Behavioral deficits induced by lead exposure are accompanied by serotonergic and cholinergic alterations in the prefrontal cortex.

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Abstract

We investigated the behavioral effects of long-term lead (Pb) exposure in adult rats producing blood Pb concentration (<20µg/dL) below those associated with neurological impairment in occupationally exposed individuals. In order to assess gender differences, we performed parallel behavioral experiments in male and female rats. Exposure to Pb acetate (50ppm in drinking water) for 6 months induced motor alterations consisting in hyperactivity in a novel environment and alteration in motor coordination. These effects were gender-dependent (only male affected). Chronic lead exposure impaired spatial learning assessed in the Morris water maze test (MWM) in both genders. Hyperactivity in male rats was accompanied by an increase in the extracellular of acetylcholine in the prefrontal cortex. Extracellular dopamine concentration was unaffected by lead exposure whereas serotonin levels were significantly decreased in both genders. These results suggest new therapeutic targets to treat neuropsychiatric deficits associated to chronic lead exposure.

Key words: lead, prefrontal cortex . acetylcholine . dopamine . serotonin . behavior

Introduction

Lead (Pb) is a non-essential toxic heavy metal widely distributed in the environment and chronic exposure to low levels of Pb has been a matter of public health concern in many countries. Despite improvements in public health policies and substantial reductions in blood Pb levels, Pb exposure remains an important health problem worldwide. Accumulating evidence suggests a link between Pb exposure and memory impairment (van Wijngaarden et al. 2009). Moreover, it has been reported that the degree of performance impairment over time increased with increasing bone lead concentration, a marker of cumulative exposure (Weisskopf et al. 2007). Additionally, increased lead concentration in bone is associated with reduced scores on the Mini Mental Status Exam (Weisskopf et al. 2004; Wright et al. 2003). A study of elderly women reported inverse associations between reaction time, digit symbol and trail making, with blood lead levels (Muldoon et al. 1996; Weuve et al. 2009) in a longitudinal community study of older women reported an inverse relationship between cumulative lead exposure using generalized estimating equations and performance in 8 cognitive tests.

For occupationally exposed individuals, there is disagreement about what exposure levels are needed to produce the earliest symptoms of neurotoxicity. Few studies examining workers with blood Pb concentrations ranging 20-40 μg/dL have associated Pb exposure with subtle cognitive alterations (Barth et al. 2002; Lucchini et al. 2000; Mansouri and Cauli 2009; Murata et al. 2009; Schwartz et al. 2001). Gender-related differences have received surprisingly little attention to date in Pb-induced neurotoxicity, particularly in studies that aimed to address the effects of Pb exposure during adulthood. Gestational exposure to Pb strongly impairs spatial learning in male offspring without affecting motor performance and visual function, whereas in female

offspring the impairment is less evident (Yang et al. 2003; De Souza et al. 2005) demonstrated that Pb exposure during pregnancy and lactation increases emotionality reactivity in male rats measured in the open-field test and depressive-like behavior in females in the forced swimming test. In rats, postnatal exposure to 500ppm Pb in the drinking water induces anxiogenic effect only in males (Soeiro et al. 2007). Short-term exposure to 50 ppm lead acetate in the drinking water alters psychomotor and cognitive functions in male rats but not in female rats (Mansouri et al. 2012). To shed some lights on sex-dependent behavioral impairment induced by lead, we performed the studies in parallel with both genders.

The cholinergic, dopaminergic and serotonergic systems are involved in specific behavioral responses and cognitive processes in both healthy subjects and those with neurological dysfunction (Bartus et al. 1982). We selected prefrontal cortex since this brain region is a part of the brain circuitry implicated in the formation of learning and memory. Additionally, medial prefrontal cortex (mPFC) has become a key focus of studies designed to elucidate the basis of behavior involving attention and decision-making, and hyperactivity (Castellanos and Proal 2012; O'Doherty 2011; Pourtois et al. 2012). It should be point out that changes of neurotransmitter concentration in the extracellular space rather those in tissue, reflects more accurately the effects of toxicants or drugs on neurotransmitter "tone" because an altered concentration in the extracellular space is accompanied by an altered stimulation of neurotransmitter receptors located in the cell membranes (Robinson and Whishaw 1988; Robinson et al. 1990; Parsons et al. 1991). The technique of in vivo brain microdialysis allows sampling and collecting neurotransmitters from the interstitial space and represents a useful tool to assess alteration of neurotransmitter concentration.

In this work we assessed:

(1) whether Pb acetate exposure (50ppm in the drinking water) for a longer period (6 months) taking into account the rats' life span in adult rats alters motor and/or cognitive functions,

(2) gender-vulnerability in Pb-induced behavioural impairment,

(3) whether Pb exposure alters acetylcholine (ACh), dopamine (DA) and serotonin (5-HT) concentration in the mPFC assessed by in vivo brain microdialysis in freely moving rats.

Motor activity and coordination were assessed by open-field and rota-rod tests, respectively. Spatial memory was evaluated by Morris water maze.

Materials and methods

Animals and Pb exposure protocol

All experiments have been conducted in accordance with the guidelines for care and use of experimental animals of the European Communities Directive (86/609/EEC; D.L., 27.01.1992, number 116). Experiments were conducted in young adult male and female Wistar rats, weighing 220±20 g at the beginning of the experiments (55-60 days of age). Animal room was maintained on a 12 light/dark cycle (lights on at 07:00 h). Food and water were freely available except during behavioural studies.

Ten days after arrival, rats were randomly divided in four groups: (1) 50ppm of Pb^{2+} male, (2) 50ppm of Pb^{2+} female, (3) control male, and (4) control female. All experimental groups contained 8 rats each. Rats were housed 3-4 per cage. All individuals making the observations were blinded to the treatment groups.

Pb-exposed rats received a 50ppm solution of Pb acetate for at least six months via their ad lib water supply, 54.7mg/L Pb acetate trihydrate from Sigma (St. Louis, MO)

dissolved in distilled water. Half of control rats received tap water and the other half received water solution containing sodium acetate (50mg/L). Pb exposure was maintained throughout the course of experiments. Drinking solution containing Pb or sodium acetate was freshly prepared every week. Data from control rats exposed to tap water or to 50 mg/L of sodium acetate produced similar results in each of the measurements performed in the study, so the data were pooled together.

