sucrose was dissolved in filtered water a relation of 1 g per 100 ml, making a solution highly palatable as demonstrated in previous reports from our laboratory (Zurita and Molina, 1999).

3-amino 1,2,4-triazole (Sigma Aldrich S.A., ~~Argentina~~) was prepared daily in SAL and injected i.p. in a dose of 250 mg/kg, 5 hour before the start of the EtOH/water choice session according to Escarabajal and colleagues (2000), and Koechling and Amit (1994). 3NPA (Sigma Aldrich S.A.) was prepared in SAL on a daily basis and administered at different concentrations. The dose and time of injection employed were based on reports from the literature (Binienda et al., 1998; Manrique et al., 2006) and on pilot experiments. It was determined that maximal EtOH intake and CAT activation associated with the least neurotoxicity were achieved when the animals were sacrificed 105 minute after 20 mg/kg 3NPA administration (see text below and Supporting information).

Behavioral Studies

Ethanol Consumption. The day before the start of the EtOH/water free-choice test, a random subset of 35-day-old male pups (Group 35d non-EtOH) was sacrificed to obtain blood and brain tissue. Other groups of rats of the same age and gender were weighed, housed in pairs, and water-restricted during the 24 hour prior to the initiation of the EtOH intake protocol, during which access to fluids was limited to 2 hour per day between 1:00 PM and 3:00 PM. Thus, on PND35, the animals were presented for 28 days to 4 graduated tubes containing either water (2 tubes) or escalating concentrations of EtOH (2 tubes) according to the following scheme (expressed in v/v): days 1 to 4: 2%; days 5 to 8: 4%; days 9 to 12: 6%; days 13 to 16: 8%; days 17 to 28: 10% EtOH, concentration frequently used to select high and low EtOH consuming rats (Cunningham et al., 2000). Thus, once EtOH intake was stabilized (on test day 21), all rats were daily injected with (i) saline (SAL; days 21 to 28), (ii) a CAT activity inhibitor: AT; days 21 to 28, or (iii) a CAT activity booster: 3NPA; days 25 to 28 (SAL on days 21 to 24). EtOH and water intake was registered daily and expressed as grams of EtOH per kilogram of body weight, while the relation EtOH over water represented the percentage of preference. At the end of the last EtOH intake session~~,~~ (60 minute in length), the animals were ether-anesthetized and cardiac blood drawn (groups 63d EtOH). Immediately after, they were sacrificed by decapitation, and several brain regions dissected out (cerebellum, frontal cortex, hippocampus, striatum, hypothalamus, and nucleus accumbens) to determine brain CAT activity. Adult control and Pb-exposed animals administered with identical vehicle or drug schemes, but not subjected to the EtOH intake protocol were sacrificed concurrently (groups 63d non-EtOH).

Sucrose Consumption. To determine whether developmental Pb exposure modifies the motivational properties of natural reinforcers and to assess whether the observed effects of AT or 3NPA on EtOH consumption would extend to other reinforcers, the total intake of a highly palatable substance, that is, a 1% sucrose solution, was registered in naive control and perinatally Pb-exposed animals. A total of twenty-four 35-day-old male pups were caged in pairs and liquid-restricted 24 hour prior to the test. The following day they were given the choice to drink water or a 1% sucrose solution for 2 hour during 12 days. Daily intake of both fluids was registered and results expressed as the percentage of preference of sucrose over water. Sucrose intake recorded from day 1 to 4 was considered baseline. Thereafter, and starting on day 5, a subset of animals was injected daily with vehicle (SAL group), while a second set was administered 250 mg/kg AT, i.p. 5 hour before the start of the free-choice test (AT group). A third group was injected with 3NPA (20 mg/kg s.c.) from days 9 to 12 immediately prior to the beginning of the free-choice test (3NPA group).

Locomotor Activity. On the basis of the psychomotor activating hypothesis (Wise and Bozarth, 1987), we aimed to assess whether the elevated amount of EtOH ingested by the Pb-exposed rats in the EtOH/water free-choice test was sufficient to induce an increase in locomotor activity, measured in actographs.

The testing apparatus consisted of 8 rectangular cages (30.5 × 19.5 × 46.5 cm) equipped with 2 parallel infrared photocell beams located 3 cm above the floor. Interruption of either beam resulted in a photocell count. On PND63, immediately after the last voluntary EtOH intake session (60 minute in length), the animals

were placed individually in each cage with motor activity counts monitored at 10 minute intervals during 1 hour under white light in a quiet room. Another group of animals designated as 63d non-EtOH was tested concurrently in the same conditions as their EtOH counterparts. All animals were habituated to the locomotor cages on the day before the experiment by free exploration of the apparatus during 60 minute (data not registered).

Biochemical Analysis

Blood Lead Levels. Lead concentration was measured in a subset of pups from the 35d non-EtOH, 63d non-EtOH-SAL, and 63d EtOH-SAL groups. Intracardiac blood from ether-anesthetized rats was drawn. Pb was measured using an atomic absorption spectrophotometer equipped with graphite furnace (Buck Scientific 200gf) according to Subramedian and Meranger (1981).

Blood Ethanol Concentration. An aliquot of the sample collected 15 minute after the end of the last free-choice EtOH/water session in a subset of the 63d EtOH-SAL, 63d EtOH-AT, and 63d EtOH-3NPA groups was used to measure blood ethanol concentration (BECs) as described in Pepino and colleagues (2002). A gas chromatograph (Hewlett-Packard model 5890; Palo Alto, CA) with butanol as internal standard was used; results were averaged for each sample and expressed as milligram of EtOH per deciliter blood.

Blood and Brain CAT Activity. Catalase activity was measured in a subset of the EtOH and nonethanol-exposed animals defined in the previous sections. Although rats were not perfused for brain CAT determinations, no blood contamination was found in brain tissue as determined by nondetectable hemoglobin content in all brain regions assayed. Whole blood samples were first used to assess hemoglobin levels by Drabkin's method. Subsequently, samples were centrifuged at 573 *g*, red cells washed, hemolyzed, and diluted. To measure brain CAT activity, dissected tissue was homogenized in phosphate buffer (50 mM; pH 7.0) with digitonin (0.01%), spun at 9,158 *g* for 10 minute, and supernatants used to determine both protein levels (Bradford, 1976) and CAT activity. Brain and blood CAT activities were assayed by measuring the decrease in H_2O_2 absorbance during 5 minute at 240 nm ($\varepsilon_{240} = 0.0394$ mmol^{-1} cm^{-1}; Aebi, 1984), and resultant activity expressed as units of CAT activity (mmol H_2O_2/min/g Hb).

Statistical Analysis

Blood Pb concentration was not statistically suitable for comparison, given that many values fell below the quantification level. Blood hemoglobin levels were analyzed by a Student's *t*-test. To analyze BECs, a 2-way ANOVA was carried out, with group (C vs. Pb) and drug (SAL, AT or 3NPA) as the variables. On the other side, weight gain across the EtOH intake test was analyzed with a 3-way repeated measures ANOVA, comparing group, EtOH consumption, and time as the repeated variable.

To facilitate statistical analysis, daily EtOH intake data were collapsed into the 4 days that belong to the same EtOH concentration and analyzed by a 2-way repeated measures ANOVA, contrasting the group against the time/EtOH concentration as the repeated variable. Once chronic AT pretreatment was in effect (test days 21 to 24 and 25 to 28), data were analyzed by a 3-way repeated measures ANOVA by comparisons between group, drug, and time/EtOH concentration as the repeated variable. On the other side, when 3NPA was administered (days 25 to 28), a 2-way ANOVA was applied, with group and drug as the variables. In all cases, when a significant interaction was found, a Tukey's test as a post hoc test was performed, with resulting *p*-values indicated in the figure legends.

Blood and brain CAT activities were first analyzed by a 2-way ANOVA: group (C vs. Pb) × age/EtOH intake as appropriate (groups 35d, 63d EtOH, and 63d non-EtOH). Subsequently, a 3-way ANOVA: group × drug × EtOH consumption was followed by a Tukey's test as a post hoc test. In the case of brain CAT activity, each brain area was compared in a separate 3-way ANOVA analyses.

