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Low-level developmental lead exposure does not predispose to adult alcohol self-administration, but does increase the risk of relapsing to alcohol seeking in mice: Contrasting role of GLT1 and xCT brain expression

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ABSTRACT

Lead (Pb) is a neurotoxic heavy metal pollutant. Despite the efforts to reduce Pb environmental exposure and to prevent Pb poisoning, exposure in human populations persists. Studies of adults with history of childhood lead exposure have consistently demonstrated cognitive impairments that have been associated with sustained glutamate signaling. Additionally, some clinical studies have also found correlations between Pb exposure and increased proclivity to drug addiction. Thus, here we sought to investigate if developmental Pb exposure can increase propensity to alcohol consumption and relapse using an alcohol self-administration paradigm. Because Pb exposure is associated with increased glutamatergic tone, we also studied the effects on the expression of synaptic and non-synaptic glutamate transporters in brain regions associated with drug addiction such as the nucleus accumbens (NAc), dorsomedial striatum (DMS), dorsolateral striatum (DLS), and medial prefrontal cortex (mPFC). We found that while developmental Pb exposure did not increase risk for alcohol self-administration, it did play a role in relapsing to alcohol. The effects were associated with differential expression of the glutamate transporter 1 (GLT1) and the glutamate/cystine antiporter (xCT). In the NAc and DLS the expression of GLT1 was found to be significantly reduced, while no changes were found in DMS or mPFC. Contrastingly, xCT was found to be upregulated in NAc but downregulated in DLS, with no changes in DMS or mPFC. Our data suggest that lead exposure is involved in relapse to alcohol seeking, an effect that could be associated with downregulation of GLT1 and xCT in the DLS.

1. Introduction

Lead (Pb) is a common neurodevelopmental toxin that causes serious harm and significant health problems, even at low levels of exposure (Canfield et al., 2003). More recently, additional neurodevelopmental and physiological alterations induced by Pb exposure have been described in detail (see Rahman, 2014; Mitra et al., 2017).

Studies in adults with a history of childhood lead exposure have consistently demonstrated cognitive impairments (Bellinger, 1995; Canfield et al., 2003; Fishbein et al., 2008), and many of the neurological alterations have been associated with disruptions of glutamate signaling (Vazquez and Pena de Ortiz, 2004; Rahman, 2014). In fact, the glutamate transport system was found affected by Pb treatment in experimental conditions as well. For example, Pb exposure significantly reduced both mRNA and protein expression of glutamate transporter-1 (GLT1) (Struzynska et al., 2005a, 2005b), which is responsible for up to 90% of synaptic glutamate removal after its release (Danbolt, 2001). Moreover, Pb exposure alters the expression of synaptic and extra-synaptic NMDA receptors (Neal et al., 2011; also see Rahman, 2014).

Abbreviations: BEC, blood ethanol concentration; BLL, blood lead levels; DLS, dorsolateral striatum; DMS, dorsomedial striatum; GLT1, glutamate transporter-1; LTP, long-term potentiation; mPFC, medial prefrontal cortex; NAc, nucleus accumbens; NMDA, N-methyl-D-aspartate; Pb, lead; ROS, reactive oxygen species; xCT, glutamate/cystine antiporter.

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In addition to the deleterious effects on cognition, clinical and preclinical studies have found correlations between Pb exposure and increased proclivity to drug addiction for cocaine and opioids (Nation et al., 2004; Fishbein et al., 2008). Accordingly, in many models of addiction, synaptic glutamate strength plays a key role on the reinforcing properties of drugs (McFarland et al., 2003; Kalivas, 2009; Gass et al., 2011). Neuroadaptive changes in glutamate transmission in the ventral portion of the striatum or nucleus accumbens (NAc), an integrative region that encodes incentive motivational behavior (Flagel et al., 2011), has been widely associated with addictive patterns including alcohol use disorders (Kapasova and Szumlinski, 2008; Pati et al., 2016; Gass et al., 2011). However, the major glutamate input onto NAc comes from the medial prefrontal cortex (mPFC), which also projects to the dorsal portion of the striatum. The dorsal striatum can be anatomically subdivided into the dorsomedial striatum (DMS) and dorsolateral striatum (DLS). This is meaningful because evidence indicates shifts in the functional topographic corticostriatal connectivity in alcohol use disorders (Gerchen et al., 2019; also see Lüscher et al., 2020). The DMS for example, mainly engages in goal-directed actions and thus, in goal-directed alcohol seeking, while the DLS plays a key role in habit formation and participates in the development of habitual alcohol use (Balleine and O'Doherty, 2010; Corbit et al., 2012; Sjoerds et al., 2013; Lüscher et al., 2020). Therefore, the complementary roles of the striatum suggest that disruption of glutamate transmission can cause a variety of functional circuitry reshaping that could, in turn, arrange the development of behavioral responses to alcohol including, self-administration, seeking and relapse.

An important consideration is that the extracellular concentration of glutamate is regulated by synaptic and non-synaptic release. At the synapse, astrocytic GLT1 plays a crucial role in glutamate removal (Danbolt, 2001), while at the extra-synaptic level, the glutamate/cystine antiporter (X_c^-) system releases glutamate (Lewerenz et al., 2006). This antiporter is a plasma membrane protein Na^+-dependent transporter located in astrocytes that exchanges extracellular cystine for intracellular glutamate in a 1:1 ratio (Bannai, 1986; Danbolt, 2001). It is estimated that ~60% of the basal extracellular glutamate is derived from the xCT system (Baker et al., 2002, 2003). Thus, together GLT1 and xCT maintain glutamate homeostasis. Importantly, it has been found that following continuous alcohol access the expression of GLT1 is downregulated in alcohol-preferring rats (Sari et al., 2013), while no changes are observed in xCT (Pati et al., 2016). Nonetheless, upregulation of both transporters GLT1 and xCT can reduce voluntary alcohol consumption (Alhaddad et al., 2014; Das et al., 2016; Rao et al., 2015), and attenuate ethanol reinstatement (Weiland et al., 2015). Similar effects have been found for other substances of abuse. For instance, alterations of these neural components have been associated with cocaine relapse (see Rangel-Barajas and Rebec, 2017), and upregulation GLT1 and xCT in the NAc attenuates reinstatement of cocaine seeking (Knackstedt et al., 2010). Furthermore, in NAc and hippocampus but not mPFC, upregulation of the transporters using ceftriaxone (a β-lactamic antibiotic that upregulates GLT1 and xCT), also attenuated opioid seeking (Alshehri et al., 2018), and while ceftriaxone had no effect on development of nicotine preference, it did reduce nicotine-induced reinstatement (Alajaji et al., 2013). Because the known effects of Pb on the expression of glutamate transporters and their relation with drug addiction, we sought to study if low-level developmental Pb exposure increases propensity to alcohol consumption and relapse using a self-administration paradigm, and the associated effects on the expression of synaptic and non-synaptic glutamate transporters in the aforementioned regions of the striatum and mPFC.

2. Materials and methods

2.1. Subjects

Male and Female C57BL/6J mice (bred on site from parents purchased from the Jackson Laboratory, Bar Harbor, ME) were housed and kept under controlled conditions of humidity (50%) and temperature (21±1 °C), with water and food available *ad libitum* (unless stated otherwise), and maintained on a 12h light/dark cycle. The experimental procedures were carried out in accord with the IUPUI School of Science Animal Care Use Committee and adhered to guidelines set forth by the National Institutes of Health.

2.2. Developmental lead exposure

Mice were weaned and grouped-housed at PND21, they were randomly assigned to either experimental group (Pb-treated; 30 ppm of lead) or control group (no-Pb treatment). We used low-level Pb exposure previously reported (Flores-Montoya et al., 2015). Our goal was to mimic the Pb exposure levels often seen in kids growing up in industrialized cities such as Indianapolis (Filippelli et al., 2015; Beidinger-Burnett et al., 2019). For the experimental group, the Pb was dissolved in glacial acetic acid (0.5%) and distilled water. The same acetate water solution was used to treat the control group. Both groups were kept with Pb-acetate or acetate water solution for three weeks (or until PND42). At that time, all cage bottles were switched to regular tap water, and left in the same housing conditions until mice reached PND60. Mice were then single-housed for at least one week before onset of self-administration training. Mice were counterbalanced by sex, family, and treatment. Forty-four mice were used (12 Pb male; 12 Pb female; 10 control male; 10 control female). Only two mice did not meet the criteria in FR1 of operant conditioning, and therefore were not included in subsequent behavioral testing. At the end of the behavioral assessment, all animals were euthanized for subsequent proteomic analysis. In a separate group of animals, blood lead levels (BLL) were determined using the Lead Care III system according to the manufacturer's instructions (which has a limit of sensitivity of 3.3 μg/dL). The BLL for Pb-treated group were lower than 10 μg/dL ($n = 16$), while all mice from the control group ($n > 50$) tested below the detectable limits (data not shown).

2.3. Self-administration apparatus

Alcohol self-administration training, extinction, reinstatement, and relapse sessions were conducted in ten operant conditioning chambers (Med Associates, St. Albans, VT). Each chamber with stainless-steel grid-floor and modular test for mouse (dimensions 21.6 × 19.7 × 12.7 cm) was placed inside a light and sound-attenuating cubicle equipped with a house fan that provided ventilation. The operant conditioning chambers were equipped with two retractable ultra-sensitive stainless-steel response levers with a yellow cue-light positioned above each lever. At the center of the two levers, a sipper tube opening where the graduated pipet sipper filled with saccharin, water, or alcohol descends when a correct response is evoked. After a correct response, the cue-light is turned-off and the retractable sipper descends for 20, 15 or 10 s depending on the training phase. When the reward access time is completed, the sipper is retracted and the cue-light turned-on until the following correct response occurs. Parameters such as session duration, number of lick contacts, and correct and incorrect lever presses were recorded using MED-PC V Software (Med Associates, St. Albans, VT). Alcohol intake was accurately measured by recording the initial and final volume in the graduated pipet sipper.