Experiments were conducted between days 150-180 after starting Pb exposure.

Motor activity (Open field)

Spontaneous motor activity was assessed by placing rats in the open-field box (76 x 76cm, divided by 8 lines into 25 squares) (8-12 rats per group). Each rat was individually placed in the centre of the open-field and allowed to explore it for 10 min. During this time the motor activity was recorded by video-taping the rat in the apparatus. Ambulatory activity was measured as "lines crossed" (horizontal movement crossing the floor lines). Other elements of exploratory activity such rearing, grooming and sniffing were carefully observed and time spent performing each behavior was recorded. These parameters were defined as follows: rearing (standing on hind legs with paws pressed against the wall of the arena); sniffing (continuous placing nose against floor for at least 2s); grooming (using paws or tongue to clean/scratch body) (Cauli and Morelli 2002). The arena was carefully cleaned with 70% ethanol solution between animal tests to eliminate any olfactory cue derived from the previous rat located in the box.

Motor coordination test (Rotarod)

The rotarod test evaluates motor coordination by measuring the ability of rats to stay on a rotating drum. An accelerating 4-lane rotarod was used (Rotomex 5, Columbus Instruments, Columbus, OH). For two consecutive days before testing, each rat was placed on the rotarod which was switched off for 3min. The day of testing, rats were placed on the rod, and the apparatus was switched on. The starting speed was set to 0, and the speed was increased by 2rpm every 5 seconds up to 30rpm. The time at which each animal fell off the rungs was recorded, with a maximum cut-off of 600s. Each animal was given 3 trials and the mean latency of three trials was calculated (Monville et al. 2006).

Morris Water Maze Test

Apparatus

The water maze was a black circular tank with 136cm in diameter and 60cm in height. The tank was filled with water (20±1°C) to a depth of 25cm. The maze was located in a room containing extra-maze cues (posters). The maze was divided geographically into four quadrants [northeast (NE), northwest (NW), southeast (SE), southwest (SW)] and starting positions [north (N), south (S), east (E), west (W)] that were equally spaced around the perimeter of the pool. A hidden circular platform (diameter: 13cm) was located in the center of the NW quadrant, 1cm below the surface of the water. A video camera was mounted directly above the water maze to record the rats' swim paths. A computer-based video tracking system (Ethovision Noldus, Waningen, The Netherlands) was used to assess escape latency, swimming speed and also the percentage of traveled distance and the time spent in the target quadrant (Morris et al. 1982).

Procedure

Thirty-two rats (eight for each experimental group) were used. The rats were given four training trials each day on 4 consecutive days. The rat's escape from the water reinforces its desire to quickly find the platform and on subsequent trials (with the platform in the same position) the rat is able to locate the platform more rapidly. This improvement in performance occurs because rats learned where the hidden platform is located relative to the conspicuous visual cues. For each training trial, the rats were placed in the water facing the pool wall at one of the four starting positions (north, south, east, or west pole) in a different order each day and allowed to swim until they reached the platform located in the NW quadrant of the maze. The latency to reach the platform was recorded for up to 90s. They remained on the platform for 30s before being removed. The recall test trial (90s) with the platform removed from the pool was conducted 24h after the last training trial to assess spatial memory. After completing the trials, rats were dried with a towel and placed in a holding cage under a heating lamp before it was returned to the home cage.

Determination of Pb levels in blood and brain

After the exposure period, to prepare the sample for blood Pb measurement, a 0.1-ml aliquot of whole blood was mixed well with 3.9 ml of 0.5 N nitric acid containing 0.01% Triton X-100. After centrifugation the supernatant was taken.

The brain Pb levels were determined by graphite furnace atomic absorption spectrometry (Yun et al. 2000). For measurement of Pb in the brain, animals were anesthetized and then perfused transcardially with 100ml of normal saline to remove blood from the brain tissue then the whole brain was collected. To prepare 10% (w/v) brain homogenates, the whole brain was homogenized at an appropriate mixture of 0.5

N nitric acid, 0.5 N perchloric acid and 0.01% Triton X-100. To determine Pb concentration in samples, the same volume of each samples and 0.2% magnesium nitrate (as a modifier) was mixed and 10µl was injected into graphite furnace of atomic absorption spectrophotometer (Perkin-Elmer 3030). Because the homogeneity of regional Pb concentration in the adult rat brain has been reported (Widsowski and Cory-Slechta 1994), we measured Pb level in the whole brain (expressed as ng of Pb/g wet tissue).

Brain microdialysis

A microdialysis guide (CMA/12 guide cannula; CMA/Microdialysis AB, Solna, Sweden) was implanted 3mm above the coordinates in the prefrontal cortex (AP 3.7, ML −0.8, DV −1.0, from brain surface) as described in Garcia-Ayllón et al. (2008). Three days later a 3mm long microdialysis probe (CMA/12, Solna, Sweden) was lowered and perfused (3µl/min) with artificial cerebrospinal fluid (145 mM NaCl, 3.0 mM KCl, 2.26 mM $CaCl_2$, 2 mM phosphate, pH 7.4). Following a stabilization period of at least 2 h, samples were collected every 20min. ACh, DA and 5-HT in the perfusate was measured using high-performance liquid chromatography (HPLC) with electrochemical detection, as previously described (Cauli et al. 2007; Garcia-Ayllón et al. 2008).

Analysis of ACh, DA and 5-HT

ACh was separated on a coiled cation exchanger ACh column (analytical column) (Sepstik 10µm ID 530′ 1.0 mm; BAS, West Lafayette, IN), followed by the post-IMER (immobilized enzyme reactor) (BAS), which consists of choline oxidase (ChO)/Ach esterase. ACh was hydrolyzed by Ach esterase to form acetate and choline in the post

IMER. Then, choline was oxidized by ChO to produce betaine and hydrogen peroxide (H_2O_2). H_2O_2 was detected and reduced to H_2O on Unijet amperometric detector cell with a peroxidase-redox-coated glassy carbon electrode (MF-2098; BAS), set at +100mV (LC-4C; BAS) versus Ag/AgCl reference electrode. This reduction was analyzed with the detector (LC-4C; BAS) as signal indicating ACh in the chromatogram. However, it is difficult in most cases to separate the ACh peak from the huge choline peak with the analytical column. Therefore, a pre-IMER (BAS), which consists of ChO and catalase, was added prior to the analytical column. Choline in the dialysate samples was oxidized by ChO to form betaine and H_2O_2 by the pre-IMER. The H_2O_2 was then converted by the catalase to H_2O before entering the analytical column. This procedure eliminates interference of the ACh peak in the chromatogram by choline, thereby decreasing the detection limit of ACh. The area and height of ACh peak showed a linear correlation with concentration. Due to the markedly increased signal-to-noise ratio, the detection limit was 2-3 fmol per sample (20µl sample loop). The mobile phase (50 mM Na_2HPO_4, pH 8.2) including ProClin (BAS), a microbiocide, was pumped at 0.5ml/min by a pump. The reagents used were analytical or HPLC grade.