RESULTS

Pb Exposure Model

As previously reported (Virgolini et al., 1999), the Pb solution administered did not modify either the fluid intake or the parameters of the dams or pups (Fig. 1).

The results presented in Table 1 demonstrate that perinatal Pb exposure elevated blood Pb concentration to 6.51 ± 0.26 μg/dl in the 35-day-old pups, levels that further decreased in the adult animals independently of their EtOH intake background. Table 1 also shows that Pb exposure decreased hemoglobin levels in the periadolescent rats when Pb traces were still present in blood ($t = -4.44$, $p < 0.01$), while no differences were observed in older animals.

Blood Ethanol Concentration

The results summarized in Table 1 (bottom) demonstrate that CAT inhibition by repeated AT pretreatment resulted in an increase in BEC in both 63d EtOH-AT groups, with a significant difference in the drug variable: $F(1, 33) = 10.60$, $p < 0.01$. In contrast, chronic 3NPA administration produced BECs below the quantification limit in both groups.

Ethanol Consumption

Effect of AT Administration on Ethanol Intake. Figure 2 (top panel) depicts a simplified EtOH metabolic pathway (Quertemont and Didone, 2006). Figure 2*A* shows EtOH intake and Fig. 2*B* the preference ratio between EtOH solutions and water. In both panels, data were collapsed in 4-day blocks corresponding to the same EtOH concentration.

Baseline—Figure 2, panel *A* (left) represents total consumption of escalating EtOH solutions until steady intake was reached at 10% EtOH (test day 20). The Pb-exposed pups showed an increase in EtOH intake from 2 to 6% EtOH, becoming relatively stable at 8 and 10%. Control animals, in contrast, demonstrate a constant low intake across all EtOH concentrations. A 2-way repeated measures ANOVA revealed a significant effect of group: $F(1, 156) = 32.21$, $p < 0.001$, the repeated variable: $F(4, 156) = 4.93$, $p < 0.001$, and an almost significance in their interaction: $F(4, 156) = 2.45$, $p = 0.050$. Figure 2*B* (left panel) depicts the preference ratio between EtOH solutions and water during days 1 to 20 of the EtOH free-choice test. As expected, preference for the drug decreased as the EtOH

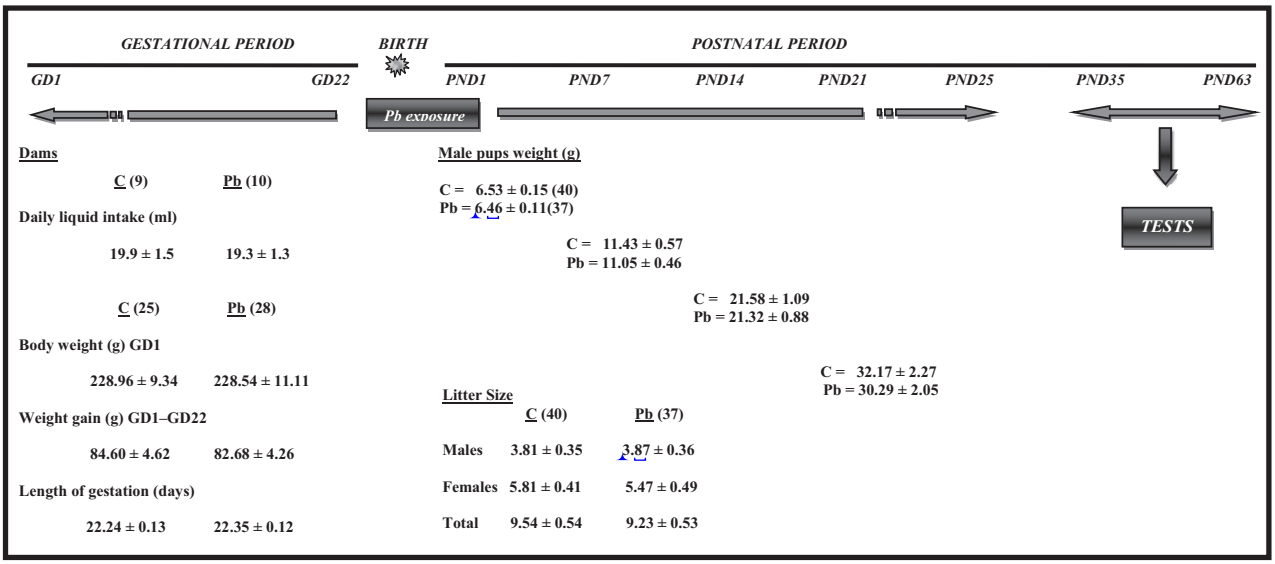

Fig. 1. Timeline for the Pb exposure protocol and data of the mothers and their litters. C, control; Pb, lead-exposed rats; GD, gestational day; PND, postnatal day. Data between parentheses indicate the number of animals in each group.

Table 1. Biochemical Determinations

	EtOH intake groups				
	35d nonEtOH	63d nonEtOH	63d EtOH-SAL	63d EtOH-AT	63d EtOH-3NPA
Blood lead levels (μg/dl)					
C	<0.75 (6)	<0.75 (5)	<0.75 (6)	—	—
Pb	6.51 ± 0.26 (5)	<0.75 (1)	<0.75 (2)	—	—
		<1.80 (5)	<1.80 (3)		
Hemoglobin levels (g/dl)					
C	14.6 ± 0.9 (8)	18.4 ± 0.6 (5)	17.8 ± 1.7 (9)	18.1 ± 1.1 (4)	18.7 ± 0.6 (4)
Pb	11.0 ± 0.4* (8)	20.8 ± 0.5 (5)	16.3 ± 1.1 (6)	20.6 ± 1.2 (4)	21.6 ± 1.3 (4)
Blood EtOH levels (mg/dl)					
C	—	—	4.1 ± 3.2 (9)	55.0 ± 11.8 (9)	4.3 (1)
					<1.0 (3)
Pb	—	—	8.0 ± 4.9 (9)	44.1 ± 21.6 (10)	<1.0 (4)

C, control; Pb, lead-exposed rats; EtOH, ethanol; SAL, saline; AT, 3-amino 1,2,4-triazole; 3NPA, 3-nitropropionic acid. Blood lead levels in μg/dl (top). 0.75 μg/dl = detection limit; 1.80 μg/dl = quantification limit. Hemoglobin levels in g/dl. *($t = -4.44$, $p < 0.01$). Blood alcohol concentrations (BACs) in mg/dl (bottom). 1.0 mg/dl = quantification limit. ANOVA: drug effect: $F(1, 33) = 10.60$, $p < 0.01$. Between parentheses is indicated the number of animals for each group. For group and drug administration protocols see the text.

concentration increased, dropping to 10 to 20% at the 10% EtOH concentrations. A 2-way repeated measures ANOVA revealed significant differences in group: $F(1, 156) = 9.94$, $p < 0.01$, and in the repeated variable: $F(4, 156) = 11.08$, $p < 0.001$.