2.4. Alcohol self-administration procedure

The purpose of the acquisition phase was to establish a relationship between an instrumental response (i.e., pressing the active lever) and its consequence (i.e., delivery of sipper with alcohol solution for a limited access). The operant training was performed on consecutive days. The timeline for the alcohol self-administration procedure is illustrated in Fig. 1. Following one week of acclimation to the single-housed

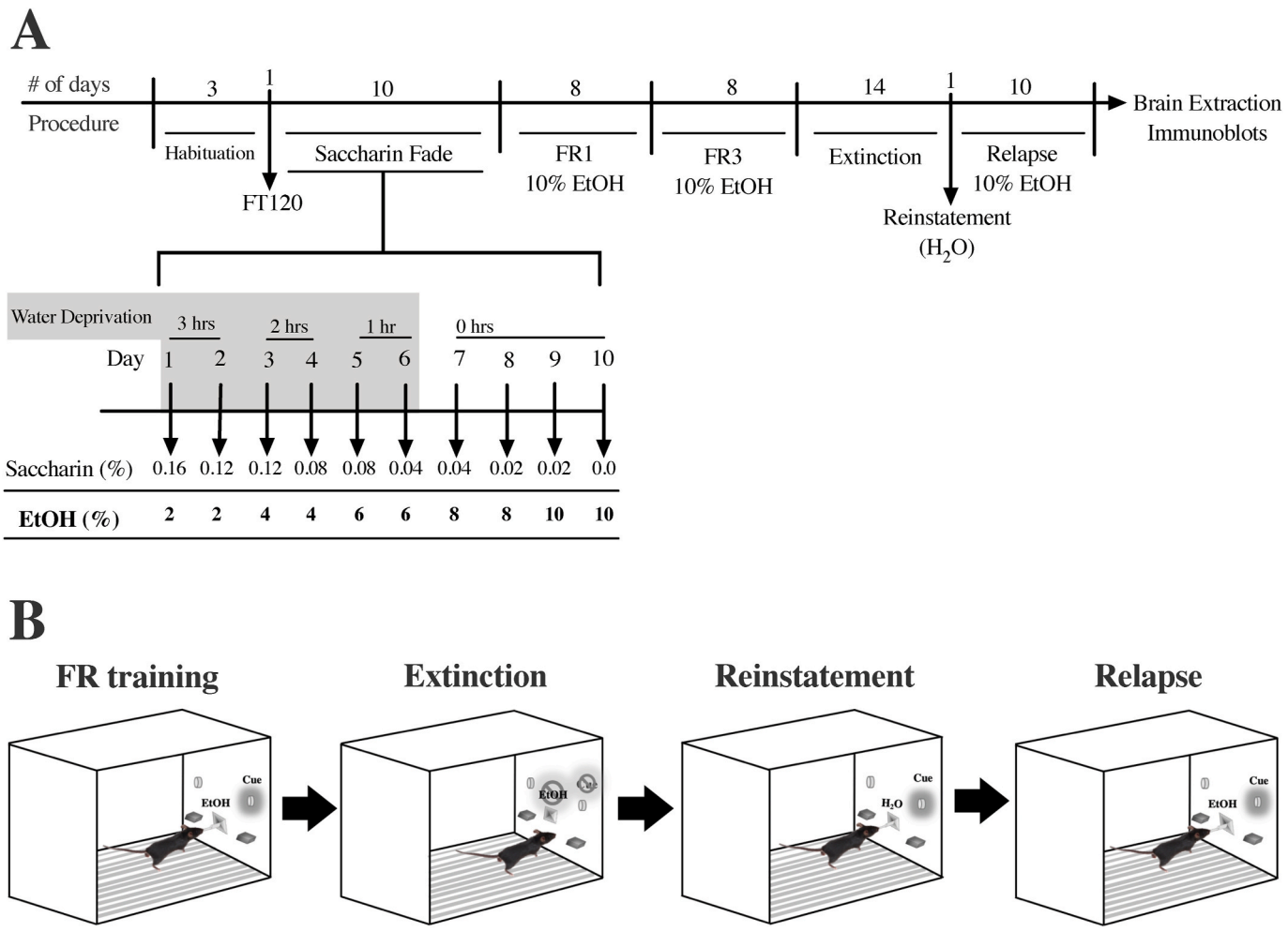

Fig. 1. Experimental procedures. A) Timeline for experimental procedure showing each training phase in days, the insert shows the saccharin fade procedure, and water deprivation scheme. B) Pictogram of self-administration chambers showing four phases: FR training, extinction, reinstatement, and relapse. The cue-light was presented during self-administration (FR training), reinstatement session, and relapse phase, but not during extinction. EtOH was available only during the self-administration training and relapse phases, but not during extinction phase or the cue-induced reinstatement session.

condition, all mice were habituated for three days to the operant chambers (30-min session). Animals were water deprived for 12-h prior to the initial training using a fixed time-120s (FT120) schedule using saccharin solution (0.2% w/v), with 20s sipper access for a single day in a 30-min session. Following the FT120 session, all animals underwent a modified fade procedure using saccharin as sweetener (McCool and Chappell, 2012). Daily saccharin concentrations were gradually decreased (from 0.16% to 0% w/v); while alcohol concentrations were gradually escalating (from 2 to 10% v/v) over 10 days (see Fig. 1A). The active lever was randomly assigned for each mouse and was reinforced on a fixed ratio 1 (FR1) in 45-min sessions with sipper access time of 15s. As the animals learned to lever press, the amount of sweetener was gradually faded out **(Fig. 1A)**, the amount of time of water deprivation progressively decreased as well, and after six days into the saccharin fade procedure no more water deprivation was used. Next, animals continued training in FR1 schedule using unsweetened alcohol (10% EtOH v/v) for eight more days. When animals showed 70% of correct response over three consecutive days, they were transitioned to fixed-ratio 3 (FR3), and the sipper access time was reduced to 10s. After eight days on FR3 training all animals exhibited a stable response (at least 80% correct responses over three consecutive days). Thus, all animals were progressed to the extinction phase **(**illustrated in **Fig. 1B)**, which consisted of removal of the discrete cue (light), and the reward (alcohol) delivery after the correct response. The extinction phase continued until all animals reached the criterion (at least 60% decrease in active lever response for at least three consecutive days). After the extinction phase, a single session of cue-induced reinstatement was performed. For this session, we used water solution in the sipper using FR3 schedule **(Fig. 1B)** and quantified consummatory behavior (i.e., volume consumed), and the number of lick contacts as the seeking response. The following day we assessed for reacquisition (or relapse), but this time using unsweetened alcohol solution (10% EtOH v/v) as contingent reinforcement again for ten days. Immediately after completion of last day of relapse, eye blood samples were taken for assessment of blood EtOH concentration (BECs).

2.5. *Eye-blood samples*

Single eye-blood samples for BECs were collected immediately after the last day of relapse testing to avoid stress-induced association with the operant procedure. Eye-blood samples were collected as previously reported (Boehm II et al., 2008). Briefly, retro-orbital sinus blood (~50 μL) was sampled immediately after completion of the last session of relapse. Blood was collected into capillary tubes and centrifuged for 5 min to separate the plasma. To determine alcohol concentrations, an alcohol assay was run on the plasma samples using the Analox alcohol analyzer (Analox Instruments, Lunenburg, MA). After 24-h all animals that were used for the behavioral studies, were euthanized by cervical dislocation, and after rapid decapitation, brains were removed and prepared for immunoblot assays.

2.6. Immunoblot assay

Expression of GLT1 and xCT were determined in all groups by Western blot. Brains were sliced and under a stereoscopic microscope, the mPFC, NAc, DMS and DLS were micro-dissected and homogenized in 500 μl ice-cold homogenizing buffer containing in mM: 320 sucrose, 1 HEPES, 1 EGTA at 7.4 pH. Homogenates were centrifuged at 1000×*g* for10 min at 4 °C to remove debris and nuclei-associated membranes. The supernatant was centrifuged at 25,000×*g* 15 min at 4 °C, the pellet was resuspended in 50 mM Tris-HCl buffer containing 1 mM of EGTA and centrifuged again. The resulting pellets were resuspended in a mixture of RIPA buffer (Thermo Scientific) and protease inhibitors (Complete mini™ tablets; Roche, Basel, Switzerland), and phenylmethanesulfonyl fluoride (PMSF). The lysates were sonicated, and aliquots (2 μl) were used to determine the protein concentration (Bio-Rad protein assay). Lysates containing equal amounts of protein (50 μg) were prepared in NuPAGE™ LSD sample buffer and heated at 95 °C for 5 min in a master cycler (Eppendorf™). The samples were loaded onto either 4–20% or 4–15% polyacrylamide precast mini gels (Mini-Protean® TGX™, Bio-Rad) and separated by SDS-PAGE electrophoresis. The resulting gels were transferred to nitrocellulose membranes using midi nitrocellulose transfer packs (Bio-Rad), and a Trans-Blot® Turbo™ transfer system (Bio-Rad) for 7-min at 25V. The transferred membranes were blotted for 2-h at room temperature with a solution of Trizma-base buffer saline (TBS) and 5% of nonfat powered milk. Membranes were incubated overnight at 4 °C with primary antibodies anti-GLT1 (~62 kDa; 1:500, Abcam ab ab178401), anti-xCT (~37 kDa and ~55 kDa; 1:1000, Abcam ab37185). The membranes were then washed with TBS-T (TBS + 0.1% Tween-20) three times (10-min each) and incubated with the secondary antibody (goat anti-Rabbit IgG (H+L); IRDye 800CW: 1:5000) for 2-h at room temperature. The immunoreactivity was visualized using ODYSSEY CLx imaging system. Densitometry analysis was corrected relative to β-actin contents using a monoclonal antibody anti-β-actin (LI-COR; 1:5000) incubated overnight, followed by the secondary antibody (anti-mouse IgG IRDye® 680RD Donkey anti-Mouse; 1:5000). Images were analyzed using quantification for western blot analysis computer-assisted imaging (LI-COR Image Studio Lite). The integrated intensity of the protein of interest was normalized calculating the ratio of optical density GLT1/actin or xCT/actin and expressed as arbitrary units (a.u.).