The content of DA, 5-HT and their major metabolites (homovanillic acid (HVA) and 5-hydroxy-indolacetic acid (5-HIAA)) in the microdialysis samples were analyzed by reverse-phase HPLC with electrochemical detection. The HPLC apparatus was equipped with a reverse phase ODS column (Symmetry C-18, 3.9mmx150mm, 5µm particle size) and a coulometric detector (Coulochem II Model 5200A; ESA) to quantitate DA. The first electrode was set at +100 mV (oxidation) and the second at –125 mV (reduction). The composition of the mobile phase was 50mM NaH2PO4, 0.1 mM Na2-EDTA, 0.5 mM n-octyl sodium sulfate, and 15% methanol; pH was adjusted

to 5.5. The sensitivity of the assay was 2 fmol/ sample. The flow rate was kept at 1ml/min. These conditions allowed monoamines to be detected within 16min. Internal standard DHBA was used for quantification and identification of the peaks. The detection limit in 20µl samples was 80 fmol/20µl for DA and 60 fmol/20 µl for 5-HT.

Verification of microdialysis probe location

After completion of the experiments, rats were anaesthetized with 4% chloral hydrate (1 mL/100g bw, i.p.) and perfused transcardially with 120 mL phosphate buffer (PB) (0.1m NaK2PO4) and 300mL of 4% paraformaldehyde in PB. The brains were removed, post fixed for 8 h in 4% paraformaldehyde in PB, and then in 30% sucrose in PB for 48 h at 4ºC. Transverse 40-µm sections were cut on a sliding microtome and stored in protecting solution (30% ethylene glycol, 30% glycerol in 0.1m NaK2PO4) at -20ºC until processing. Sections were observed under light microscopy for verification of the cannulae and microdialysis probe.

Statistical analysis

The results are presented as mean±SEM. The data were analyzed by one-way ANOVA followed by Dunnett's test or two-way ANOVA followed Tukey's post-hoc test. P values lower than 0.05 were considered to be statistically significant. Statistical analysis was performed using the Graph Pad Prism4 software (GraphPad Software Inc., San Diego, USA).

Results

Body weight was measured at the beginning of experiments and at the time of sacrifice. During the treatment all animals showed a normal and gradual increase of the body. Two-way ANOVA showed a significant effect of time $P<0.0001$, of group $P<0.0001$ and interaction $P<0.01$. At the end of experiment the weight of female rats were significant different from male ($P<0.05$). No significant effect of Pb exposure was observed in the weight gain (Table 1).

Pb level in blood and brain

As expected, Pb-exposed rats showed significant more Pb concentration in the blood, and in the brain. Two-way ANOVA showed a significant effect of treatment $P<0.0001$, of gender $P<0.05$ and interaction $P<0.05$ for both blood and brain (Fig. 1). In control groups there was no statistical difference of Pb concentration in the blood and brain between female and male rats. In contrast, Pb-exposed female rats showed significant lower Pb concentration than Pb-exposed male rats either in blood (10.6±0.7 μg/dl and 18.9±1.1 μg/dl, $P<0.05$) (Fig. 1A) and in the brain (350±11 ng/g and 415±15 ng/g, $P<0.01$) (Fig. 1B).

Motor activity

Ambulatory activity

Two-way ANOVA showed a significant effect of treatment ($P<0.05$), no significant effect of gender ($P=0.25$) and no significant treatment X gender interaction ($P=0.21$). Male rats exposed to Pb displayed significantly increased ambulatory activity counts (333±17) compared to control rats (266±15, $P<0.05$). There was no significant difference in ambulatory activity counts between female rats exposed to Pb (309±16) as compared to corresponding control (332±20) (Fig. 2A).

Rearing

Two-way ANOVA showed a significant effect of treatment ($P<0.05$), no significant effect of gender ($P=0.67$) and no significant treatment X gender interaction ($P=0.15$). Male rats exposed to Pb displayed significantly increased rearing activity counts (44±3) compared to control rats (33±3, $P<0.05$). There was no significant difference in rearing activity counts between female rats exposed to Pb (41±2) as compared to corresponding control (39±3) (Fig. 2B).

Grooming

Two-way ANOVA showed a significant effect of treatment ($P<0.01$), no significant effect of gender ($P=0.47$) and no significant treatment X gender interaction ($P=0.13$). Male rats exposed to Pb displayed significantly increased grooming activity counts (65±4) compared to control rats (48±4, $P<0.05$). There was no significant difference in grooming activity counts between female rats exposed to Pb (56±3) as compared to corresponding control (51±4) (Fig. 2C).

Sniffing activity

Two-way ANOVA showed no significant effect of treatment ($P=0.11$), of gender ($P=0.40$) and no significant treatment X gender interaction ($P=0.26$) for sniffing activity. No significant effect of Pb exposure was observed in sniffing activity (control male 37±3; Pb male 45±4; control female 42±2; Pb female 44±3) (Fig. 2D).

Rotarod test

Two-way ANOVA showed a significant effect of treatment ($P<0.01$), a significant effect of gender ($P<0.01$) and a significant treatment X gender interaction ($P<0.05$). Male rats exposed to Pb displayed significantly reduced latency to fall (237±10 sec) compared to control rats (315±16 sec, $P<0.01$). There was no significant difference in

the latency to fall between female rats exposed to Pb (234±14 sec) as compared to corresponding control (244±9 sec) (Fig. 3).

Morris water maze

Learning, assessed by the reduction in latency to find the hidden platform over days, was clearly evident in all groups.