AT Administration—Starting on test day 21, when 10% EtOH intake was stabilized and demonstrated to be significantly higher in the Pb-exposed rats, CAT inhibition ~~was in effect~~. Accordingly, as indicated in Fig. 2*A* by 2 arrows, 250 mg/kg AT was injected for 8 days (test days 21 to 28) 5 hour before the start of the daily 2-hour free-choice session. The results show that a daily AT injection blunted the elevated EtOH consumption observed in Pb-exposed animals, while a nonsignificant difference was manifested in controls treated with AT compared to the same group vehicle-injected animals. A 3-way repeated measures ANOVA indicates a significant effect of group: $F(1, 37) = 27.80$, $p < 0.001$, drug: $F(1, 37) = 4.28$, $p < 0.05$, and their interaction: $F(1, 37) = 6.74$, $p < 0.02$, as well as a marginal effect for the interaction between the repeated variable and the group: $F(1, 37) = 4.10$, $p = 0.05$. In the same way, AT pretreatment decreased EtOH preference in Pb-exposed rats (Fig. 2*B*). The statistical analysis revealed a significant effect of group: $F(1, 37) = 6.27$, $p < 0.02$, an almost significant difference in drug: $F(1, 37) = 4.09$, $p = 0.05$, a signifi-

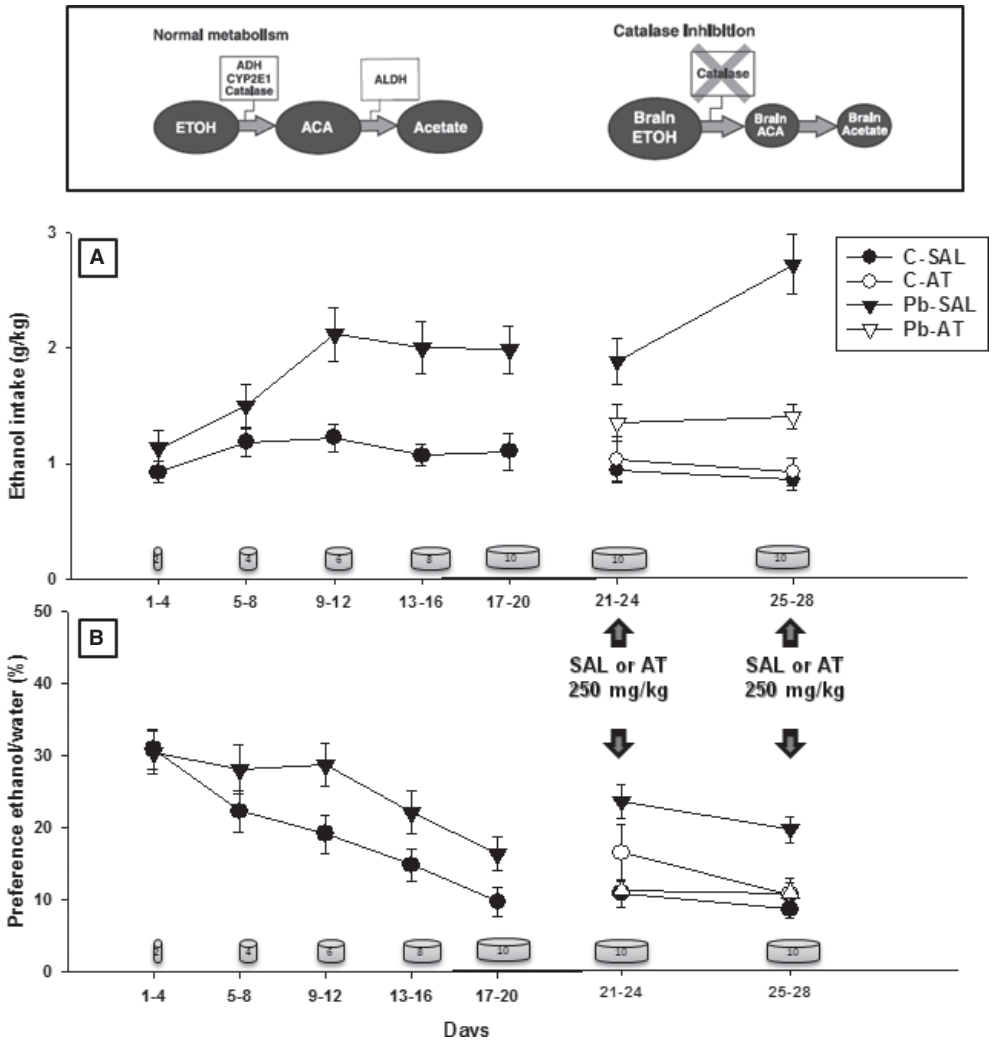

Fig. 2. Voluntary ethanol (EtOH) intake in response to 3-amino 1,2,4-triazole (AT) administration. At the top is depicted the EtOH metabolism in physiological conditions (left) or after CAT pharmacological blockade (right; Quertemont and Didone, 2006). (**A**) EtOH consumption expressed as grams of EtOH per kilogram of body weight. (**B**) Preference ratio EtOH/water. Data (mean ± SE) grouped in 4-day blocks along the horizontal axis correspond to EtOH intake in response to increasing EtOH concentrations symbolized as cylinders (days 1 to 4: 2%; days 5 to 8: 4%; days 9 to 12: 6%; days 13 to 16: 8%, and days 17 to 20: 10%). EtOH intake (g/kg) on days 17 to 20 was C-SAL = 1.13 ± 0.29; C-AT = 1.10 ± 0.17; Pb-SAL = 1.87 ± 0.26; Pb-AT = 2.12 ± 0.32. The arrows signify the start of SAL or AT administration (days 21 to 24 and 25 to 28; 250 mg/kg i.p.) under a 10% EtOH concentration. C-SAL = 10; C-AT = 11; Pb-SAL = 11; Pb-AT = 9 animals per group. CAT, catalase; SAL, saline.

cant interaction between group and drug: $F(1, 37) = 15.48$, $p < 0.001$, as well as in the interaction between group and the repeated variable: $F(1, 37) = 4.59$, $p < 0.05$.

Effects of 3NPA Administration on Ethanol Intake. As presented before, Fig. 3 (top panel) symbolizes a simplified EtOH metabolic pathway (Quertemont and Didone, 2006). The left end represents EtOH oxidation under physiological conditions, while the right denotes CAT activation. Figure 3*A* shows EtOH consumption and Fig. 3*B* the preference ratio between EtOH and water. In all cases, data were collapsed in 4-day blocks corresponding to the same EtOH concentration.

Baseline—Figure 3*A* (left) represents voluntary consumption of the escalating EtOH solutions over time (test days 1 to 20). Increased drug intake was evident in Pb-exposed pups as early as the 2% concentration, while control animals displayed a relatively low and steady EtOH intake for the whole experiment. This is supported by the statistical analysis that revealed a significant effect of group: $F(1, 175) = 111.50$, $p < 0.0001$, the repeated variable: $F(5, 175) = 7.22$, $p < 0.0001$, and their interaction: $F(5, 175) = 2.57$, $p < 0.05$, results that were extensive to the preference to drink EtOH over water (Fig. 3*B*). Thus, a 2-way repeated measures ANOVA reveals a significant effect of group: $F(1, 175) = 99.88$, $p < 0.0001$, and the repeated variable: $F(5, 175) = 6.52$, $p < 0.0001$.

3NPA Administration—In Fig. 3*A* (right), the effect of chronic 3NPA administration on EtOH intake is plotted. As indicated by an arrow, a 4-day injection scheme was chosen