2.7. Statistical analysis

All data were expressed as mean ± SEM. Analysis of variance (ANOVA) was used for all analyses, including treatment (control and Pb-treated mice) and sex (male and female). Sex was found no to be a significant factor; therefore, data were collapsed across sex. Active, inactive lever presses, correct response, number of lick contacts, and alcohol intake during self-administration, and extinction training sessions were analyzed with factorial two-way ANOVAs, with Pb-treatment × training sessions as factors by repeated-measures (RM) ANOVA. Significant interactions were followed up with post hoc Sidak's multiple comparisons test. Immunoblots were analyzed with unpaired *t*-test comparing control versus Pb-treated mice. Descriptive correlational determinations were performed with linear regression analysis to determine the best fit and to obtain the coefficient of determination (r^2). Normality tests were performed for all the data, and parametric statistical tests were used as appropriate. Two-tailed test were performed for all the studies. The analysis was performed using Prism 8.0a GraphPad Software (San Diego, CA). Significance for all statistical comparisons was set at $p \leq 0.05$. Relevant statistical analysis information can be found in the supplemental material **(Tables S1 and S2).**

3. Results

3.1. Acquisition of alcohol self-administration

Mice were trained to orally self-administer alcohol using a saccharin fade procedure to facilitate the acquisition of the behavior and alcohol intake (McCool and Chappell, 2012). The number of lever presses increased as a function of sessions during the saccharin fade training. Statistical analysis revealed that both groups Pb-treated, and control mice progressively increased the number of active lever presses across sessions [$F_{5.99,239.7} = 38.94$, $p < 0.0001$, two-way ANOVA], but no effect was found for developmental Pb exposure [$p = 0.94$; Fig. 2A]. There was no difference between Pb-treated and control groups on the number of incorrect lever presses over the time course of the saccharin fade (Fig. 2B). The number of lick contacts gradually decreased as a function of time as alcohol concentration increased across sessions [$F_{4.88,185.6} = 21.43$, $p < 0.0001$, RM two-way ANOVA; Fig. 2C], but no differences were detected between control and Pb-treated mice [$p = 0.074$]. Consistent with the active responding to the lever press, the alcohol self-administration significantly increased as a function of time as well [$F_{10,410} = 2.12$, $p = 0.02$, RM two-way ANOVA], but again no main effects in alcohol consumption during saccharin fade were found between the Pb-treated group of mice when compared to control group [$p = 0.271$; Fig. 2D].

To consolidate the acquisition, we continued on FR1 schedule training for an additional eight days using the unsweetened alcohol solution (EtOH 10% v/v). Stable responding was observed during all FR1 sessions, and therefore, animals were transitioned to FR3 reducing the time availability of the sipper to 10s. As expected the number of lever presses increased as function of time from 43 ± 3.5 to 78 ± 7 in the control group from 47 ± 2.6 to 69 ± 7 in the Pb-treated group, with a significant change across sessions, but no increase in the responding towards the incorrect lever was observed over time [$F_{15,600} = 62.04$, $p < 0.0001$, RM two-way ANOVA, Fig. 3A]. The percentage of correct response was calculated during both FR1 and FR3. All mice reached a 70% of correct responding during FR1 and 80% of correct lever presses during FR3 training (Fig. 3A). Taken together, these data suggest that mice acquired operant self-administration for alcohol, and that the acquisition was not impaired by the developmental Pb exposure under these operant conditions.

We also measured the number of lick contacts and we did not find differences in treatment as a factor, but increased number of licks as function of time (in sessions) was detected [$F_{9.26,388.9} = 14.48$, $p < 0.0001$, two-way ANOVA, Fig. 4A]. Similar results were found for alcohol intake [$F_{8.94,362} = 7.01$, $p < 0.0001$, two-way ANOVA, Fig. 4B]. Correlational analysis contrasting number of lick contacts in all sessions versus the alcohol consumption showed a significant positive correlation between the variables in both control and Pb-treated groups during FR1 [control: $r^2 = 0.143$, $p < 0.0001$; Pb-treated: $r^2 = 0.129$, $p < 0.001$, Fig. 4C and E respectively], and the FR3 training phase [control: $r^2 = 0.279$, $p < 0.0001$; Pb-treated: $r^2 = 0.216$, $p < 0.0001$, Fig. 4D and F respectively].

To determine if the level of intoxication could be a confounding variable using this model of operant alcohol self-administration, we correlated the number of incorrect responses with alcohol intake in both FR1 and FR3 training sessions and we found no positive correlation between the number of inactive lever presses and the amount of alcohol consumed per session (FR1: control: $r^2 = 0.008$, $p = 0.25$; Pb-exposed $r^2 = 0.0002$, $p = 0.84$; FR3: control: $r^2 = 0.075$, $p = 0.20$; Pb-exposed $r^2 = 0.00017$, $p = 0.86$; data not shown).

3.2. Extinction, cue-induced reinstatement, and relapse

Overall, the extinction training resulted in a gradual decrease of the active lever press responses. Sessions continued until the animals no longer showed clear preference for the active lever for at least three

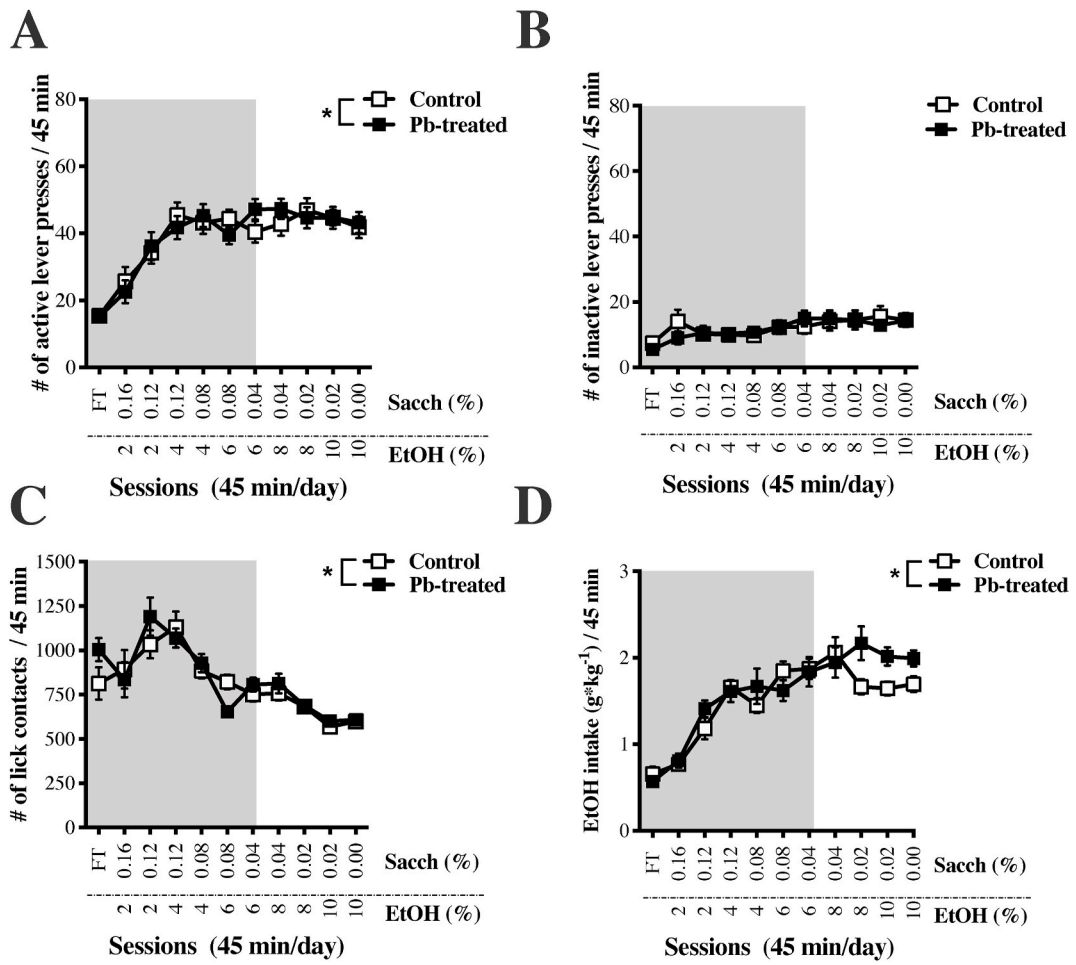

Fig. 2. Operant responding in Pb-treated and control mice during the saccharin fade self-administration training. Gradual increase in the operant responding on the active (A) but not inactive (B) lever presses in 45-minute/day (10 sessions). Gray shadow marks the self-administration sessions in which water deprivation was used. Gradual decrease in the number of lick contacts (C) as the EtOH concentration increased (*p < 0.0001; RM two-way ANOVA), but no significant effect by treatment. There was a gradual increase as a function of time in EtOH intake (D) but not main effect by treatment (*p < 0.0001; RM two-way ANOVA). Data are plotted as mean ± SEM. Developmental Pb-exposure (closed squares) and control (open squares). Data represent performance of n=21 per group.

consecutive days. It took 11 sessions to achieve the extinction criterion (at least 60% reduction of lever press responding). Extinction was also confirmed by the lack of significant difference between active and inactive lever presses by RM two-way ANOVA ($p = 0.85$; data not shown). Similar to the acquisition phase, no interactions of Pb-treatment × sessions were found, but a significant reduction in the active lever responding over time was observed [$F_{14,560} = 37.7$, $p < 0.0001$, RM two-way ANOVA, Fig. 5A].