Two-way ANOVA showed significant effect of days ($P<0.0001$), significant effect of group ($P<0.001$) and no significant days X group interaction ($P=0.95$). Rats learn the task, needing every day less time to find the platform. During the learning phase of the spatial task no gender difference was observed in both control and Pb group. However, Pb exposure impaired learning ability in MWM task at day 2, 3 and 4 in both genders (Table 2 and Fig. 4A). At day 2, both male and female Pb exposed rats needed more time to find the platform compared to respective control ($p<0.05$). At day 3, control female and male rats needed significant less time ($p<0.001$ and $p < 0.01$, respectively) to find the platform as compared to day 1. At day 3, both male and female Pb exposed rats needs more time to find the platform compared to respective control ($p<0.05$). At day 4, control female and male rats needed significant less time ($p<0.001$) to find the platform as compared to day 1. At day 4, only male Pb exposed rats needed more time to find the platform compared to respective control ($p<0.01$). At day 4, female Pb exposed rats tended, although no significantly, to need more time to find the platform later compared to respective control ($p=0.08$). Twenty-four hours after the last trial, the platform was removed from the pool and the rats were allowed to swim.

The percentage of time spent swimming in the quadrant where the platform was located during the learning phase (days1-4) was measured as an index of memory recall. Two-way ANOVA showed significant effect of treatment ($P<0.0001$), no significant effect of gender ($P=0.13$) and significant treatment X gender interaction ($P<0.50$). In the control

group, female rats spent significant less time in the target quadrant than male (47±3 sec and 36±2 sec respectively, P<0.05). In the Pb-treated groups, no significant difference was observed between female and male rats (30±2 sec and 27±3 sec respectively). Pb exposure significantly decreased spatial memory in both male and female rats as compared to the corresponding control (P<0.01 and p< 0.05, respectively; Fig. 4B).

For the swimming speed, two-way ANOVA showed no significant effect of treatment (P=0.1), no significant effect of gender (P=0.07) and no significant treatment X gender interaction (P=0.52) (data not shown). Pb exposure tended to decrease swim speed in male rats but it did not reach statistical significance (p=0.09).

Extracellular ACh, dopamine and serotonin concentration in prefrontal cortex after lead exposure

One-way ANOVA showed a significant effect for ACh concentration in prefrontal cortex [F(3,80), p<0.05]. Dunnett's test showed that lead exposure significantly (p<0.05) increased extracellular ACh concentration in male rats compared to control (5.6±0.4 nM and 3.8±0.2 nM, respectively, Fig. 5A). No significant difference in extracellular ACh concentration was observed between Pb-exposed and control female rats (4.1±0.4 nM and 5.0±0.6 nM, respectively Fig. 5A).

One-way ANOVA showed no significant effect for DA concentration in prefrontal cortex among experimental groups (control male: 6.2±1.4 nM, Pb-exposed male: 9.0±1.1 nM, control female: 8.1±1.9 nM, Pb-exposed female: 9.2±0.8 nM, Fig. 5B).

One-way ANOVA showed a significant effect for 5-HT concentration in prefrontal cortex [F(3,92), p<0.001]. Dunnett's test showed that lead exposure significantly (p<0.05) decreased extracellular serotonin concentration in male rats compared to control (6.0±0.8 nM and 10.2±1.8 nM, respectively, Fig. 5C). Dunnett's test showed that lead exposure significantly (p<0.001) decreased extracellular serotonin concentration in

female rats compared to control (7.5±0.9 nM and 16.0±1.3 nM, respectively, Fig. 5C). Dunnett's test also showed that extracellular serotonin concentration in prefrontal cortex is significantly ($p<0.05$) increased in control female rats compared to control male rats (Fig. 5C).

The extracellular concentration of monoamines metabolites HVA and 5-HIAA were unaffected by lead exposure (data not shown).

Discussion

Our results showed that in adult rats long-term exposure to Pb, producing blood Pb concentration (10-20 μg/dL) below those associated with overt neurological impairment in occupational exposed individuals (Barth et al. 2002; Lucchini et al. 2000; Murata et al. 2009; Schwartz et al. 2001; Shih et al. 2007), induced behavioural alterations. These data support the following main ideas: (i) chronic exposure to Pb, mimicking exposure occurring in occupational settings, impairs motor and cognitive functions, (ii) some of the deleterious effects induced by chronic lead exposure depend on gender (iii).

Motor and cognitive deficits induced by chronic lead exposure are accompanied by changes in extracellular neurotransmitter concentration (ACh, DA, 5-HT) in the prefrontal cortex.

We discuss these ideas below:

(i) Although the mean blood Pb levels of the entire population is relatively low (1-3 μg/dL), thousands of adult workers continue to be exposed to higher concentrations of Pb in many industries including battery manufacturing, painting, non-ferrous smelting, radiator repair, brass and bronze foundries, pottery production, scrap metal recycling, firing ranges, and wrecking and demolition (Mansouri and Cauli 2009; Rybicki et al.

1997). Centers for Disease Control and Prevention (CDC) recommended as a preventive health measure, a reduction of the concentration of the metal below <25 μg/dl in subjects occupationally exposed (CDC 2009). In this study, we demonstrated that long-term (6 months) exposure to 50ppm of Pb acetate in young adult rats producing blood Pb levels ranging 10-20 μg/dl induced mild but significant behavioural (motor and cognitive) alterations. It should be noted that hyperactivity and motor coordination deficits induced by chronic Pb exposure were observed only in male rats, whereas deficits in spatial learning and memory were observed in both genders. One can speculate that these differences might be due to the increased Pb concentration in brain of male rats compared to female. Gender differences in Pb distribution in other organs and species have been observed (Lanszki et al. 2009; Nwokocha et al. 2012; Sobekova et al. 2009). Gender differences in Pb metabolism/accumulation have been also observed in humans. Young or premenopausal women retain Pb more avidly or release Pb more slowly than do men (Popovic et al. 2005; Theppeang et al. 2008). The lower brain Pb concentration in female rats exposed to Pb is unlikely responsible for the lack of impairment in motor activity or coordination (Mansouri et al. 2012). Difference in brain region sensitivity to Pb could be likely due to differences in the interactions of Pb with biochemical or cellular targets modulated by sex hormones.