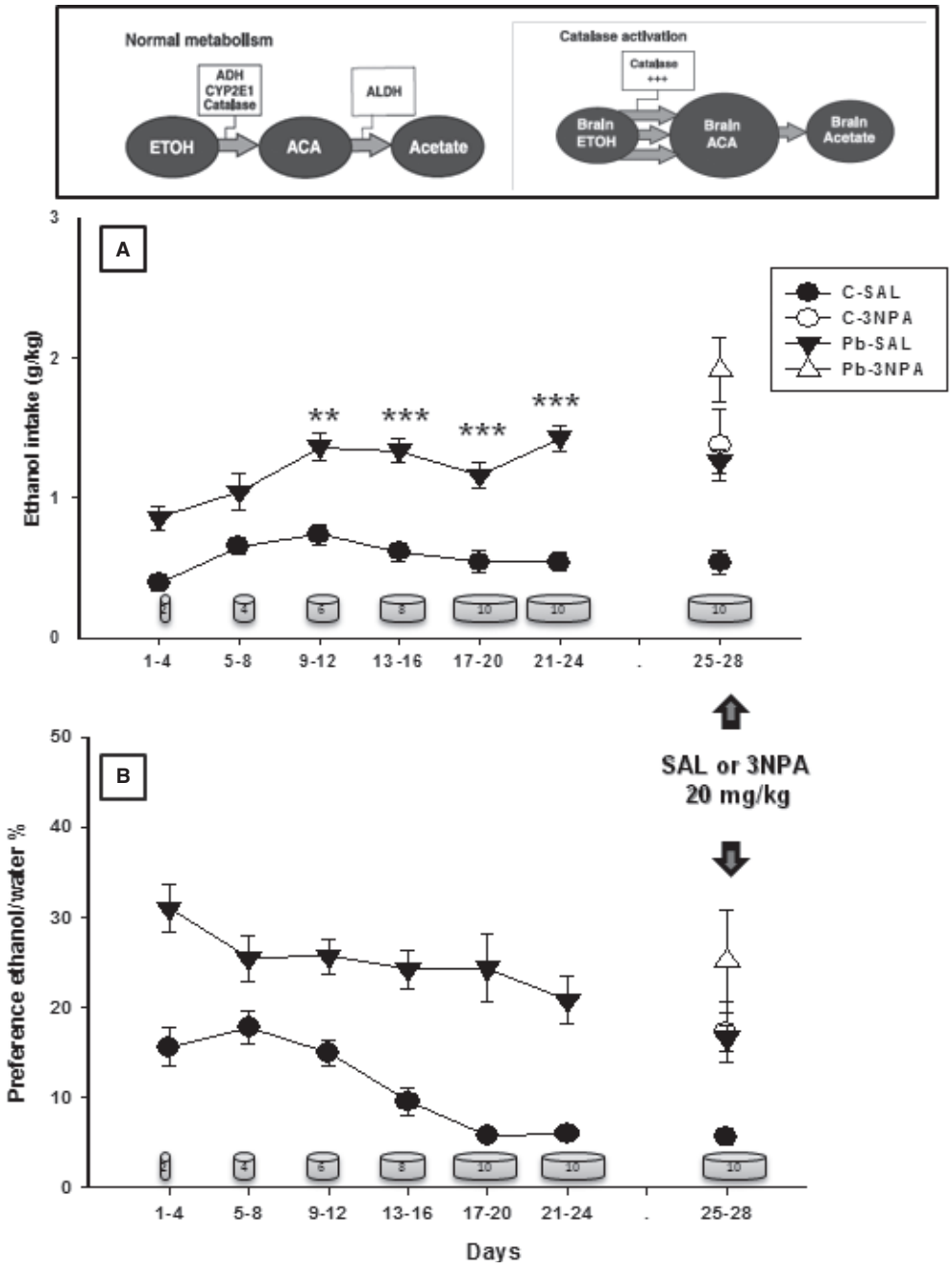

Fig. 3. Voluntary ethanol (EtOH) intake in response to 3NPA administration. At the top is depicted the physiological EtOH metabolism (left) or after CAT pharmacological agonism (right; Quertemont and Didone, 2006). (**A**) EtOH consumption expressed as grams of EtOH per kilogram of body weight. (**B**) Preference ratio EtOH/water. 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 20 and 21 to 24: 10%). EtOH intake (g/kg) on days 21 to 24 was C-SAL = 0.53 ± 0.08; C-3NPA = 0.55 ± 0.10; Pb-SAL = 1.49 ± 0.15; Pb-3NPA = 1.40 ± 0.11. The arrow signifies the start of SAL or 3NPA administration (days 25 to 28; 20 mg/kg s.c.) under a 10% EtOH concentration. Baseline: *denotes statistical difference compared to controls at $^{**}p < 0.01$ and $^{***}p < 0.001$. C-SAL = 8; C-3NPA = 11; Pb-SAL = 9; Pb-3NPA = 9 animals per group. CAT, catalase; SAL, saline.

to avoid neurotoxicity. It is noteworthy that at the end of the study, all animals were alive and no indications of evident toxicity were manifested. Thus, starting on test day 21, total fluid intake was registered for 4 additional days; and thereafter 3NPA administration was implemented (days 25 to 28). The data demonstrate that 3NPA was able to enhance EtOH intake in both control and Pb-exposed animals compared to SAL-injected rats. A 2-way ANOVA reflects a significant effect of group: $F(1, 33) = 9.97$, $p < 0.01$, and drug: $F(1, 33) = 14.43$, $p < 0.001$. A similar effect was attained in the EtOH/water preference ratio, showing high EtOH preference in the Pb-exposed group with a significant difference in group: $F(1, 33) = 6.88$, $p < 0.02$, and in drug: $F(1, 33) = 7.90$, $p < 0.01$.

Sucrose Preference Test. Vehicle Administration—Although all animals showed a clear preference to consume the sweet solution, no differences between groups were observed during the first 4 days of the test (Fig. 4). Subsequent SAL injections (days 5 to 12) did not modify sucrose intake, while a slight enhancement in the preference was seen in both groups, as revealed by a 2-way repeated measures ANOVA: $F(2, 12) = 6.12$, $p < 0.05$, (Fig. 4*A*).

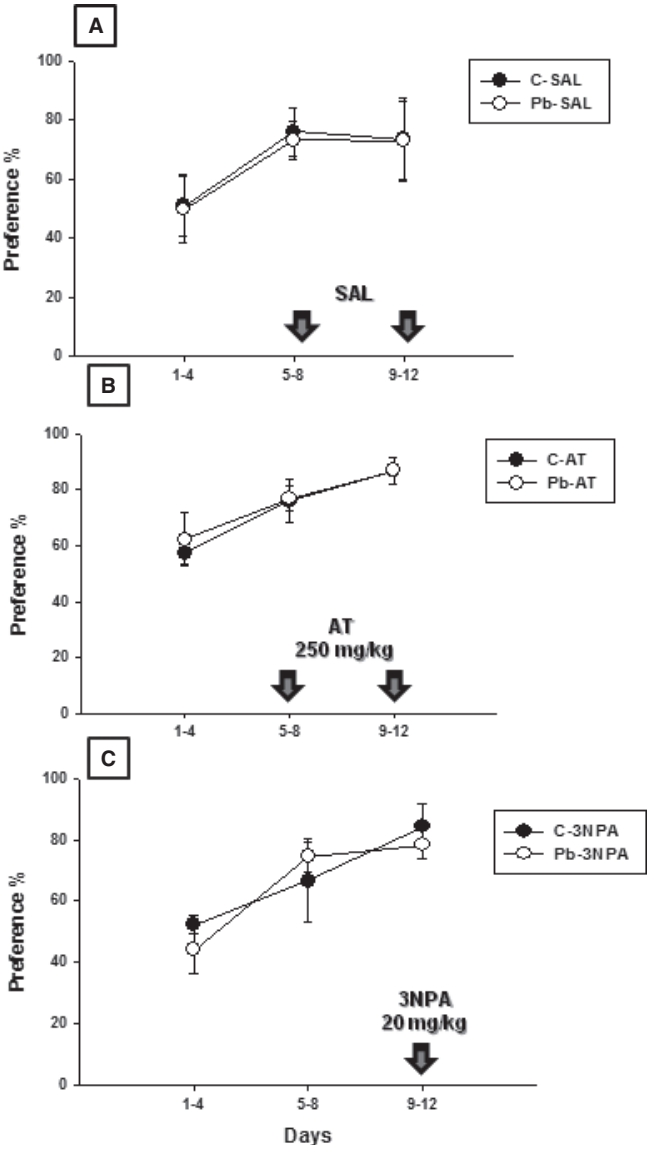

Fig. 4. Voluntary sucrose intake in saline (SAL), 3-amino 1,2,4-triazole (AT), or 3NPA-treated rats. Data (mean ± SE) are grouped in 4-day blocks along the horizontal axis. Sucrose intake is represented as the percent of preference of sucrose over water. The arrows symbolize the start of SAL, AT, or 3NPA administration, as appropriate. (**A**) Sucrose consumption in drug-free conditions (days 1 to 4) and after SAL administration (days 5 to 12). C-SAL = 4; Pb-SAL = 4 animals per group. (**B**) Sucrose consumption in noninjection conditions (days 1 to 4) and after AT administration (days 5 to 12; 0.25 mg/kg i.p.). C-AT = 4, Pb-AT = 4 animals per group. (**C**) Sucrose consumption in noninjection conditions (days 1 to 8) and after 3NPA administration (days 9 to 12; 20 mg/kg s.c.). C-3NPA = 4, Pb-3NPA = 4 animals per group.

AT Administration—3-amino 1,2,4-triazole failed to induce a decrease in preference for the sweet solution; on the contrary, both control and Pb-exposed animals showed a small increase in sucrose intake over time: $F(2, 12) = 14.16$, $p < 0.001$ (Fig. 4*B*).