Re-exposure of mice to the cues (e.g., cue light and the noise of the retractable sipper) reliably reinstated responding on the active lever in both groups Pb-treated and control [$F_{2,80} = 30.48$, $p < 0.0001$; RM two-way ANOVA, Fig. 5B). For the cue-induced reinstatement test, the sipper tube was filled with water, and the following day all animals were tested again but this time with alcohol solution (10% EtOH v/v) as a contingent reward to test relapse. While no difference in the number of active lever presses was found when comparing Pb-treated and control groups, a factorial analysis of Pb treatment × training sessions showed that Pb-treated mice displayed a higher number of lick contacts than control group when the sipper tube was filled with alcohol solution as reward [$F_{2,80} = 20.41$, $p < 0.0001$; Pb-treated vs control $p < 0.0001$; Sidak's multiple comparison test, Fig. 5C]. Accordingly, the total volume of alcohol consumed during relapse session was also significantly higher in Pb-treated than control mice [$F_{2,80} = 31.34$, $p < 0.0001$; Pb-treated vs control relapse $p < 0.0001$; Sidak's multiple comparison test; Fig. 5D].

To study if the effect observed on the first day of EtOH relapse persisted over time, we tested nine additional days on the same conditions. We measured the alcohol consumption, number of lick contacts per session, and the number of active lever presses (Fig. 6). Surprisingly, both alcohol intake and number of lick contacts (Fig. 6A and B) were significantly lower in the control group when compared to the Pb-treated mice across the ten sessions [EtOH: Pb-treatment × sessions: $F_{25,995} = 10.79$, $p < 0.0001$; lick contacts: Pb-treatment × sessions: $F_{25,995} = 10.60$, $p < 0.0001$]. The decreased consummatory effect on the control group persisted for the ten sessions of the relapse phase [$p < 0.0001$; Sidak's multiple comparison tests]. An interesting observation is that in the Pb-treated group, the amount of alcohol intake and the number of lick contacts observed throughout the relapse phase remained unaffected, and were consistent with the previous consumption seen over FR3 schedule sessions (Fig. 6A and B gray shadow). There was significant interaction between relapse Pb-treatment × sessions [$F_{25,995} = 2.98$, $p < 0.0001$], and a main effect in the number of active lever presses [$F_{25,995} = 22.61$, $p < 0.0001$; Pb-treated vs control relapse, $p < 0.05$ in Sidak's multiple comparison test on days 36, 37, 38, Fig. 6C].

3.3. Blood EtOH concentrations and alcohol intake on relapse phase

To estimate the strength of association between the alcohol consummatory behaviors and BEC, we performed correlational analysis taking the data from the last session of the relapse phase. A strong and significant correlation between the alcohol intake and the BEC was

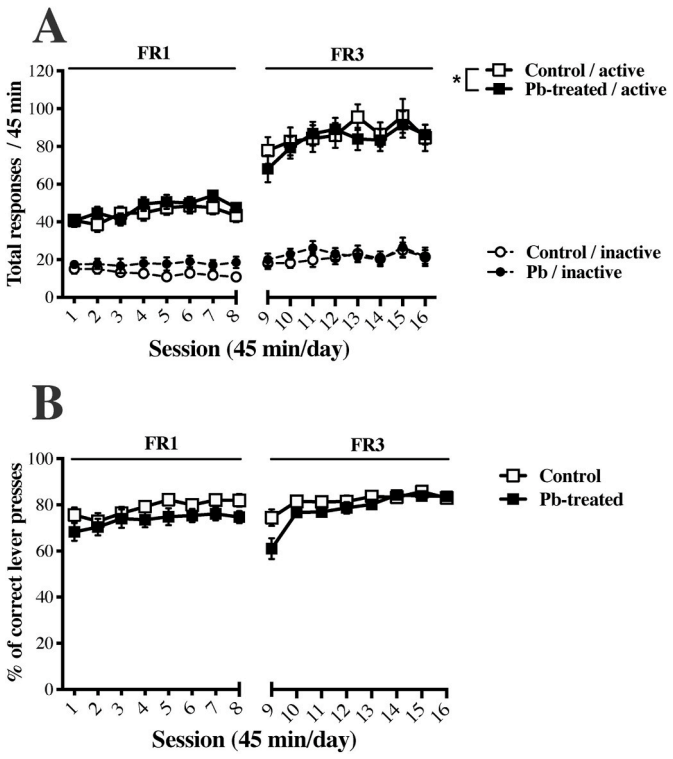

Fig. 3. Acquisition of alcohol self-administration in Pb-treated and control mice. Total responses per session on the active and inactive levers during alcohol self-administration FR1 and FR3 training phases (A), no changes in the rate of responding towards the inactive lever press, but significant increase as a function of time in FR3 training (*p < 0.0001; RM two-way ANOVA). Similar acquisition in Pb-treated and control mice (B), showing at least 70% of correct response in FR1 and 80% of correct response during FR3. Data are plotted as mean ± SEM. Developmental Pb-exposure (closed squares/circles) and control (open squares/circles). Data represent performance of n=21 per group.

detected for both groups [control: $r^2 = 0.786$, $p < 0.0001$; Pb: $r^2 = 0.564$, $p < 0.0001$; Table 1]. We also found a positive and significant correlation between sipper lick contacts and BECs [control: $r^2 = 0.560$, $p < 0.0001$; Pb exposed: $r^2 = 0.660$, $p < 0.001$, Table 1]. Interestingly, when contrasting the number of rewards earned during the last relapse session and BECs, we found no significant association between the variables in the control group, but did find a significant and strong correlation in the Pb-treated group [control: $r^2 = 0.064$, $p = 0.27$; Pb exposed: $r^2 = 0.736$, $p < 0.001$, Table 1]. Additionally, we estimated correlations between alcohol intake and parameters of alcohol self-administration; we found that all parameters associated with consummatory behaviors predicted the EtOH intake (Table 1).

3.4. GLT1 and xCT protein expression

The increase of alcohol intake in the Pb-treated group during the relapse phase, was accompanied by downregulation of GLT1 in NAc and DLS, [NAc: $t = 3.05$, $df = 18$, $p = 0.006$; DLS: $t = 2.39$, $df = 18$, $p = 0.027$, Fig. 7 B and D respectively], but not mPFC and DMS [mPFC: $t = 0.49$, $df = 18$, $p = 0.62$; DMS: $t = 0.83$, $df = 18$, $p = 0.41$, Fig. 7A and C respectively]. In contrast, the glutamate/cysteine antiporter xCT was differentially affected. It is noteworthy to mention that the xCT has a predicted molecular weight of ~55 kDa; however, it has a high number of hydrophobic residues, which may affect the migration of the protein in the SDS-PAGE. The monomeric form of xCT is expected to migrate at ~37 kDa, while the modified-xCT is expected to migrate at ~55 kDa. We analyzed both predicted molecular weight bands to avoid misinterpretation as has been pointed out previously (Van Liefferinge et al., 2016). Interestingly, no changes were found on the ~55 kDa predicted band ($xCT_{\sim 55kDa}$) in mPFC [$t = 0.91$, $df = 37$, $p = 0.36$, Fig. 8A], or in any of the regions of the striatum studied, NAc [$t = 0.21$, $df = 36$, $p = 0.83$, Fig. 8B], DMS [$t = 1.3$, $df = 35$, $p = 0.18$, Fig. 8C], or DLS [$t = 0.49$, $df = 36$, $p = 0.62$, Fig. 8D]. However, we found that the $xCT_{\sim 37kDa}$ was increased in the NAc [$t = 2.34$, $df = 36$, $p = 0.02$, Fig. 9B], but decreased in DLS [$t = 2.24$, $df = 37$, $p = 0.03$, Fig. 9D], while no changes were observed in mPFC or DMS [mPFC: $t = 1.7$, $df = 37$, $p = 0.096$, Fig. 9A; DMS: $t = 0.59$, $df = 35$, $p = 0.55$, Fig. 9C].

4. Discussion

The main conclusion that emerges from these observations is that although low-level developmental Pb exposure does not increase the risk of alcohol consumption in adult mice, it seems to play an important role in relapse to alcohol seeking, an effect that was associated with differential expression of synaptic and non-synaptic glutamate transporters in the striatum.

4.1. Effect of developmental lead exposure on alcohol self-administration and relapse

Here we used the operant self-administration paradigm paired with extinction and cue-induced reinstatement to study if low levels early-life Pb exposure affects motivation, seeking, and relapse to alcohol in adult mice. During operant self-administration training, both control and Pb-treated mice learned the correct choice during the saccharin fade training (Fig. 1A and B), with a clear consolidation of the acquisition during FR1 schedule in which a plateau of responses on the active lever was observed across eight days. Similar results were found for the FR3 schedule, with an increase in the number of responses on the active lever as expected, but no changes in the number of inactive lever responses (Fig. 3A). Accordingly, during both training phases all animals met similar percentage of correct response (Fig. 3B). Consistent with the responding measures (i.e., active lever presses and number of lick contacts), intake of alcohol was not different between control and Pb-treated mice in either FR1 or FR3 (Fig. 4B). Based on these results we concluded that there was no evidence of impairment on the acquisition process. However, to estimate if alcohol intoxication could be associated with a reduced instrumental learning, we evaluated the strength of association between the number of incorrect responses and alcohol intake rates for all the training sessions, and we found no correlation between these variables (data not shown). Thus, these data indicate that developmental Pb exposure did not impair the learning process, nor did it predispose to a greater alcohol intake. Contrasting results have been found previously. A series of studies determined that Pb exposure increased alcohol intake in rats (Virgolini et al., 1999; Mattalloni et al., 2017, 2019). Nonetheless, it should be noted that there were three important differences in the experimental designs of these works: 1) the Pb treatment took place during the perinatal period; 2) the Pb concentration used was much higher at 220 ppm; and 3) the times at which the voluntary alcohol self-administration took place were different. The studies using perinatal Pb exposure made escalating alcohol concentrations available beginning at PND35, a time considered periadolescence in rats (Mattalloni et al., 2017, 2019). Importantly, using the same conditions, there was a significant difference in alcohol consumption at PND35 but not at PND70 (Virgolini et al., 1999), which is in agreement to our study.