(ii) Chronic lead exposure impaired learning ability in the MWM to a similar extent in both genders. The impairment in memory in the spatial task induced by lead exposure was more significant in male compared to female rats. However, looking at the values of Pb-induced memory impairment (Fig. 4B), no differences were observed in Pb-exposed male and female rats, rather the performance of female rats in the control group was significant reduced compared to male rats in the control group. The latter observation agrees with other reports showing a better performance of male in MWM

test (Galea et al. 1995; Roof and Stein 1999, Woolley et al. 2010). Morris water maze task depend from hippocampus (Morris et al. 1982) and related neuronal circuitry included prefrontal cortex which subserves attention and cognition (Leon et al. 2010; Silachev et al. 2009). Hippocampus is a brain area particularly sensitive to neurotoxic effect induced by postnatal Pb exposure since it induces astrogliosis (Selvin-Testa et al. 1994, 1997), increases the expression of astrocyte marker glial-fibrillary acidic protein (GFAP) (Selvin-Testa et al. 1994) or mRNA for GFAP (Stoltenburg-Didinger et al. 1996) and increase myo-inositol concentration (Mansouri et al. 2012). Alteration in hippocampus and prefrontal cortex (see later) might be at basis of impairment of learning and memory abilities induced by chronic lead exposure. In contrast to the equal memory impairment elicited by lead exposure in both genders, motor activity and motor coordination were only impaired in male rats after chronic Pb exposure.

Exposure to Pb during pregnancy and lactation also increased emotionality state detected in the open-field test only in male rats (de Souza et al. 2005). According to our findings about chronic lead-induced impairment of motor coordination, male mice gestationally exposed displayed shorter latencies to fall from the rotarod compared with controls, whereas females were unaffected (Leasure et al. 2008). Other studied using much higher doses of Pb exposure and at various speeds of rotation yielded mixed results (Kishi et al. 1983; Grant et al. 1980; Ma et al. 1999; Moreira et al. 2001; Overmann 1977). The impairment of motor coordination in male rats exposed to lead during adulthood is evident only after long-term (6 months) exposure whereas short-term (1 month) exposure does not (Mansouri et al. 2012) whereas hyperactivity induced by lead appears since shorter time of exposure. These results likely suggest that lead exposure may affect striatal (relevant for modulation of motor activity) and cerebellar (playing a pivotal role in the modulation of motor coordination) neuronal circuit with

different sensitivity being the former more vulnerable to lead-induced alteration of brain function.

The reason why female rats did not display changes in motor activity and coordination needs further studies taking into account also data showing an increased risk to develop neuromotor deficits in male children exposed to lead (Ris et al. 2004).

An increased vulnerability in male rats to some effects induced by lead exposure such as increased in corticosterone concentration and decreased fixed-interval response rates have been previously reported (Cory-Slechta et al. 2004). In addition, analyses of brain images obtained with magnetic resonance imaging (MRI) indicate that childhood lead exposure is associated with region-specific reductions in adult gray matter volume (frontal lobe) mainly in males (Cecil et al. 2008). Extracellular acetylcholine concentration was increased in the prefrontal cortex only in male rats suggesting that this may be another marker of gender-dependent effects induced by lead exposure at least for adult exposure similar to that observed in occupational exposed individuals.

(iii) Our study is, to our knowledge, the first to examine extracellular neurotransmitter concentration in the prefrontal cortex of chronically exposed rats. It should be point out that changes of neurotransmitter concentration in the extracellular space rather those in tissue, reflect more accurately the effects of toxicants or drugs on neurotransmitter "tone" because an altered concentration in the extracellular space is accompanied by an altered stimulation of neurotransmitter receptors located in the cell membranes (Robinson and Whishaw 1988; Robinson et al. 1990; Parsons et al. 1991). The technique of in vivo brain microdialysis allows sampling and collecting neurotransmitters from the interstitial space and represents a useful tool to assess alteration of neurotransmitter concentration. We selected to study the prefrontal cortex because this brain region mediates executive functions, a set of control processes that

optimize performance on cognitive tasks, regulate decision-making and mediate adapted behaviors. In experimental animals it has been showed that cholinergic and serotonergic neurotransmission in prefrontal cortex is involved in spatial memory assessed by MWM (Blin et al. 2009; Millian et al. 2004; Nieto-Escamez et al. 2002). Either alterations in dopaminergic, serotonergic and cholinergic neurotransmission in the prefrontal cortex have been shown to play a role in hyperactivity and attentional deficits (Arnsten and Pliszka 2011; Dalley et al. 2008; Del Campo et al. 2011). Chronic lead exposure increased extracellular ACh concentration in the prefrontal cortex only in males. Earlier studies showed changes in the cholinergic system in this brain area accompany hyperactive behaviour in rodents (Giovannini et al. 1998; Robbins 2002). Even we cannot rule out that other brain areas are responsible to lead-induced hyperactivity in male rats, the increase in ACh in males might likely play a role. This suggestion is supported by the fact that both extracellular ACh in prefrontal cortex and motor activity is not increased in female rats exposed to lead. Chronic lead exposure in adult rats decreases extracellular 5-HT neurotransmission in the prefrontal cortex whereas DA concentration was unaffected. Although lead exposure has been repeatedly showed to impair dopaminergic neurotransmission, it seems that this effect is brain-region dependent. For instance Nowak et al. (2008) showed that Pb exposure during intrauterine life did not substantially affect cortical dopaminergic neurotransmission in adult offspring rats in mPFC. Prenatal Pb intoxication produces long-term changes in monoamine turnover, striatal DA in particular, without markedly affecting basal levels of monoamine in striatum and prefrontal cortex (Szczerbak et al. 2007). It is likely that striatum, nucleus accumbens or other DA rich brain areas are more intricately associated with Pb precipitated behavioral, DA-dependent impairments observed in mammalians.

Concentration of extracellular DA and 5-HT metabolites, HVA and 5-HIAA were unaffected by lead exposure suggesting that the mono amine oxydase enzymes (catabolise both DA and 5-HT) activity is unlike impaired by lead exposure. Further results should address if the selective reduction of extracellular 5-HT levels in the prefrontal cortex by lead exposure are due to an increased 5-HT uptake or to reduced firing of serotonergic fibers that project to prefrontal cortex. A major source of serotonergic innervation in the prefrontal cortex comes from fibers originating from the reticular formation. Interestingly, exposure to 50ppm lead acetate for 12 weeks alters the apparent diffusion coefficient of water (an index obtained by MRI analysis, sensitive to water content and white matter integrity) and blood brain barrier permeability in the reticular formation (López-Larrubia and Cauli 2011) suggesting a possible explanation for the reduced 5-HT release in the prefrontal cortex.