3NPA Administration—As before, a slight increase in sucrose preference was observed over time in both groups: $F(2, 12) = 15.33$, $p < 0.001$, while no effects can be ascribed to 3NPA administration when compared to baseline values (Fig. 4*C*).

Locomotor Activity. Figure 5 shows total locomotor activity counts (insert) and counts in 10 minute bins of

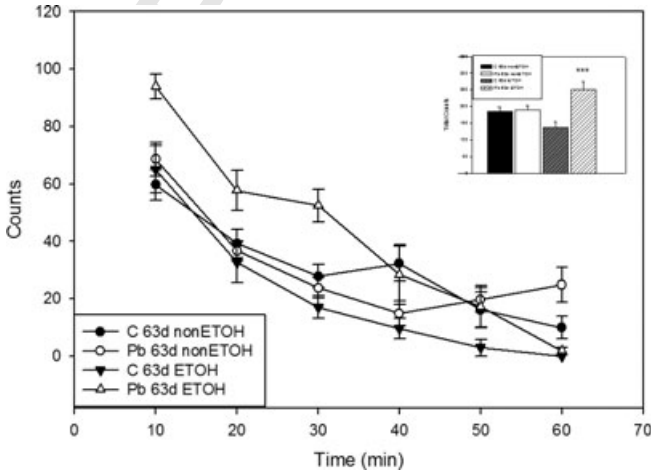

Fig. 5. Effect of voluntary ethanol (EtOH) (10%) intake on total locomotor activity (insert) and on counts measured in 10 minute bins across time. The graphic represents data from both the animals submitted to a 60-minute free-choice EtOH/water protocol immediately before the locomotor test and their nonethanol counterparts, all habituated to the locomotor cages on the previous day. Insert: *denotes significantly different from the controls in the same EtOH condition at ***$p < 0.001$. C 63d EtOH (EtOH) = 11; C 63d non-EtOH = 14; Pb 63d EtOH = 12; Pb 63d non-EtOH = 14 animals per group.

. 6. Acquisition of oral 10% ethanol (EtOH) self-administration under an across-session progressive FR (1 to 20) schedule of reinforcement in control and Pb-exposed rats. (**A**) Mean responses per session (total 28 sessions) on the EtOH-associated lever. (**B**) Mean responses averaged for each FR requirement. The graph depicts the effects of escalating the work requirement for EtOH (FR1 increased to FR2, FR5, FR10, and FR20). Data (mean ± SE) represent *n* = 8 animals per group.

animals submitted to voluntary EtOH intake as well as their nonethanol counterparts habituated to the locomotor cages on the previous day. A repeated measures ANOVA of the 10-minute bins registered on test day revealed a significant effect of group: $F(1, 47) = 11.24$, $p < 0.01$, time: $F(5, 235) = 82.61$, $p < 0.001$, group × EtOH consumption: $F(1,47) = 9.745$, $p < 0.005$, time × EtOH consumption: $F(5,235) = 6.36$, $p < 0.001$, and the overall interaction between group × EtOH consumption × time $F(5, 235) = 3.45$, $p < 0.01$. Furthermore, the ANOVA performed to analyze total activity counts revealed a significant interaction between group and EtOH consumption $F(3, 47) = 7.22$, $p < 0.001$, evidencing that the increase in locomotor activity assessed immediately after the EtOH intake session was observed selectively in the Pb-exposed animals.

CAT Activity. Table 2 show blood and brain CAT activity in control and Pb-exposed animals belonging to the 35d non-EtOH group and to the animals that were submitted to voluntary EtOH intake for 28 days and injected with SAL, AT or 3NPA, as well as to their nonethanol counterparts.

Blood CAT Activity. Firstly, CAT activity was compared at the 2 different ages used in this study in a nonethanol condition (group 35d non-EtOH vs. group 63d non-EtOH-SAL). A 2-way ANOVA demonstrated a significant effect of group: $F(1, 22) = 13.84$, $p < 0.01$, age/EtOH intake: $F(1, 22) = 17.92$, $p < 0.001$, and their interaction: $F(1, 22) = 16.96$, $p < 0.001$.

A separate 2-way ANOVA was performed to compare blood CAT activity in periadolescent animals against adult rats submitted to the voluntary EtOH intake test (group 35d non-EtOH vs. group 63d EtOH-SAL), revealing a significant effect of group: $F(1, 27) = 52.40$, $p < 0.0001$, age/EtOH intake: $F(1, 27) = 10.77$, $p < 0.01$, and their interaction: $F(1, 27) = 12.00$, $p < 0.01$.

On the other hand, data from adult animals were compared by a 3-way ANOVA, which showed a significant effect of group: $F(1, 43) = 11.70$, $p < 0.01$, drug: $F(2, 43) = 13.09$, $p < 0.001$, EtOH: $F(1, 43) = 10.22$, $p < 0.01$, group × drug: $F(2, 43) = 9.72$, $p < 0.001$, group × EtOH: $F(1, 43) = 10.99$, $p < 0.001$, drug × EtOH: $F(1, 43) = 10.86$, $p < 0.001$, and the overall interaction between the 3 factors: $F(2, 43) = 11.27$, $p = 0.001$.

Brain CAT Activity. The data presented in Table 2 reflect Pb-associated increases in CAT activity in most cases, with the exception of those animals treated with AT. In relation to their nonethanol counterparts, it seems that the absence of EtOH intake accounts for mostly Pb-related reductions in CAT activity, which present differences according to the drugs administered to these animals. These observations are supported by the statistical data presented below.

The comparison of the 35d non-EtOH and the 63d non-EtOH groups revealed significant differences in the age variable in the cerebellum: $F(1, 27) = 4.05$, $p < 0.05$, frontal cortex: $F(1, 26) = 13.91$, $p < 0.001$, striatum: $F(1, 26) = 19.36$, $p < 0.001$, and hypothalamus: $F(1, 26) = 33.62$, $p < 0.001$, indicating a differential pattern for CAT enzymatic activity over the lifespan in these areas. The analysis of brain CAT in the hippocampus showed a significant effect of age/EtOH intake: $F(1, 26) = 13.39$, $p < 0.01$, and in the interaction: $F(1, 26) = 10.82$, $p < 0.01$, suggesting a differential response of hippocampal enzyme activity at the ages under study.

Secondly, comparison between the 35d non-EtOH and the 63d EtOH-SAL groups showed a significant effect of age in the cerebellum: $F(1, 27) = 643.45$, $p < 0.001$, striatum: $F(1, 27) = 49.00$, $p < 0.001$, hypothalamus: $F(1, 27) = 18.46$, $p < 0.001$, and nucleus accumbens: $F(1, 9) = 12.67$, $p < 0.01$, with no differences manifested in the frontal cortex, and a significant effect of group: $F(1, 27) = 10.15$, $p < 0.01$, in the hippocampus.

Finally, brain CAT activity for adult animals was analyzed by 3-way ANOVAs that were carried out separately for each brain region. In the cerebellum, significant effects of drug: $F(2, 59) = 19.81$, $p < 0.0001$, EtOH: $F(1, 59) = 19.81$, $p < 0.01$, and the interaction drug × EtOH: $F(2, 59) = 4.92$, $p < 0.01$, were revealed. The statistical analysis of the frontal cortex data showed a significant effect in the group variable: $F(1, 59) = 4.82$, $p < 0.05$, and in the interaction drug × EtOH: $F(2, 59) = 7.00$, $p < 0.002$. The data comparison for the hippocampus showed a significant effect of drug: $F(2, 59) = 15.82$, $p < 0.0001$, group × EtOH: $F(1, 59) = 4.61$, $p < 0.05$, drug × EtOH: $F(2, 59) = 9.68$, $p < 0.001$, and the overall interaction between the 3 factors: $F(2, 59) = 9.49$, $p = 0.001$. In the striatum, there was a significant effect of drug: $F(2, 59) = 18.62$, $p < 0.0001$, EtOH: $F(1, 59) = 4.42$, $p < 0.05$, and drug × EtOH: $F(2, 59) = 5.24$, $p < 0.01$, while the analysis of the hypothalamus results revealed a significant effect of drug: $F(2, 59) = 38.05$, $p < 0.0001$, and drug × EtOH: $F(2, 59) = 13.81$, $p < 0.0001$. Finally, in the nucleus accumbens, only a drug × EtOH interaction emerged: $F(2, 39) = 6.21$, $p < 0.01$.