A major finding of this study is that while Pb-treatment did not influence the self-administration of alcohol, it did significantly change the consummatory behavior after alcohol deprivation. Removing the discrete drug-paired cue (i.e., cue light), and programed consequences of lever press responding is a common model to study motivation and seeking behavior. Thus, after all the animals extinguished lever press responding (Fig. 5A), we tested cue-induced reinstatement. Control and Pb-treated groups underwent a single reinstatement session in which the

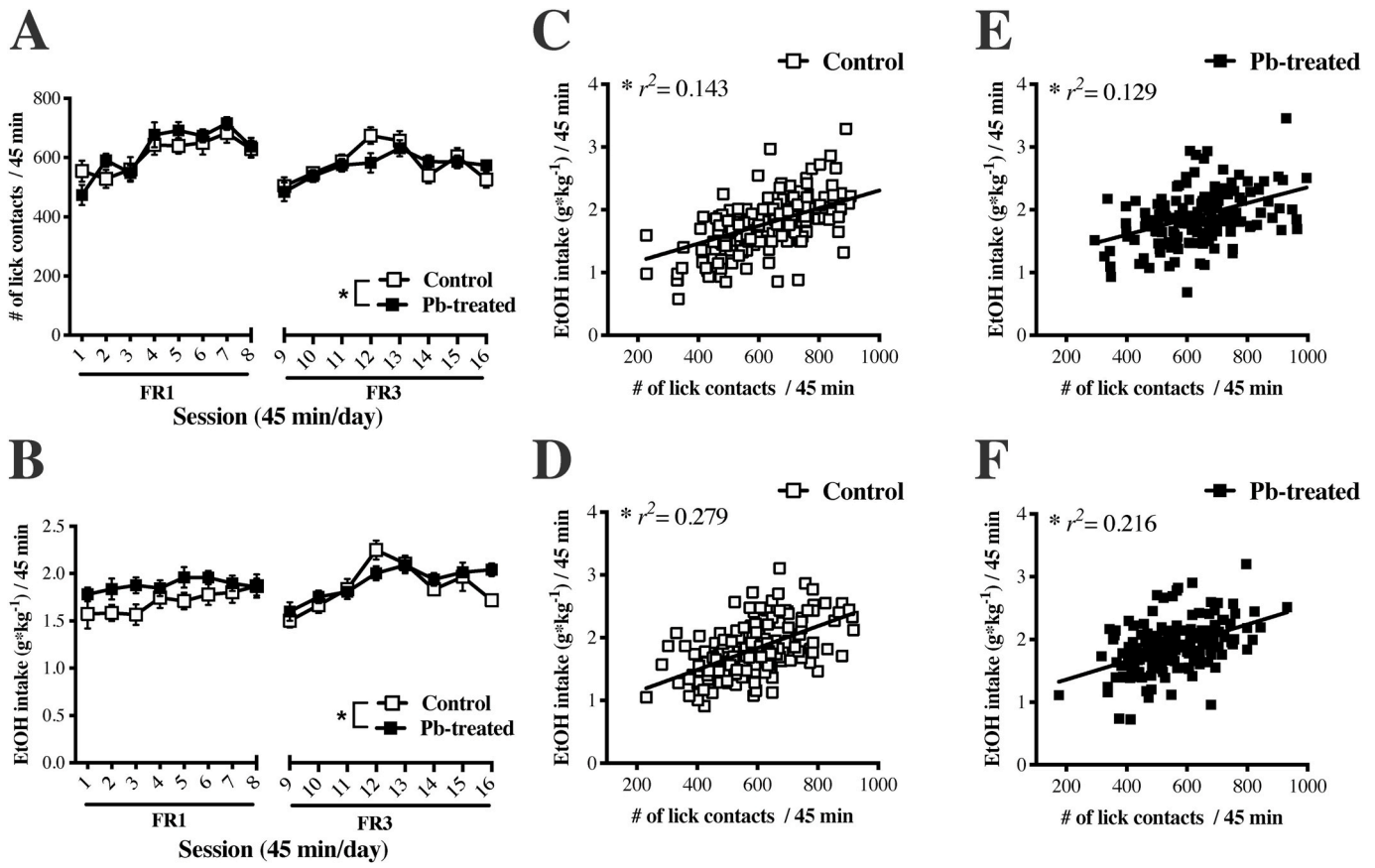

Fig. 4. Appetitive and consummatory parameters on alcohol self-administration in Pb-treated and control mice. (A) Shows the number of lick contacts and (B) the EtOH intake (gr*kg-1) across FR1 and FR3 daily training sessions, significant effect as a function of time (*p < 0.0001; RM two-way ANOVA), but no significant effect by treatment. Correlational analysis contrasting the number of lick contacts and the EtOH intake in both control (open squares) and Pb-treated mice (closed squares) during FR1 (C and E) and FR3 (D and F) training schedules. Each symbol represent one measure/session per subject during eight days of each training phase. The slope was significant deviated from zero for both groups and training phases (*p<0.001).

cue was reintroduced, and the responses to the active lever resulted in reinforcing stimuli (i.e., retractable sipper sound and sipper tube delivery). For the cue-induced reinstatement test, the sipper tube was loaded with water, the following day (or relapse); the sipper tube was filled with alcohol (10% EtOH v/v) instead. Under this paradigm, we found that Pb-treated mice showed significantly greater fluid intake and number of lick contacts in comparison with the control group when they received alcohol as a reward (Fig. 5C and D). Thus, our data suggest that while low-level early-life Pb-exposure does not contribute to the development of alcohol abuse, it may play an important role in the maintenance of alcohol use disorder once it develops.

The analysis of alcohol self-administration in operant conditioning has been distinguished between 'appetitive' and 'consummatory' processes (Samson and Hodge, 1996; Cunningham et al., 2000). An interesting observation was that Pb-treated mice consumed as much alcohol as they were consuming before the alcohol deprivation (or extinction phase). Measuring consummatory behavior (i.e., intake) and appetitive or 'seeking' (i.e., lever pressing to gain access to alcohol solution) after deprivation of alcohol can give us an idea of relapse, but relapse after long periods of abstinence is frequently associated with increased alcohol drinking or alcohol deprivation effect (Oster et al., 2006; Vengeliene et al., 2006; Rotermund et al., 2017). Our data does not support that notion. Pb-treated mice consumed as much alcohol during relapse as they had during the FR3 phase, while the control group did not show resumption of alcohol-taking behavior to the same extent.

To study the strength of relationship between consummatory behaviors and the actual blood alcohol level achieved during the last day of relapse phase, we performed correlational analysis (Table 1). As expected, a strong and significant correlation between alcohol intake and BECs was found, which was also true for the association of BECs by volume consumed. It has been suggested that appetitive processes control the alcohol-seeking behaviors (i.e., lever pressing), in a way that they motivate and direct behaviors toward sources of alcohol, therefore, influencing the initiation of consumption (Cunningham et al., 2000). Accordingly, in the control group the number of rewards earned and BECs were not significantly correlated, while a significant correlation was determined in consummatory behaviors such as volume consumption by number of lick contacts, and by alcohol intake (Table 1). The fact that Pb-treated mice also exhibited a reduced number of lever presses on relapse phase without changing the alcohol consumption or the number of lick contacts, indicates that the number of active lever presses may not be always a good predictor for seeking as was recently reported (Blegen et al., 2018).

Cue-associated seeking and relapse have been attributed to hippocampus-mediated learning (Kutlu and Gould, 2016; Lüscher et al., 2020). On the other hand, many observations support that Pb exposure produced aberrant hippocampal LTP, neurochemical and epigenetic modifications (Sadiq et al., 2012; Luo et al., 2014; also review Rahman, 2014). Given the ability of this brain region to support context-induced memories (Kim and Lee, 2011; Loureiro et al., 2012), disrupted hippocampal function by Pb exposure could facilitate drug-context associations and contribute to the maintenance of alcohol addiction (i.e., relapse to alcohol seeking), as we found here. Although the study of hippocampal LTP and developmental Pb exposure is beyond the scope of our study, we believe this might be an interesting venue to further investigate the underlying mechanisms by which Pb exposure induces