The reduction of 5-HT in the extracellular space may contribute to the impairment in the MWM (Markowska and Wenk 1991; Lehmann et al. 2000, 2002) test in both male and female rats suggesting that drugs that increase extracellular 5-HT may be useful in the treatment of cognitive impairment in adults occupationally exposed to lead.

Acknowledgements

This work was supported by the grant from the Ministerio de Ciencia y Innovacion CTQ2010-20960-C02-02.

Conflict of Interest Statement

The authors declare that there are no conflicts of interest

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Legends to tables and figures

Table 1 Weight gain of rats exposed to 50 mg/L Pb in the drinking water for six month. Data are expressed as mean±SEM for n=8/group. There was no statistically significant effect of Pb acetate on body weight (P>0.05)

Table 2 Time spent by rats exposed to 50 mg/L Pb in the drinking water to find the platform during the acquisition of spatial learning task.

Data are expressed as mean±SEM of time (sec) spent to find the platform for n=8/group. *: differs from corresponding control; [a]: differs from male rats, Dunnett's test

Figure 1 Concentration of Pb in the blood (A) and in brain (B) of control and Pb-exposed (50 mg/L in the drinking water) rats.

Values are mean±SEM (μg/dL for blood, ng/g wet weight for brain) for n=8/group(n=8). *: differs from corresponding control; a: differs from male rats, Dunnett's test.

Figure 2 Effect of exposure to 50 mg/L Pb in the drinking water in motor and exploratory activity: (A) Ambulatory activity; (B) frequency of rearing response; (C) frequency of grooming response; and (D) frequency of sniffing response

Data are expressed as mean ± SEM for n=8/group. *: differs from corresponding control; [a]: differs from male rats, Dunnett's test

Figure 3 Effect of exposure to 50 mg/L Pb in the drinking water in motor coordination measured as latency time to fall from rota-rod test.

Data are expressed as mean ± SEM for n=8/group. **$p < 0.01$: differs from corresponding control, Dunnett's test

Figure 4 Effect of exposure to 50 mg/L Pb in the drinking water in learning and memory. (A) Time to find the platform during the acquisition of the spatial navigation task. (B) Memory retrieval as indicated by time spent in the target quadrant after the acquisition phase.

Data are expressed as mean±SEM for n=8/group. *: differs from day 1 corresponding control; [a]: differs from control rats

Figure 5 Effect of exposure to 50 mg/L Pb in the drinking water in the extracellular concentration of acetylcholine, dopamine and serotonin. Acetylcholine (A), dopamine (B) and serotonin (C) were assessed by in vivo brain microdialysis in freely moving rats as described in Methods. Values (nM range) are reported uncorrected from probe recovery. Data are expressed as mean±SEM for n=8/group. *: differs from corresponding control, [a]: differs from male. Post-hoc Dunnett's test

Figure 1.

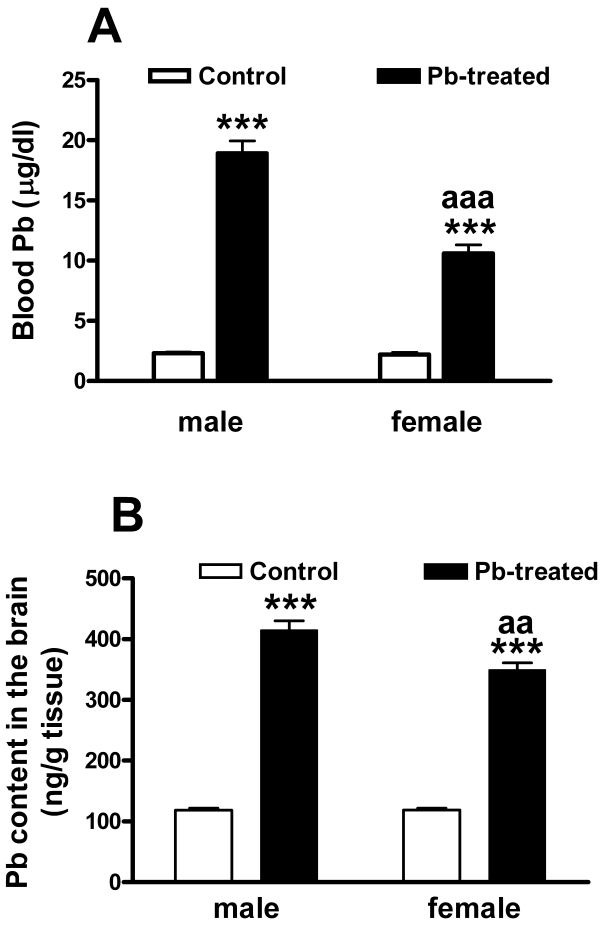

A
Control
Pb-treated
Blood Pb (μg/dl)
25
20
15
10
5
0
***
aaa
***
male
female
B
Control
Pb-treated
Pb content in the brain (ng/g tissue)
500
400
300
200
100
0
***
aa
***
male
female

Figure 2.