DISCUSSION

These results provide evidence of the participation of the enzyme CAT in the increased voluntary EtOH intake that we have previously reported in developmentally Pb-exposed rats (Virgolini et al., 1999). In effect, pretreatment with the CAT antagonist AT was able to blunt the increased voluntary EtOH intake, as well as the elevated blood and brain (cerebellum and hippocampus) CAT activity observed in perinatally Pb-exposed rats after EtOH consumption. In contrast, CAT overactivation correlated well in these animals with the increased EtOH intake shown after 3NPA administration. The operant EtOH self-administration and the EtOH-induced hyperlocomotion results further establish the interaction between perinatal Pb exposure and the behavioral effects observed after EtOH consumption in adult animals. Importantly, these effects were associated with blood Pb levels nearly the threshold considered safe in developing organisms (Canfield et al., 2003; CDC, 2012).

Table 2. Blood CAT Activity Expressed as mmol H_2O_2/min/g Hemoglobin

	SAL			AT		3NPA	
	35d non-EtOH	63d non- EtOH	63d EtOH	63d non- EtOH	63d EtOH	63d non- EtOH	63d EtOH
Blood CAT activity							
C	47.3 ± 8.0 (8)	45.1 ± 10.6 (5)	40.2 ± 8.1 (9)	32.7 ± 1.9 (4)	0.47 ± 0.07 (4)	69.4 ± 5.9 (3)	25.8 ± 5.9 (4)
Pb	191.5 ± 26.5 (8)###	37.8 ± 6.1 (5)$$$	449.5 ± 91.6 (6)###,$$$	33.6 ± 7.3 (4)	0.26 ± 0.05 (8) ***	60.7 ± 6.3 (3)	28.3 ± 5.7 (4)***
Brain CAT activity							
Cerebellum							
C	0.49 ± 0.08 (8)	1.90 ± 0.48 (8)	0.56 ± 0.11 (8)^^^	0.39 ± 0.06 (4) ***	0.47 ± 0.07 (8)	2.72 ± 0.14 (5)***	0.38 ± 0.05 (4)
Pb	1.08 ± 0.21 (7)	1.97 ± 0.32 (7)	1.02 ± 0.12 (8) ^^^	0.32 ± 0.15 (3) ***	0.26 ± 0.05 (8)	2.36 ± 0.07 (4)***	0.45 ± 0.05 (4)
Cortex							
C	0.59 ± 0.03	0.34 ± 0.07	0.50 ± 0.06	0.66 ± 0.07	0.86 ± 0.12	0.60 ± 0.08	0.45 ± 0.05
Pb	0.58 ± 0.05	0.34 ± 0.09	0.44 ± 0.08	0.25 ± 0.06	0.30 ± 0.10	0.73 ± 0.04	0.29 ± 0.03
Hippocampus							
C	0.37 ± 0.03	1.24 ± 0.19$$$	0.33 ± 0.05^^^	0.14 ± 0.09***	0.63 ± 0.06	0.21 ± 0.03***	0.18 ± 0.02
Pb	0.54 ± 0.10	0.58 ± 0.10##	0.90 ± 0.18	0.04 ± 0.00	0.42 ± 0.10	0.22 ± 0.00	0.19 ± 0.01*
Striatum							
C	0.48 ± 0.09	2.15 ± 0.52	1.98 ± 0.20	0.20 ± 0.04***	2.16 ± 0.40^^	0.38 ± 0.04**	0.45 ± 0.07**
Pb	0.82 ± 0.20	2.75 ± 0.61	2.12 ± 0.27	0.24 ± 0.08***	2.76 ± 0.62^^	0.34 ± 0.02**	0.50 ± 0.07**
Hypothalamus							
C	1.07 ± 0.05	4.47 ± 0.90	2.04 ± 0.34^^^	0.43 ± 0.16***	1.75 ± 0.76	0.54 ± 0.07***	0.41 ± 0.05*
Pb	0.64 ± 0.11	3.68 ± 0.50	1.88 ± 0.23^^^	0.32 ± 0.07***	1.33 ± 0.15	0.48 ± 0.04***	0.38 ± 0.06*
Nucleus accumbens							
C	0.12 ± 0.03 (4)	0.11 ± 0.03 (8)	0.43 ± 0.03 (2)	0.20 ± 0.04 (4)	0.27 ± 0.12 (6)	0.62 ± 0.06 (5) ***	0.42 ± 0.07 (4)^
Pb	0.14 ± 0.06 (4)	0.27 ± 0.04 (7)	0.29 ± 0.10 (3)	0.24 ± 0.01 (3)	0.20 ± 0.06 (4)	0.79 ± 0.11 (4) ***	0.22 ± 0.03 (3)^

3NPA, 3-nitropropionic acid; AT, 3-amino 1,2,4-triazole; CAT, catalase; SAL, saline.

The numbers between parentheses indicate the number of animals per group. $denotes a significant difference compared to periadolescent animals from the same group belonging to the SAL EtOH and nonethanol conditions at $$$p < 0.001. *denotes a significant difference from the SAL-injected animals in the same group and EtOH condition at ***$p < 0.001$. #denotes a significant difference from the control in the same drug and EtOH condition at #$p < 0.05$, and ##$p < 0.001$. Brain CAT activity expressed as mmol H_2O_2/ml/mg protein. The numbers between parentheses in the cortex indicate the number of animals per group and are extensive to all other regions of the same group, except for the nucleus accumbens, where the corresponding number of animals is indicated. $denotes a significant difference in animals from the same group at different ages in either the EtOH or nonethanol conditions at $$p < 0.05$; $$$p < 0.01$, and $$$$p < 0.001$. *denotes a significant difference from the SAL-injected animals in the same EtOH condition at *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$. ^significantly different from the same drug nonethanol condition at ^$p < 0.05$, ^^$p < 0.01$, and ^^^$p < 0.001$. #denotes a significant difference from the control in the same drug and EtOH condition at #$p < 0.05$, ##$p < 0.01$, and ###$p < 0.001$.

Thus, either the presence of the metal in the brain at the beginning of the test, the initiation of EtOH consumption in adolescence (considered a highly risky period for the initiation of drug addiction (Spear, 2000)), or the neurotoxic imprint caused by developmental Pb exposure may be responsible for the permanent alterations in EtOH intake. In light of the results obtained, particularly in BECs and blood and brain CAT activity, and considering that both drugs were administered systemically, whether CAT-mediated Pb effects are central or systemic is an issue that remains to be investigated.

Several considerations indicate that EtOH's pharmacological effects rather than its orosensory confounding factors are affected by Pb exposure. First, we have previously reported that Pb-exposed rats demonstrated a higher sensitivity to the anxiolytic, motivational, and sedative effects of EtOH (Virgolini et al., 1999). Secondly, it is known that the free-choice test shows a positive correlation with operant oral self-administration procedures (Green and Grahame, 2008), a relationship that we also report in this study. In the third place, the finding that both groups drink similar amounts of sucrose provides additional evidence of the selectivity of EtOH as a reinforcer in the Pb-exposed rats. In addition, according to the "psychomotor activation" hypothesis, the hyperactivity induced by EtOH has been proposed to reflect the positive stimulating properties of the drug (Wise and Bozarth, 1987). Accordingly, the amount of EtOH consumed during the free-choice test seemed enough to induce hyperlocomotion selectively in Pb-exposed animals, results that are in agreement with studies in AA and P rats (Colombo et al., 1998; Päivärinta and Korpi, 1993). Finally, it has been suggested that the higher the activity of CAT, the higher the rate of acetaldehyde formation in the brain and the higher the increase of locomotion produced by EtOH (Correa et al., 2012), a hypothesis that requires future consideration in our animals.