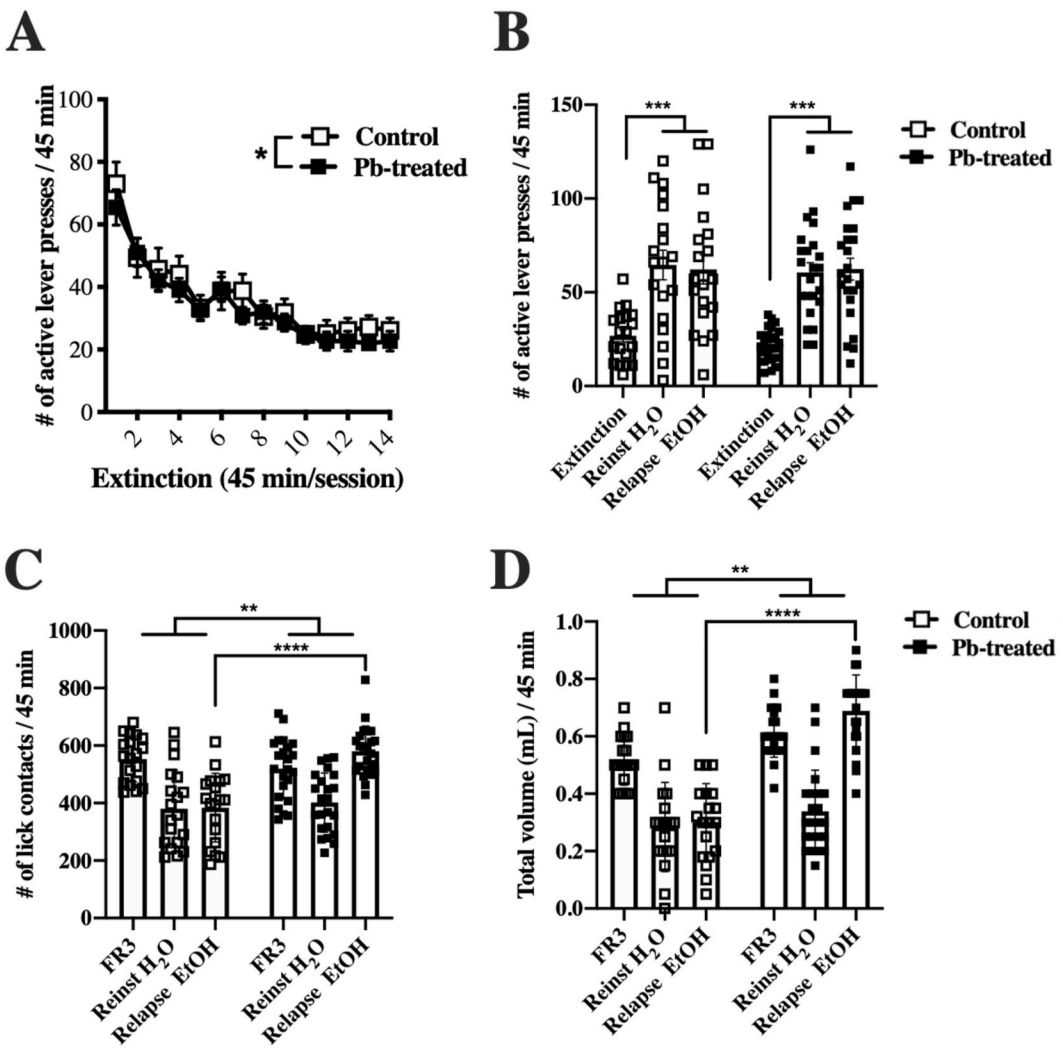

Fig. 5. Effect of developmental Pb exposure on extinction, cue-induced reinstatement, and relapse. Rate of responding on the active lever over 14 training sessions in extinction phase (A). There was a significant reduction of active lever presses as a function of time in both control and Pb-treated group ($p < 0.0001$; RM two-way ANOVA). There was a main effect in the number of active lever presses during the cue-induced reinstatement and relapse sessions (B) when compared with the last day of extinction, in both control and Pb-treated groups ($p < 0.0001$; RM two-way ANOVA). The number of lick contacts was significantly higher in the first session of relapse to alcohol (C) in the Pb-treated group, but not in the cue-induced reinstatement session (Pb-treated vs control $p < 0.0001$; Sidak's multiple comparison test). The total volume consumption (D) was significantly higher in the Pb-treated group during the relapse to alcohol session, but not different from the cue-induced reinstatement (Pb-treated vs control relapse $p < 0.0001$; Sidak's multiple comparison test). Data are plotted as mean ± SEM and represent performance of n=21 per group.

relapse to alcohol seeking behavior.

4.2. Lead exposure differentially affects the expression of synaptic and non-synaptic glutamate transporters

A glutamatergic component is undoubtedly involved in Pb-induced neurotoxicity. For instance, reduced expression of glutamate transporters has been demonstrated after Pb exposure (Struzynska et al., 2005a, 2005b), along with dysregulated extracellular levels of glutamate that secondarily affect glutamate receptors expression (Neal et al., 2011; Rahman, 2014). Such neurotoxic changes in early-life exposure have not only immediate, but also long-term consequences (Patrick, 2006; Mitra et al., 2017). On the other hand, alterations in the glutamatergic system also play a crucial role in addiction. Drug-evoked plasticity of glutamate synapses has been largely implicated with addictive behaviors (Lüscher and Bellone, 2008; Kalivas, 2009). Accordingly, alcohol drinking enhances glutamatergic plasticity and excitotoxicity (Joffe et al., 2018; Blaker et al., 2019) accompanied with dysregulation of glutamate homeostasis, which is driven by disrupted expression of synaptic and non-synaptic glutamate transporters in alcohol abuse (Gass and Olive, 2008; Kalivas, 2009).

Considering these findings, we investigated whether alterations in the expression of synaptic and non-synaptic glutamate transporters (GLT1 and xCT respectively) could be associated with alcohol relapse observed in the Pb-treated mice. We focused on three regions of the striatum highly associated with drug addiction and seeking behaviors. The NAc that is the most ventral region of the striatum, and considered crucial in the reward circuit pathways and alcohol sensitization (Abrahao et al., 2011). We also analyzed the DMS that has been associated with goal-directed behaviors (Balleine and O'Doherty, 2010), and mediates initial reinforcement of alcohol drinking (Lüscher et al., 2020), as well as the DLS, which is associated with the formation and expression of stimulus-response learning (Featherstone and McDonald, 2004), and mediates drug-seeking habits (Sjoerds et al., 2013; Everitt and Robbins, 2013). All these regions of the striatum receive glutamatergic inputs from the mPFC. We therefore also studied protein expression in this brain region, as these corticostriatal circuits are linked to drugs of abuse and alcohol addiction (Kalivas and Kalivas, 2016; Lüscher et al., 2020).

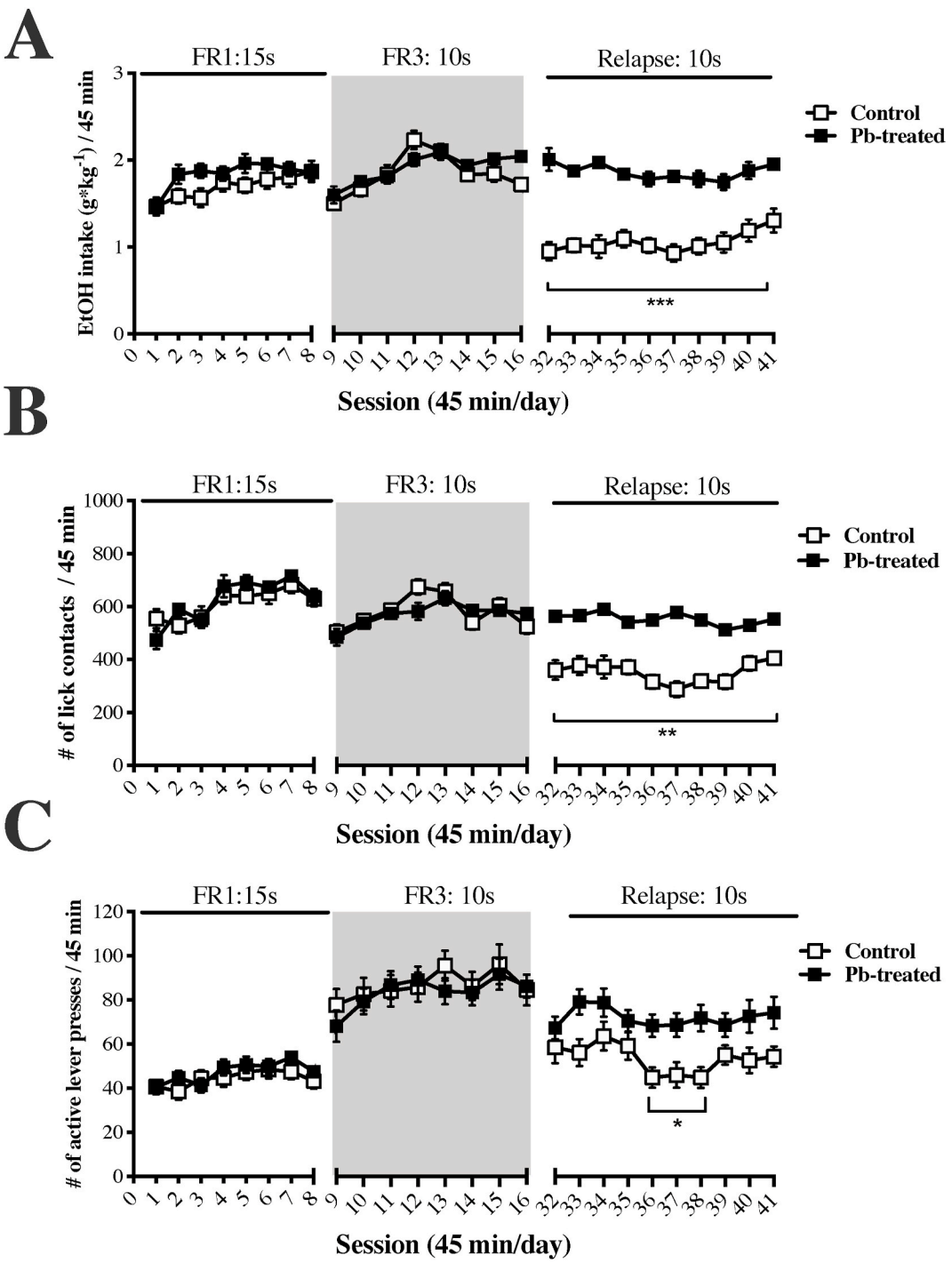

Fig. 6. Comparison of appetitive and consummatory parameters on FR1, FR3 and relapse phases. EtOH consumption (gr*kg-1) time course of relapse to alcohol seeking (A) shows that low-level Pb exposure induced relapse across sessions on the Pb-treated, but not in the control group ($p < 0.0001$; RM two-way ANOVA). Gray shadow marks the change to FR3 training schedule and the reduction of sipper availability time to10s. The number of lick contacts (B) was significantly lower in the control group ($p < 0.0001$; RM two-way ANOVA). (C) Shows a significant interaction of sessions × Pb-treatment in the number of lever presses ($p < 0.0001$; RM two-way ANOVA), with a significant reduction in number of lever presses in the control group on days 36-38 ($p < 0.05$; Sidak's multiple comparison test). Data are plotted as mean ± SEM and represent performance of n=21 per group.

In accord to previous studies (Sari et al., 2013; Hakami et al., 2016), we found that chronic alcohol intake resulted in a significant down-regulation of GLT1 in NAc. Additionally, we found significant reduction of GLT1 expression in DLS but not in DMS (Fig. 7). Operant responding for ethanol in training procedures is considered a goal-directed behavior, thus, DMS dependent (Corbit et al., 2012; Cheng et al., 2017). Consistent with this, we did not find differences in either responding or alcohol intake over the acquisition phase, but rather on relapse to alcohol in the Pb-treated group (Fig. 6). Glutamatergic strength of communication in the DLS could account for these effects. In fact, studies have shown an overreliance on DLS-dependent stimulus response habit in alcohol dependence, which is paired with decreased engagement of brain areas implicated with goal-directed behaviors (Corbit et al., 2012; Sjoerds et al., 2013). The role of DLS on alcohol

Table 1
Summary of correlations of alcohol blood concentration, self-administration parameters, and recorded consummatory behaviors on relapse phase.

Variables	Control			Pb-treated		
	EtOH intake (g*kg-1)	# of lick contacts	BEC (mg/dL)[a]	EtOH intake (g*kg-1)	# of lick contacts	BEC (mg/dL)[a]
# of lick contacts	r2 = 0.416***			r2 = 0.175***		
BEC (mg/dL)[a]	r2 = 0.786***	r2 = 0.560***		r2 = 0.564***	r2 = 0.660***	
Volume consumed (mL)	r2 = 0.889**	r2 = 0.507***	r2 = 0.782*	r2 = 0.703**	r2 = 0.297***	r2 = 0.702*
# of rewards earned	r2 = 0.027*	r2 = 0.316***	r2 = 0.064	r2 = 0.028*	r2 = 0.221**	r2 = 0.736*

[a] All correlational analysis using BEC as a variable, include only data collected from the last day of relapse training phase. The rest of the correlations were performed using data from all sessions on relapse training phase. Asterisks indicate the statistical significance ***p < 0.0001; **p < 0.001; *p < 0.05.

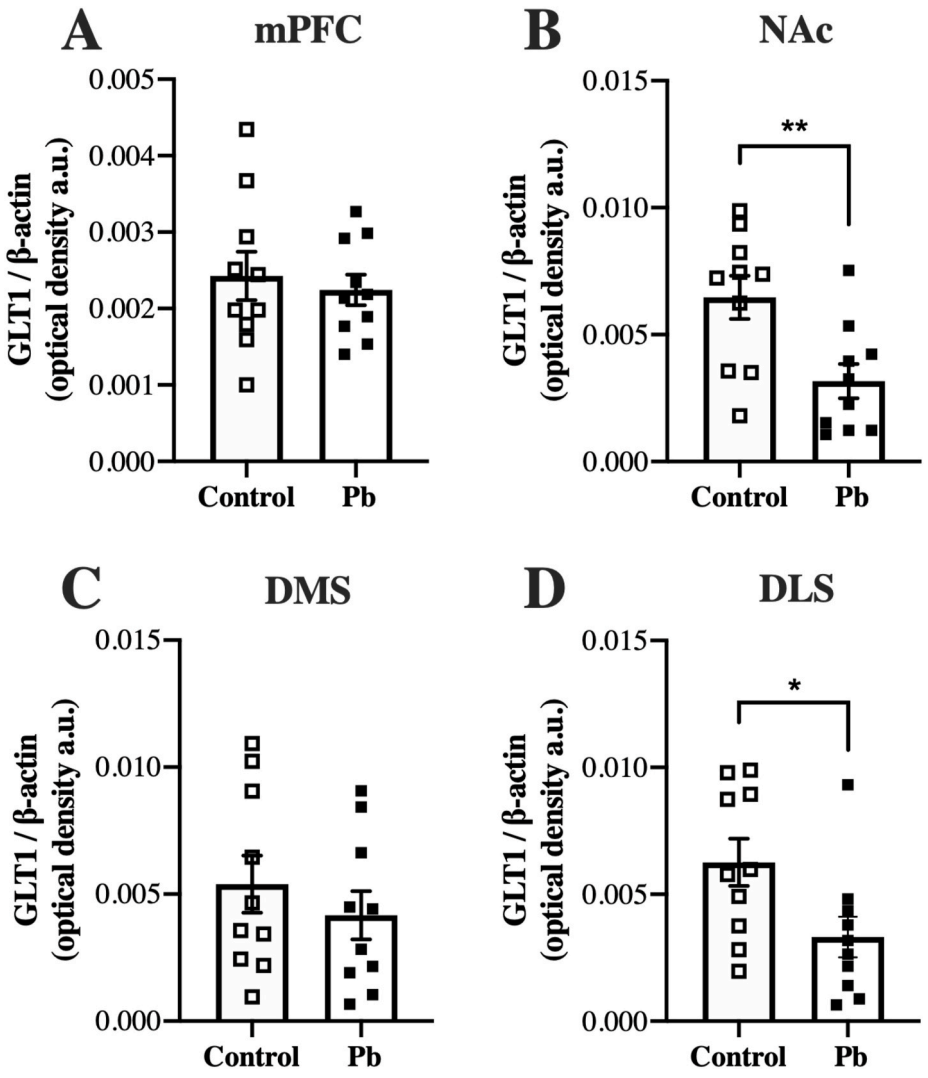

Fig. 7. Effect of developmental Pb exposure and relapse on GLT1 expression. Immunoblot analysis of GLT1 expression on A) mPFC, B) NAc, C) DMS, and D) DLS. Pb exposure and relapse to alcohol induced downregulation of GLT1 in NAc and DLS, but not mPFC and DMS. Relative optical density data are plotted as mean ± SEM. Unpaired parametric t test (**p < 0.001; * p < 0.01, n=12-15)

drug-seeking habits has recently been reviewed in detail (Lüscher et al., 2020).

Although previous reports have found significant downregulation of GLT1 in the mPFC as well, we did to detect such changes. A possible explanation could be attributed to methodological differences in the alcohol drinking procedures, and the time elapsed between last alcohol intake and the tissue harvesting. For example, in conditions of tissue harvesting 24-h after the last alcohol intake, no differences in GLT1 expression were found in mPFC (Alhaddad et al., 2014; Hakami et al., 2016), which is in agreement to our findings. However, downregulation of GLT1 expression has been demonstrated in conditions of alcohol withdrawal, (Abulseoud et al., 2014; Das et al., 2016). At the synapse, GLT1 is the major transporter in the brain removing up to 90% of the glutamate. However, at extra-synaptic sites another important contributor in the extracellular glutamate homeostasis is the antiporter xCT. It has been reported that xCT is a major source of non-synaptic glutamate (Baker et al., 2002, 2003), and largely involved in alcohol addiction and other substances of abuse such as cocaine and opioids (Weiland et al., 2015; LaCrosse et al., 2017; Alshehri et al., 2018).

To evaluate the expression of xCT, we took into consideration both

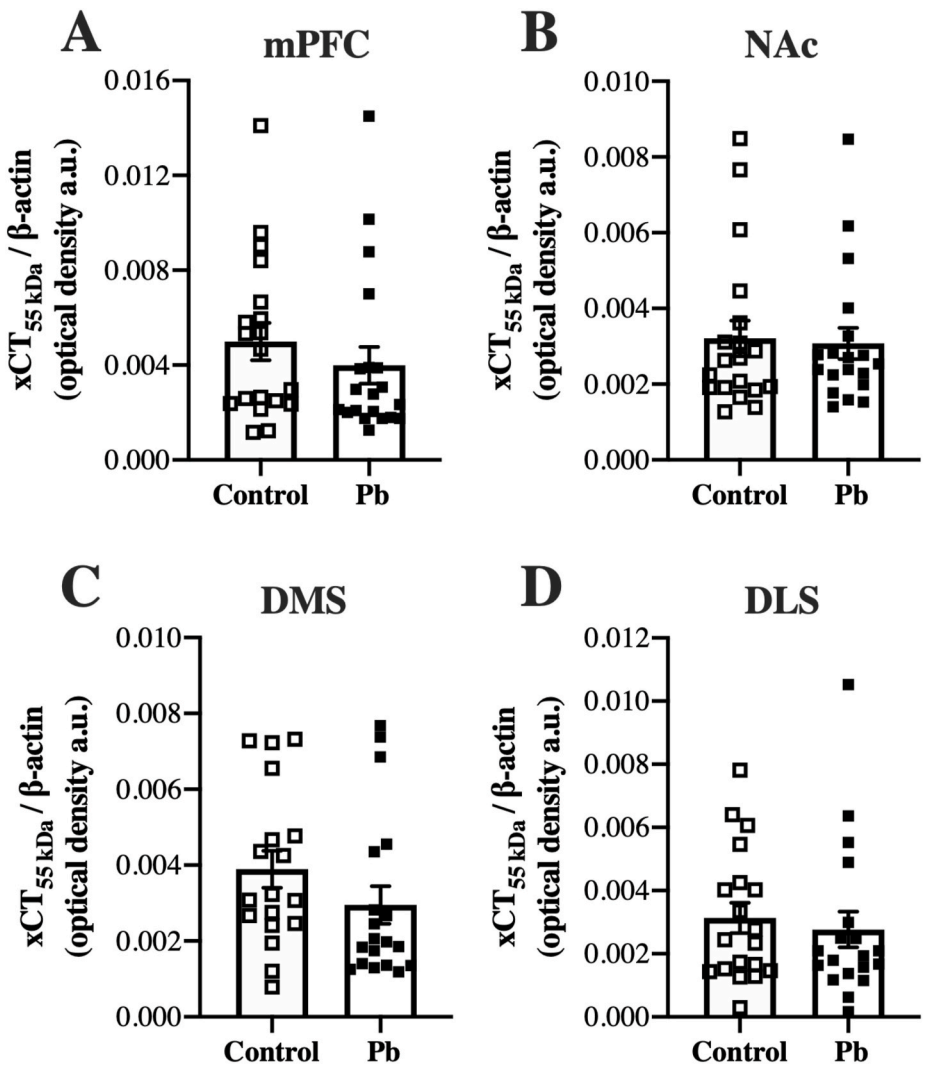

Fig. 8. Effect of developmental Pb exposure and relapse on modified-xCT expression. Immunoblot analysis of xCT (~55 kDa band) expression on A) mPFC, B) NAc, C) DMS, and D) DLS. Pb exposure and relapse to alcohol did not affect the expression of xCT~55kDa. Relative optical density data are plotted as mean ± SEM. Unpaired parametric t test (n=20 each group).

the monomeric xCT at ~37 kDa, and the modified xCT at ~55 kDa predicted band weights, with the understanding that there is a debate over the correct western blot band for xCT (Van Liefferinge et al., 2016). We found that the $xCT_{\sim 55kDa}$ band was not affected in any of the brain structures studied here (Fig. 8). However, the monomeric band $xCT_{\sim 37kDa}$, exhibited contrasting and significant differences in the NAc and DLS. While there was an upregulation in NAc, in DLS the expression of $xCT_{\sim 37kDa}$ was downregulated (Fig. 9). Many reports have found that upregulation of both GLT1 and xCT using β-lactamic antibiotics can reduce significantly home cage alcohol intake (Sari et al., 2013; Alhaddad et al., 2014; Rao et al., 2015), alcohol self-administration (Stennett et al., 2017), and reinstatement of operant alcohol seeking (Weiland et al., 2015). Given that the Pb-treated mice did relapse to alcohol (Fig. 6), increased xCT expression on NAc was unpredicted, but it was interesting that changes on $xCT_{\sim 37kDa}$ levels occurred in the same striatal regions in which GLT1 was downregulated (i.e., NAc and DLS).

The effect of Pb exposure on glutamatergic synapses have been largely studied (review Rahman, 2014). Pb excitotoxicity can be attributed not only to excessive glutamate that overstimulate glutamate ionotropic receptors inducing massive calcium intracellular influx (Guilarte, 1997; Lasley and Gilbert, 1999), but also to altered signaling and expression of NMDA receptors (Neal et al., 2011; Rahman, 2014). Additionally, Pb itself can produce oxidative stress, which leads to free radical damage through the generation of reactive oxygen species (ROS) (Nava-Ruiz et al., 2012; Sharma et al., 2015; Lopes et al., 2016). Interestingly enough, xCT maintains redox balance by introducing cystine into the cells and protecting cells from oxidative glutamate toxicity (Sato et al., 2005; Lewerenz et al., 2006). Intracellularly, cystine is required for the synthesis of glutathione, which is an important antioxidant in the brain that removes ROS (Schulz et al., 2000; Conrad and Sato, 2012). In conditions of excitotoxicity both the xCT mRNA and protein have been found to be upregulated (Soria et al., 2014). Further studies are needed to investigate if the upregulation of xCT observed in NAc is associated with Pb-induced toxicity.

Intriguingly, we found opposite effects in the expression of xCT in the DLS (Fig. 9B and D), in which both GLT1 and xCT were downregulated. One important feature of the xCT function is that the non-synaptic glutamate released by xCT, which accounts for 60% of the extracellular glutamate, can regulate glutamate transmission through metabotropic glutamate autoreceptors (mGluR2/3) due to the extra-synaptic localization (Baker et al., 2002). Thus, downregulation of xCT could prevent the inhibition of synaptic glutamate at presynaptic sites. Additionally, the downregulation of GLT1 would result in exacerbated extracellular glutamate concentrations. The downregulation of both synaptic and non-synaptic transporters could be favoring the strength of glutamate communication in DLS and contributing to alcohol relapse.

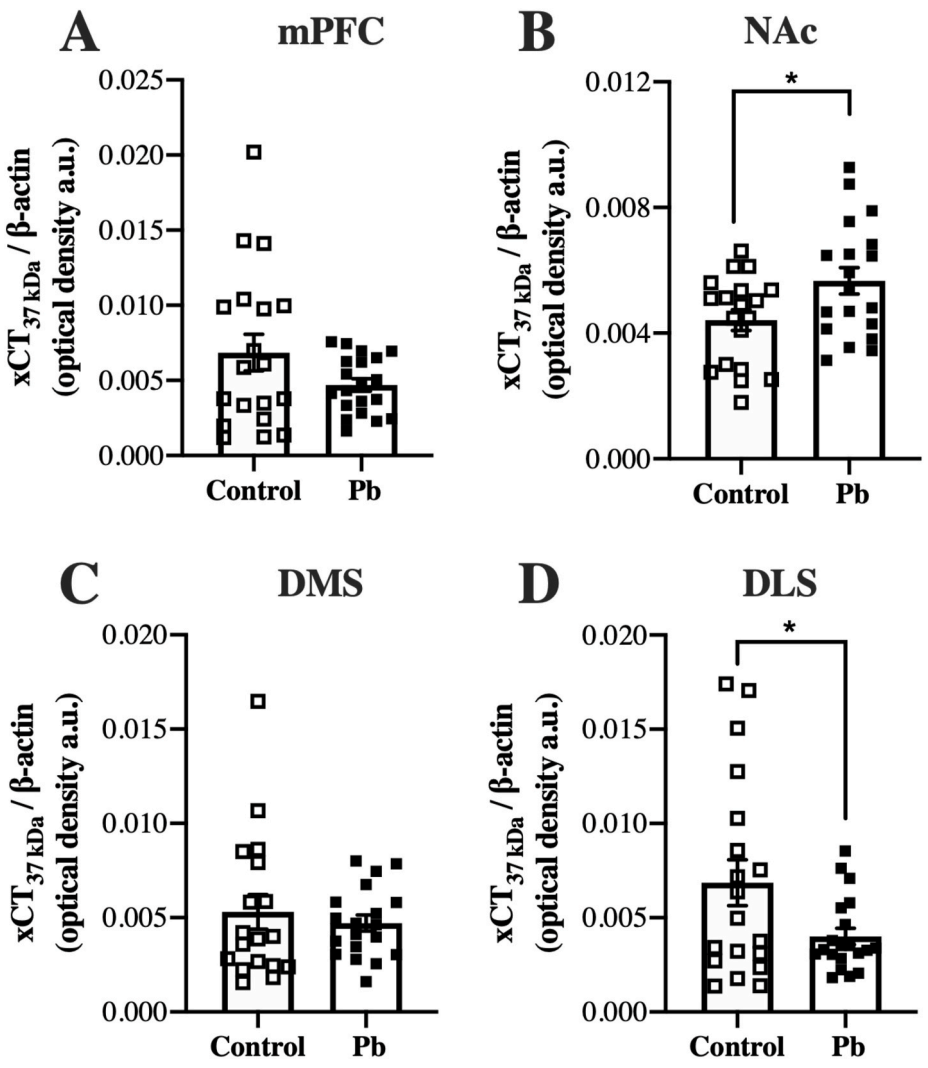

Fig. 9. Effect of developmental Pb exposure and relapse on monomeric xCT expression. Immunoblot analysis of xCT (~37 kDa band) expression on A) mPFC, B) NAc, C) DMS, and D) DLS. Pb exposure and relapse to alcohol induced upregulation of xCT~37kDa expression in NAc, but downregulation on DLS, while no changes were found in mPFC or DMS. Relative optical density data are plotted as mean ± SEM. Unpaired parametric t test (* $p < 0.01$, n=20 each group).

5. Conclusions

In summary, the current data show that although low-level child- and adolescent-equivalent Pb exposure does not alter the acquisition of alcohol self-administration, it enhances relapse responding when alcohol is reintroduced after extinction. The effect is accompanied by altered expression of transporters known to regulate synaptic and non-synaptic glutamate levels in the dorsal and ventral striatum. These results are consistent with a number of studies in which developmental Pb exposure is associated with altered brain glutamate signaling, and is striking that the effects are observed at Pb exposure levels currently deemed safe by state and federal authorities. More studies will be needed to determine the exact mechanism by which developmental Pb exposure increases risk of relapse, as well as the role of region-specific disrupted expression of synaptic and non-synaptic glutamate transporters. The participation of synaptic and extra-synaptic NMDA receptors is also of interest as they may also play an important role on Pb toxicity and alcohol relapse.

CRediT authorship contribution statement

Claudia Rangel-Barajas: Conceptualization, Methodology, Formal analysis, Investigation, Writing - original draft, Writing - review & editing, Project administration. **Israel Coronel:** Investigation, Validation, Resources, Writing - review & editing. **Yanping Zhang:** Investigation, Resources. **Maribel Hernández:** Resources, Investigation. **Stephen L. Boehm II:** Conceptualization, Project administration, Supervision, Funding acquisition.

Declaration of competing interest

The authors have no conflicts to disclosure.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.neuropharm.2020.108339.

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