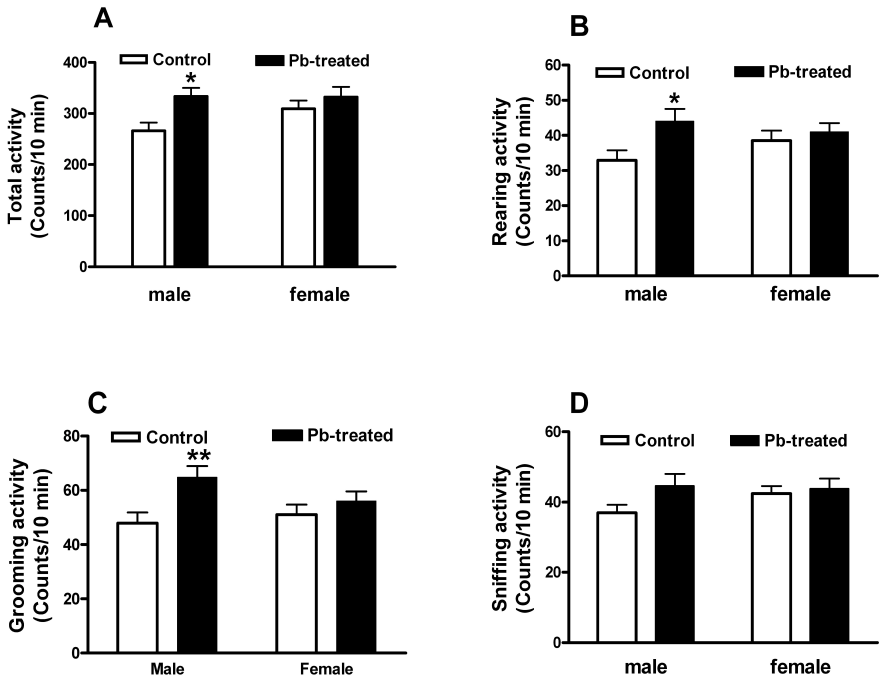

A
Control
Pb-treated
*
Total activity
(Counts/10 min)
400
300
200
100
0
male
female
B
Control
Pb-treated
*
Rearing activity
(Counts/10 min)
60
50
40
30
20
10
0
male
female
C
Control
Pb-treated
**
Grooming activity
(Counts/10 min)
80
60
40
20
0
Male
Female
D
Control
Pb-treated
Sniffing activity
(Counts/10 min)
60
40
20
0
male
female

Figure 3.

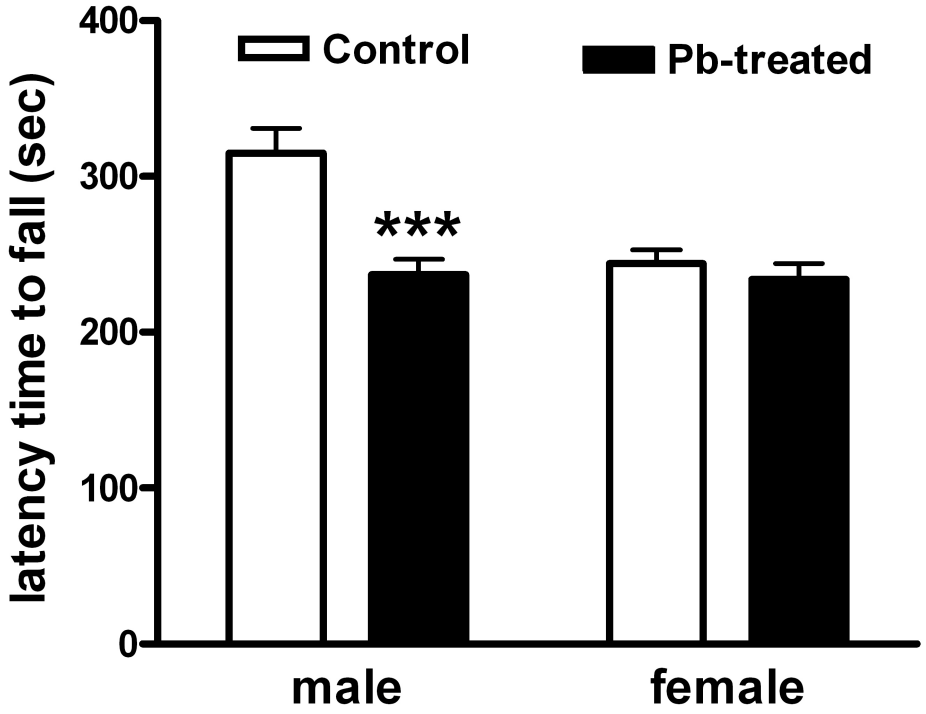
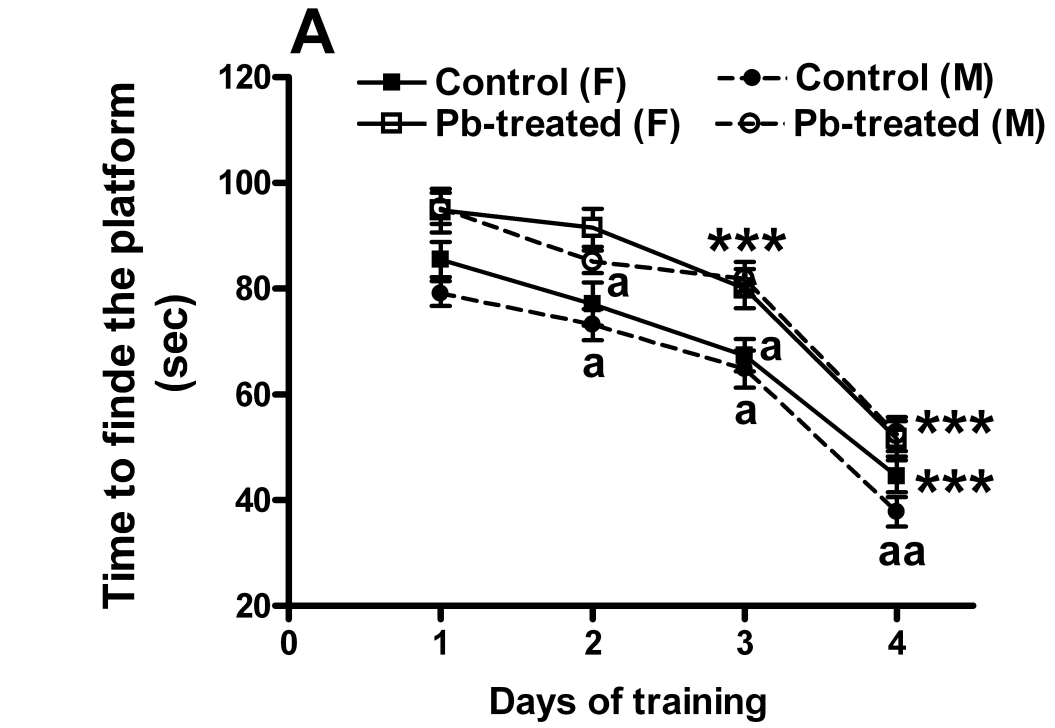

400
300
200
100
0
latency time to fall (sec)
Control
Pb-treated
***
male
female

Figure 4.


A
Control (F)
Control (M)
Pb-treated (F)
Pb-treated (M)
Time to finde the platform (sec)
120
100
80
60
40
20
0
1
2
3
4
Days of training
a
a
a
aa
***
***
***

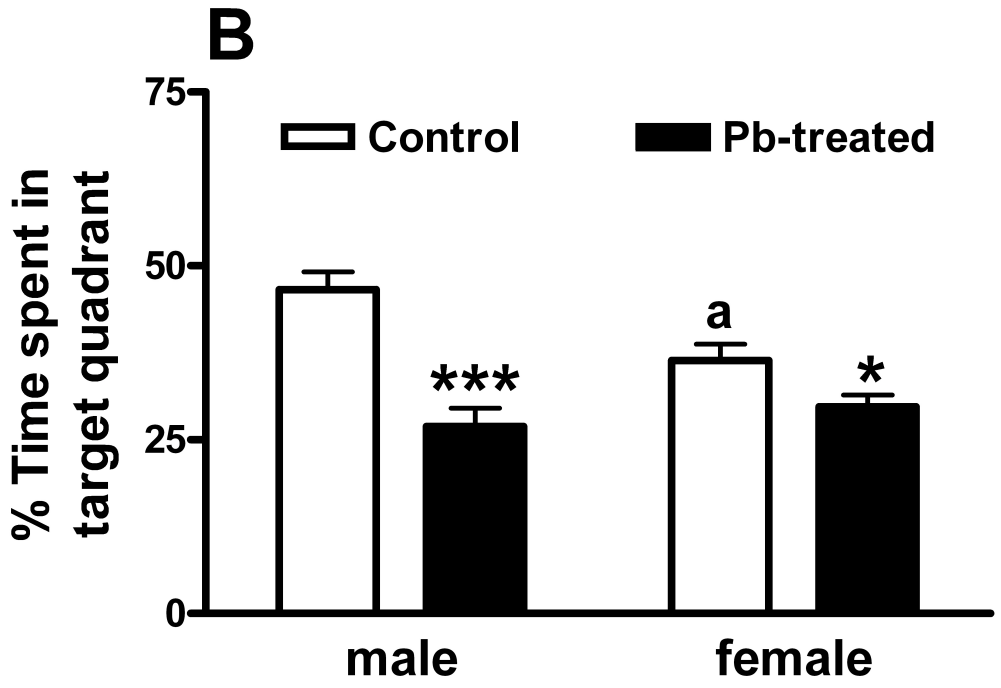
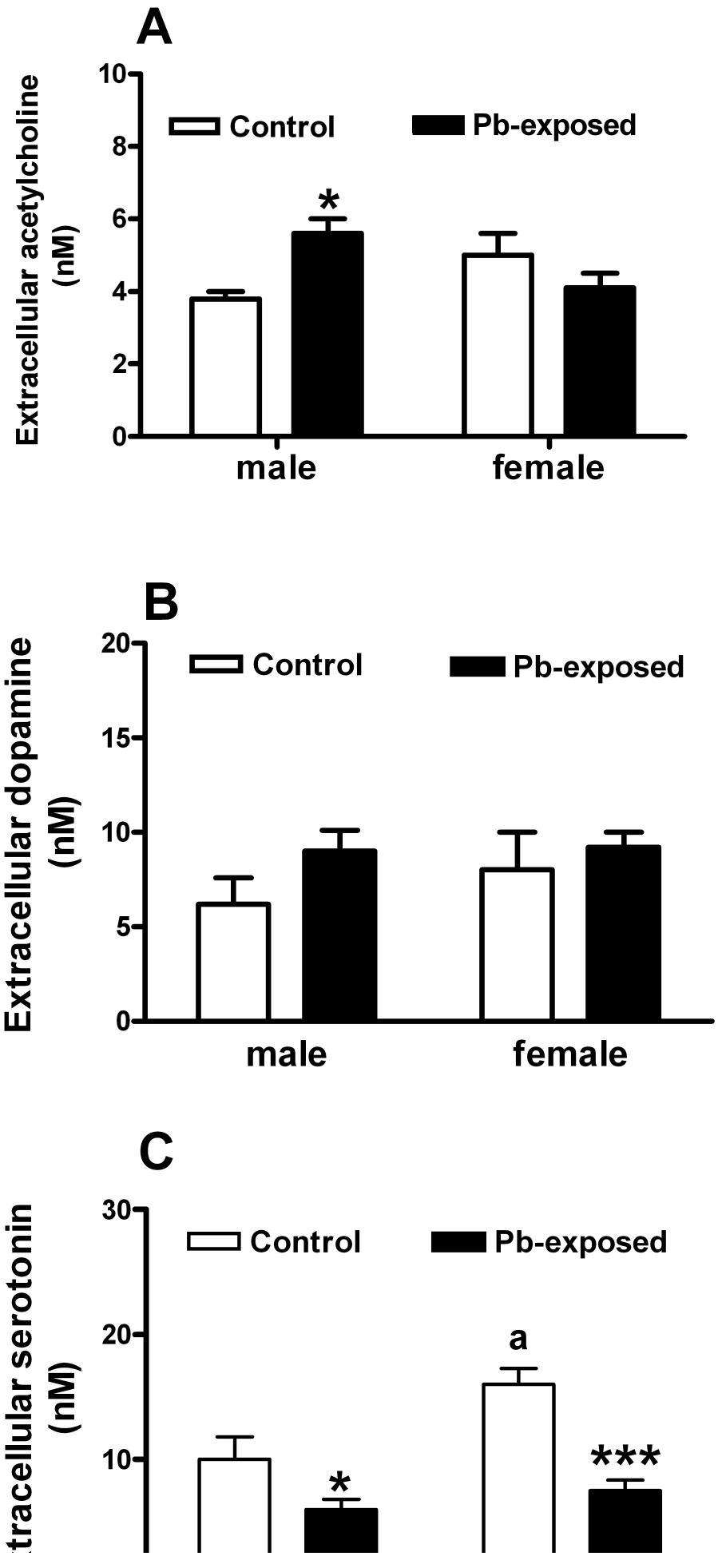

B
Control
Pb-treated
% Time spent in target quadrant
75
50
25
0
male
female
***
a
*

Figure 5.


A
Extracellular acetylcholine (nM)
10
8
6
4
2
0
Control
Pb-exposed
*
male
female
B
Extracellular dopamine (nM)
20
15
10
5
0
Control
Pb-exposed
male
female
C
tracellular serotonin (nM)
30
20
10
Control
Pb-exposed
*
a
***