In relation to the pharmacological approaches, AT is an herbicide that has been used as a CAT antagonist because it induces a slowly developing irreversible inhibition of enzyme catalytic activity by reacting with the CAT-H_2O_2 complex. Therefore, we can postulate that the higher AT inhibition exerted on EtOH intake might be related to an enhanced ROS (H_2O_2) production in the Pb-exposed rats. Moreover, the lack of effect of AT in control animals suggests that the inhibition of the enzyme requires either an elevated EtOH intake or high H_2O_2 content for the pharmacological antagonism to be evident. These data contrast with evidence suggesting that the AT effect on EtOH consumption is secondary to a reduction in the appetite for calories and not related to CAT inhibition (Rotzinger et al., 1994; Tampier et al., 1995), an assertion that diverges from both, reports from Aragon and Amit (1992) in which AT reduces EtOH intake (and brain CAT) without affecting total fluid consumption, and from the sucrose intake experiment presented

here. However, a recent report elegantly resolves these uncertainties (Karahanian et al., 2011). These authors microinfused a lentiviral vector that encodes a shRNA against CAT synthesis into the ventral tegmental area (VTA) from the low and high drinking strain of rats (UChA and UChB rats, respectively), a strategy that abolishes voluntary consumption of alcohol in these animals.

On the other hand, only few studies have addressed the consequences of pharmacological elevation in CAT activity. Although using 2 different behavioral outputs, our results match those of Manrique and colleagues (2006). In both studies, either EtOH-induced hyperlocomotion (their data) or voluntary intake (our results) was accompanied by an increase in CAT activity after 3NPA administration. Given that 3NPA is a neurotoxin that produces Huntington's disease-like symptoms in animal models by damaging striatal neurons (Kumar et al., 2010), we were able to achieve CAT activation (although modest and selectively in Pb-exposed animals) with minimal 3NPA-associated neurotoxicity (see Supporting Information).

Finally, and with reference to anatomical considerations, we found that Pb exposure alone increases CAT activity in the cerebellum, a finding that parallels the higher erythrocyte CAT activity measured in the same animals. Additionally, it is noteworthy that the Pb-exposed groups' EtOH intake at a later age not only further elevated blood CAT activity, but was also accompanied by higher CAT activity in the cerebellum, which along with the hippocampus is considered one of the preferential sites for Pb deleterious effects (Finkelstein et al., 1998; Oberto et al., 1996). Indeed, hippocampal CAT activity also shows a Pb and EtOH interaction in the groups that have consumed EtOH injected with either vehicle or 3NPA. It is also remarkable that brain CAT activity in this area showed opposite directions according to the EtOH consumption background. Yet more notable is the fact that the striatum, the region most consistently reported affected by 3NPA evidenced the maximal 20 mg/kg 3NPA-induced CAT activity in Pb-exposed animals in relation to the control counterparts. The finding of high CAT expression in aminergic neurons (Moreno et al., 1995; Zimatkin and Lindros, 1996) suggests that the acetaldehyde produced in these structures could mediate some of the activation of the dopaminergic neurons occurring as part of EtOH effects on the central nervous system. Overall, it appears that EtOH intake stimulates, while its absence decreases brain CAT activity in selected regions, an effect which is restricted to perinatal Pb exposure and persists in spite of the cessation of Pb exposure.

In conclusion, these results provide additional evidence of an increased EtOH self-administration in the Pb-exposed animals and support the participation of CAT in Pb effects on EtOH intake, an interaction that may be mediated by both the antioxidant and metabolic properties of the enzyme. Further investigations are necessary to better clarify Pb effects on CAT, to differentiate central versus systemic CAT participation, and to ascribe these alterations to the putative participation of centrally-formed acetaldehyde.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Acquisition of oral 10% ethanol self-administration under an across-session progressive FR (1 to 20) schedule of reinforcement in control and Pb-exposed rats.

Fig. S2. Body weight slope for the animals that have consumed ethanol and their non-ethanol counterparts.

Fig. S3. Voluntary ethanol intake in response to 3NPA administration (10 mg/kg s.c. for 4 days).

Fig. S4. Voluntary ethanol intake in response to 3NPA administration (30 mg/kg s.c. for 4 days).

Fig. S5. Nissl stain of representative saline and AT-treated rats (250 mg/kg i.p. 8 days).

Fig. S6. Nissl stain of representative saline and 3NPA-treated rats (20 mg/kg s.c. 4 days).

Data S1. Material and methods.

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10	**AUTHOR: Table 1 has been changed to Figure 1. Similar changes are made in text also. Please check.**	
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17	**AUTHOR: Please provide Fig. 6 and also cite the figure in the appropriate location in the text.**	

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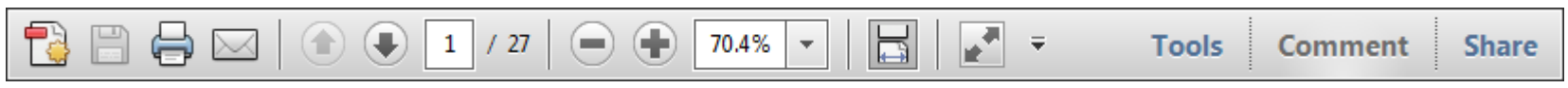
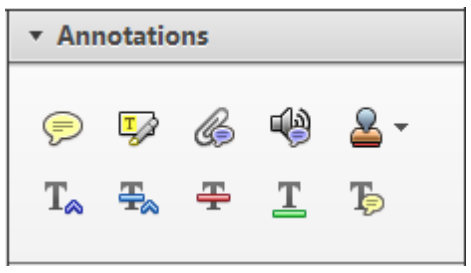
USING e-ANNOTATION TOOLS FOR ELECTRONIC PROOF CORRECTION

Required software to e-Annotate PDFs: Adobe Acrobat Professional or Adobe Reader (version 7.0 or above). (Note that this document uses screenshots from Adobe Reader X)
The latest version of Acrobat Reader can be downloaded for free at: http://get.adobe.com/uk/reader/

Once you have Acrobat Reader open on your computer, click on the Comment tab at the right of the toolbar:



This will open up a panel down the right side of the document. The majority of tools you will use for annotating your proof will be in the Annotations section, pictured opposite. We've picked out some of these tools below:

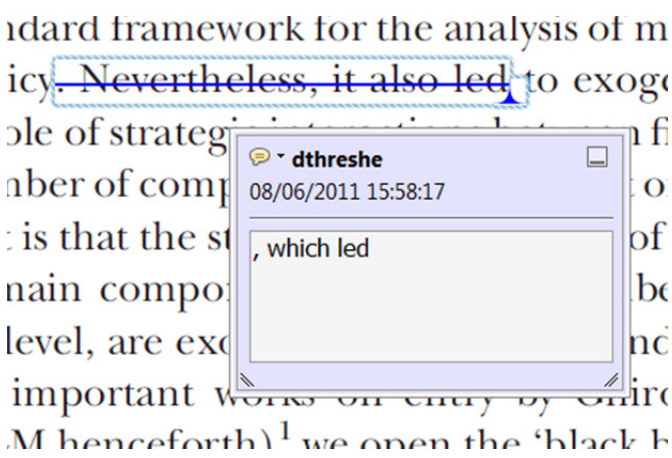


1. Replace (Ins) Tool – for replacing text.

Strikes a line through text and opens up a text box where replacement text can be entered.

How to use it

- Highlight a word or sentence.
- Click on the Replace (Ins) icon in the Annotations section.
- Type the replacement text into the blue box that appears.

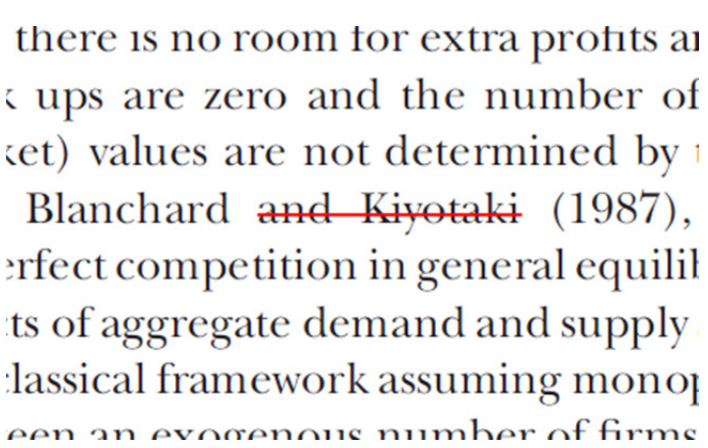


2. Strikethrough (Del) Tool – for deleting text.

Strikes a red line through text that is to be deleted.

How to use it

- Highlight a word or sentence.
- Click on the Strikethrough (Del) icon in the Annotations section.

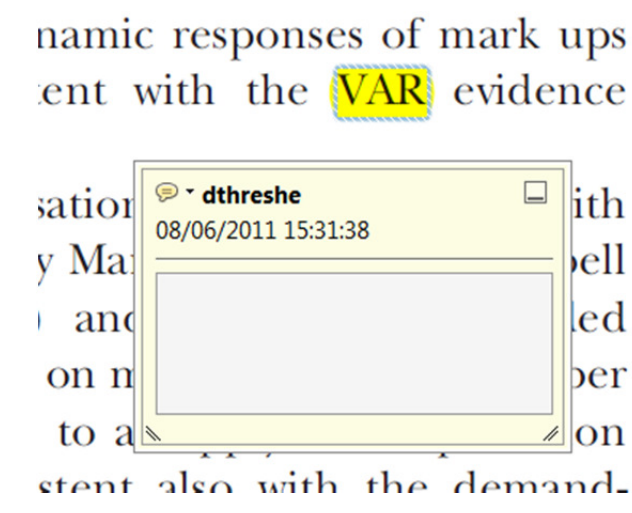


3. Add note to text Tool – for highlighting a section to be changed to bold or italic.

Highlights text in yellow and opens up a text box where comments can be entered.

How to use it

- Highlight the relevant section of text.
- Click on the Add note to text icon in the Annotations section.
- Type instruction on what should be changed regarding the text into the yellow box that appears.

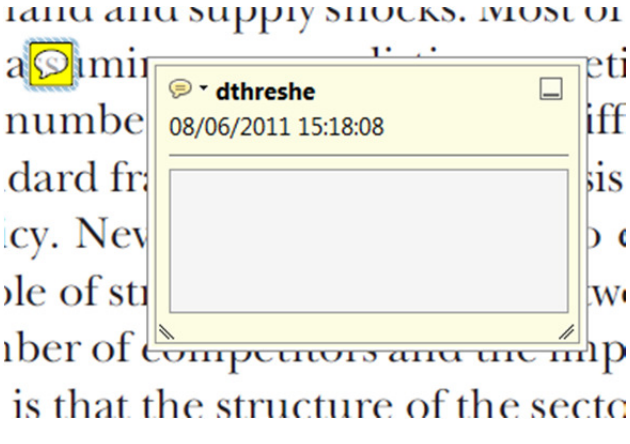


4. Add sticky note Tool – for making notes at specific points in the text.

Marks a point in the proof where a comment needs to be highlighted.

How to use it

- Click on the Add sticky note icon in the Annotations section.
- Click at the point in the proof where the comment should be inserted.
- Type the comment into the yellow box that appears.



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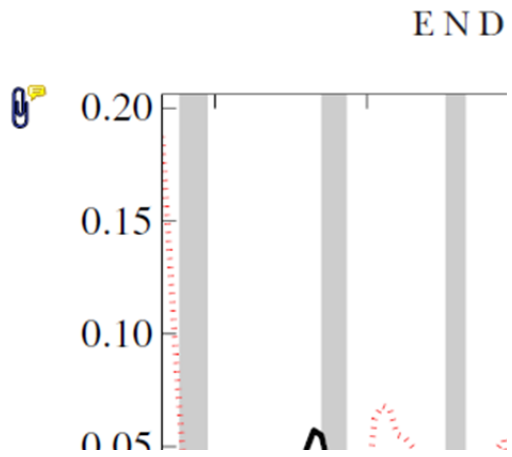
USING e-ANNOTATION TOOLS FOR ELECTRONIC PROOF CORRECTION

5. Attach File Tool – for inserting large amounts of text or replacement figures.

Inserts an icon linking to the attached file in the appropriate pace in the text.

How to use it

- Click on the Attach File icon in the Annotations section.
- Click on the proof to where you'd like the attached file to be linked.
- Select the file to be attached from your computer or network.
- Select the colour and type of icon that will appear in the proof. Click OK.

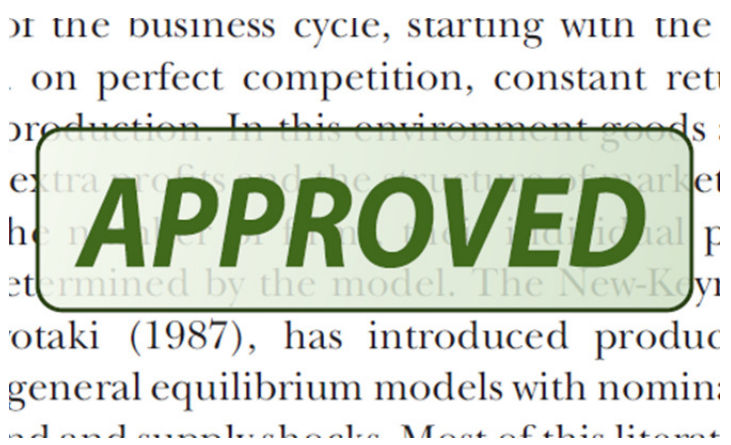


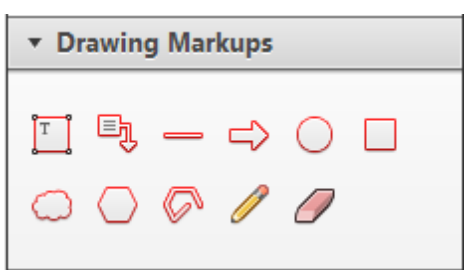
6. Add stamp Tool – for approving a proof if no corrections are required.

Inserts a selected stamp onto an appropriate place in the proof.

How to use it

- Click on the Add stamp icon in the Annotations section.
- Select the stamp you want to use. (The Approved stamp is usually available directly in the menu that appears).
- Click on the proof where you'd like the stamp to appear. (Where a proof is to be approved as it is, this would normally be on the first page).



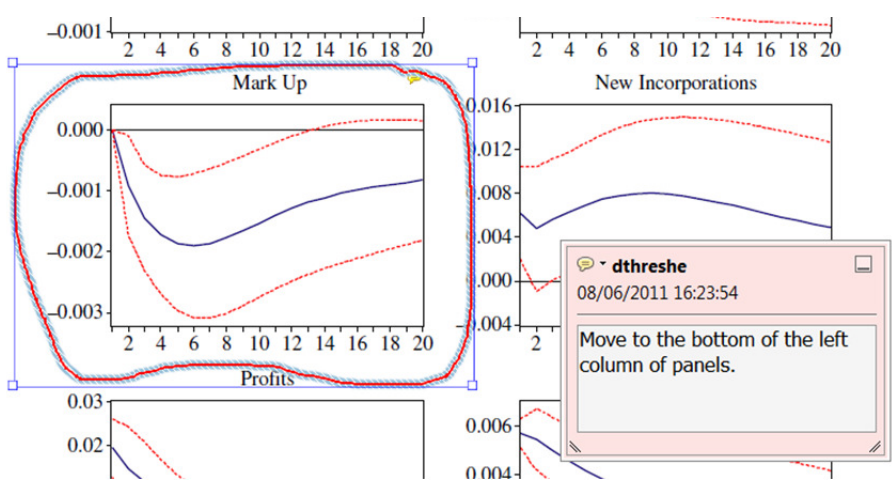


7. Drawing Markups Tools – for drawing shapes, lines and freeform annotations on proofs and commenting on these marks.

Allows shapes, lines and freeform annotations to be drawn on proofs and for comment to be made on these marks..

How to use it

- Click on one of the shapes in the Drawing Markups section.
- Click on the proof at the relevant point and draw the selected shape with the cursor.
- To add a comment to the drawn shape, move the cursor over the shape until an arrowhead appears.
- Double click on the shape and type any text in the red box that appears.



For further information on how to annotate proofs, click on the Help menu to reveal a list of further options: