UNIVERSIDADE DE LISBOA

Faculdade de Medicina de Lisboa

Comparative study on the physiological dynamics evoked by different profiles of lead exposure

Liana Shvachiy

Orientadores:

Professora Doutora Vera Geraldes

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Dissertação especialmente elaborada para obtenção do grau de Mestre em Neurociências

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'Everything is theoretically impossible, until it is done'

(Robert A. Heinlein)

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List of Abbreviations

Abbreviations used more than once are listed.

Ab antibody
AT angiotensin
ATP adenosine triphosphate
BBB blood brain barrier
BLL blood lead level
BP blood pressure
bpm beats per minute
BRG baroreceptor reflex gain
CA *Cornu Ammonis*
CDC *Center of Disease Control*
ChS chemoreflex sensitivity
CNS central nervous system
cpm cycles per minute
CTL control
DAPI 4',6-Diamidino-2-Phenylindole, Dihydrochloride
dBP diastolic blood pressure
DG dentate gyrus
DWT wavelet transform
ECG electrocardiogram
EPM Elevated Plus Maze
GABA Gamma-aminobutyric acid
GFAP Glial fibrillary acidic protein
GSH glutathione in oxidized state
GSSG glutathione in reduced state
HF high frequency
HR heart rate
HTN arterial hypertension
Iba-1 Ionized calcium binding adaptor molecule 1
Ig immunoglobulin
IL interleukin
ip intraperitoneal
IV intravenous
LF low frequency
mBP mean blood pressure
N novel object
na not available
NeuN Neuronal Nuclei
NMDA N-methyl-D-aspartate
NO nitric oxide
NOR Novel object recognition test
NTS nucleus tractus solitarius
OFT Open Field Test
PbI intermittent lead exposure
PbP permanent lead exposure
PBS phosphate-buffered saline
PbS single lead exposure
PbS/I single/intermittent lead exposure
PVN paraventricular nucleus of hypothalamus
RF respiratory frequency
RGB read, green and blue
ROI region of interest
ROS reactive oxygen species
S sample object
sBP systolic blood pressure
Syn Synaptophysin
TBS Tris-buffered saline
TNF Tumor necrosis factor
w weeks
WHO World Health Organization

Abstract

Lead is a toxic metal which widespread use has resulted in environmental contamination and significant health problems. It is a cumulative toxicant that affects multiple body systems, including the cardiovascular, hematopoietic, reproductive, and renal systems. Lead is also a well-known neurotoxin, inducing changes in neurogenesis, neurodegeneration and changes on glial cells. These changes in the molecular and cellular processes lead to cognitive and behaviour alterations, particularly during developmental phases, persisting throughout the lifetime.

Most of the studies that have been performed in both humans and animals were focused in a continuous chronic exposure to lead. This lead exposure causes behavioural changes, cognitive impairment and hypertension associated with sympathoexcitation, baroreceptor reflex hyposensitivity and increased chemoreceptor reflex sensitivity. But the effects of an intermittent lead exposure are scarse and standardized animal models are non-existent. This pattern of exposure has been increasing in the last years due to migrations, implementation of school exchange programs and/or residential changes.

Therefore, the overall purpose of this work was to evaluate lead effects on mammal's physiology along different profiles of lead exposure, including a new animal model of intermittent low-level lead exposure.

Animal models of lead exposure were developed by replacing the tap water of seven-day pregnant Wistar females with 0.2% (p/v) solution of lead acetate. After being weaned at 21 days, rat pups, both sexes, were divided into 3 groups of lead exposure: long-term (exposure from foetal period until 28 weeks of age), short-term (exposure from foetal period until 12 weeks) and intermittent (exposure from foetal period until 12 weeks, lead-free period until 20 weeks and a second exposure between 20 and 28 weeks of age).

At 12, 20 and 28 weeks of age, behavioural tests were performed for anxiety (Elevated Plus Maze Test), locomotor activity (Open Field Test), spatial working memory (Y-Maze) and episodic long-term memory (Novel Object Recognition test) assessment. Blood pressure (BP), electrocardiogram (ECG), heart rate (HR) and respiratory frequency (RF) were recorded at the same timepoints in the acute experiment. Baroreflex gain (BRG), chemoreflex sensitivity (ChS), cardiovascular variability were also evaluated.

Immunohistochemistry studies for neuronal nuclear antigen (NeuN), Synaptophysin (Syn), ionized calcium binding adapter molecule-1 (Iba-1) and Glial fibrillary acidic protein (GFAP) stainings were performed in brain slices, and confocal imaging acquired and stainings quantified at dentate gyrus

(DG) of the hippocampus. Blood lead levels were assessed by atomic absorption spectroscopy and metabolic evaluation of all groups was done using metabolic cages. A control group of Wistar rats without lead exposure, of both sexes, with the same number of individuals, underwent the same protocol and were evaluated in the same time points. Student T-test and one-way ANOVA with Tukey's multiple comparison between means were used (significance $p<0.05$) for statistical analysis.

Our data showed a clear association between lead exposure, hypertension and cardiorespiratory reflexes impairment, without heart rate changes, independently of the type of lead exposure profile. We also demonstrated for the first time that lead intermittent exposure causes adverse health effects, i.e, hypertension, sympathetic overactivity, increased chemoreflex sensitivity and baroreflex impairment, similar to a chronic exposure, however less pronounced. In fact, at 28 weeks, PbI group, the intermittent animal model of lead exposure developed, had a less severe hypertension when compared to the long-term exposure group (PbP), which might suggest that the duration of Pb exposure is more relevant than the time of exposure. Moreover, the effect on diastolic blood pressure produced by lead exposure was more evident than that of systolic blood pressure.

Lead exposure from foetal period until 12 weeks of age causes long lasting hypertension and chemoreceptor reflex dysfunction even after a 16 weeks period without exposure. However, the clearance of lead promoted an improvement in baroreceptor reflex function, with repercussions on blood pressure values, since these values decreased, but did not reached the normotensive values.

Regarding the autonomic data, in our study, the overactivity of the sympathetic nervous system, evaluated by the LF band, is concomitant with baroreceptor reflex impairment and/or hypertension. This means that the sympathetic nervous system may be involved in the modulation of the baroreceptor reflexes responses or in the hypertension development due to lead exposure.

Concerning the effect of lead at behavioural level, all groups exposed to lead, evaluated in the three different time points, had behavioural changes, namely anxiety, hyperactivity and/or long-term memory impairment and molecular changes in the hippocampus region, more specifically, reactive astrogliosis and microgliosis were detected, indicating the presence of neuroinflammation. However, these alterations seem to reverse after lead abstinence for a certain period (single exposure) and are enhanced when a second exposure occurs (intermittent exposure), along with a synaptic loss.

In summary, this study shows, that exposure to lead during the developmental phase can alter the normal course of development, with lifelong health consequences. Since all exposed Pb groups had the same route of exposure (i.e. exposure to lead by water) and the same dose and, despite the different time of exposure, all were exposed to lead since foetal period until adulthood, the most

susceptible period to adverse health effects. Therefore, we can conclude that the different effects of lead toxicant between groups mainly depends on the total duration of lead exposure.

This comparative study brings new insights on the environmental factors that influence nervous and cardiovascular systems during development, which can help creating public policy strategies to prevent and control the adverse effects of Pb toxicity.

Key-words: lead toxicity; autonomic dysfunction; hypertension; behavioural and cognitive changes; gliosis

Resumo

A identificação de agentes potencialmente tóxicos e a avaliação dos seus efeitos sobre o organismo humano constituem um tema importante de saúde pública. O chumbo encontra-se neste grupo de agentes, sendo bastante utilizado em todo o mundo, devido às suas propriedades únicas, como a alta maleabilidade, baixo ponto de fusão, suavidade, ductilidade e resistência à corrosão. O vasto uso deste metal pesado em indústrias, como a automóvel, cerâmica, de tintas e do plástico levou ao aumento da quantidade de chumbo livre no ambiente e a sua ocorrência nos sistemas biológicos, devido à sua natureza não biodegradável.

A toxicidade do chumbo, como resultado da sua ingestão, inalação ou por contacto direto, mesmo em pequenas quantidades, pode evocar efeitos adversos irreversíveis em várias funções do corpo, afetando principalmente os sistemas cardiovascular (sendo uma das causas da hipertensão, promovendo aterosclerose, trombose, arteriosclerose e doenças cardiovasculares), hematopoiético, reprodutivo e renal. O chumbo é também uma neurotoxina já bem estudada, que induz alterações na neurogénese, nas células gliais e neurodegeneração. Estas alterações nos mecanismos celulares e moleculares, quando ocorrem durante as fases de desenvolvimento, provocam alterações cognitivas e comportamentais, que persistem durante toda a vida.

Em termos de classificação, dois tipos de toxicidade de chumbo podem ser definidos: a toxicidade aguda, que geralmente ocorre pela exposição ocupacional a níveis elevados de chumbo, sendo esta bastante incomum, e a toxicidade crónica, uma exposição a níveis baixos de chumbo, mais comum no ambiente familiar. A maioria dos estudos realizados até à data em seres humanos e animais, focam-se na exposição crónica contínua e/ou permanente ao chumbo e nas consequências adversas na saúde deste tipo de exposição. Existem já, vários modelos animais descritos para a exposição contínua a níveis baixos de chumbo. No entanto, em determinadas situações, como nas migrações, nos programas de intercâmbio escolar e/ou nas mudanças residenciais, a exposição intermitente ao chumbo pode ocorrer, mas os estudos disponíveis em seres humanos são escassos e os modelos animais padronizados inexistentes para este tipo de exposição, que tem vindo a crescer exponencialmente nos últimos anos.

Posto isto, o objetivo geral deste trabalho consistiu em avaliar os efeitos de diferentes perfis de exposição a níveis baixos de chumbo na fisiologia de ratos *Wistar*, incluindo o desenvolvimento de um novo modelo animal de exposição intermitente a chumbo.

Os modelos animais de exposição ao chumbo foram desenvolvidos substituindo a água dos biberões das fêmeas *Wistar* grávidas de sete dias por uma solução de acetato de chumbo a 0,2% (p/v).

Após os 21 dias de desmame, as crias, de ambos sexos, foram divididas em 3 grupos de exposição ao chumbo: de longo prazo (PbP - exposição do período fetal até às 28 semanas de idade), de curto prazo (*Short-term* → PbS - exposição do período fetal até às 12 semanas, com abstinência ao chumbo até às 28 semanas) e intermitente (PbI - exposição do período fetal até 12 semanas, seguida por um período sem chumbo até 20 semanas e uma segunda exposição entre 20 e 28 semanas de idade). Em três diferentes pontos temporais (12, 20 e 28 semanas de idade), os diferentes grupos de animais foram sujeitos a testes comportamentais, para a avaliação dos níveis de ansiedade (EPM), da atividade locomotora (OFT), da memória espacial de trabalho (Y-Maze) e da memória episódica de longo prazo (NOR). Para avaliação dos parâmetros fisiológicos nos diferentes pontos temporais, os animais foram sujeitos a uma experiência aguda, onde foram registados os seguintes parâmetros: pressão arterial (PA), eletrocardiograma (ECG), frequência cardíaca (FC) e frequência respiratória (FR). Nesta experiência também se avaliaram os reflexos baro- e quimiorrecetores e obtiveram-se registos para a análise da variabilidade da FC e da PA sistólica. Após o término da experiência aguda, os animais foram sacrificados e os cérebros extraídos para estudos de imunohistoquímica em secções coronais, nas quais se analisou a morfologia das células e se quantificou a perda neuronal (*neuronal nuclear antigen* - NeuN), a astrogliose (*Glial fibrillary acidic protein* – GFAP) e a microgliose (*ionized calcium binding adapter molecule-1* - Iba-1), bem como alterações na transmissão sináptica (*Synaptophysin* – Syn) no girus dentado do hipocampo. Os níveis de chumbo no sangue foram avaliados por espectroscopia de absorção atómica e a avaliação metabólica realizada através do uso de gaiolas metabólicas. Um grupo controlo de ratos *Wistar* sem exposição ao chumbo, de ambos os sexos e com o mesmo número de indivíduos, foi submetido ao mesmo protocolo e foi avaliado nos mesmos pontos temporais (12, 20 e 28 semanas de idade). Para a análise estatística foi utilizado o teste T de Student e a análise de Variância (ANOVA) unidirecional com o teste *post-hoc* de Tukey, considerando-se significativas diferenças com $p < 0,05$.

Os resultados deste estudo mostram que, independentemente do tipo de perfil de exposição ao chumbo, existe uma associação clara entre exposição a chumbo, hipertensão e diminuição do ganho do barorreflexo, sem alterações de frequência cardíaca.

Também demonstramos, pela primeira vez, que uma exposição intermitente a chumbo provoca efeitos adversos para a saúde, como hipertensão, hiperatividade simpática, aumento da sensibilidade quimiorreflexa e diminuição do ganho do barorreflexo, efeitos adversos semelhantes ao de uma exposição crónica permanente (PbP), porém menos pronunciada. De facto, às 28 semanas, o grupo PbI, o modelo animal intermitente de exposição ao chumbo desenvolvido, apresentou uma hipertensão menos grave em relação ao grupo de exposição de longo prazo (PbP), o que pode sugerir que a duração da exposição ao chumbo é mais relevante do que o tempo de exposição. Além disso, o

efeito da exposição ao chumbo sobre a pressão arterial diastólica foi mais evidente do que sobre a pressão arterial sistólica. A exposição ao chumbo, desde o período fetal até as 12 semanas de idade, provoca hipertensão e disfunção quimiorreflexa duradoura, mesmo com um período de 16 semanas sem exposição. No entanto, a abstinência do chumbo promoveu uma melhoria na função barorreflexa, com repercussões nos valores da pressão arterial, uma vez que estes valores diminuíram, apesar de não atingirem os valores de normotensão.

Em relação à avaliação autonómica, os dados indicam que quando existe um aumento do tónus simpático, avaliado pela banda LF, este é concomitante com disfunção barorreflexa e/ou hipertensão arterial. Isso significa que o sistema nervoso simpático deve estar envolvido na modulação da resposta barorreflexa ou no desenvolvimento da hipertensão decorrente da exposição ao chumbo.

Relativamente ao efeito do chumbo a nível comportamental, todos os grupos expostos ao chumbo, avaliados nos diferentes pontos temporais, apresentaram alterações comportamentais, nomeadamente ansiedade, hiperatividade e / ou défices de memória a longo prazo, bem como alterações moleculares, mais especificamente, astrogliose e microgliose reativa, que indicam a presença de neuroinflamação. No entanto, estas alterações parecem reverter após a abstinência do chumbo durante um determinado período (PbS - exposição de curta duração), sendo mais evidentes quando ocorre uma segunda exposição a chumbo (PbI - exposição intermitente), levando mesmo a perda sináptica mais pronunciada.

Em resumo, este estudo mostra, que exposições a chumbo durante as fases de desenvolvimento podem alterar o seu curso normal, com consequências adversas para a saúde que podem persistir para toda a vida. Uma vez que todos os grupos expostos a chumbo tiveram a mesma via de exposição (isto é, exposição ao chumbo através da água) e a mesma dose e, apesar do tempo de exposição diferente, todos foram expostos ao chumbo desde o período fetal até a idade adulta, período em que são mais suscetíveis a efeitos adversos na saúde. Portanto, podemos concluir que os diferentes efeitos tóxicos do chumbo entre os grupos dependem principalmente da duração total da exposição ao chumbo.

As novas evidências obtidas por este estudo comparativo permitem-nos contribuir para o esclarecimento sobre os fatores ambientais que influenciam os sistemas nervoso e cardiovascular durante o desenvolvimento, o que pode ajudar a criar estratégias de políticas públicas para prevenir e controlar os efeitos adversos da toxicidade do chumbo.

Palavras-chave: toxicidade do chumbo; disfunção do sistema nervoso autónomo; hipertensão; alterações comportamentais e cognitivas; gliose

Authorship

The work described in this thesis resulted in the following publications and communications:

Peer-reviewed journal articles:

- Shvachiy, L., Geraldes, V., Amaro-Leal, Â., Rocha, I. (2018). *Intermittent low-level lead exposure provokes anxiety, hypertension, autonomic dysfunction and neuroinflammation.* Under review: Neurotoxicology.

Congress paper:

- Shvachiy, L., Geraldes, V., Carvalho, M., Rocha, I. (2018). *Autonomic Function Evaluation in an Intermittent Lead Exposure Animal Model*. i-ETC: ISEL Academic Journal of Electronics Telecommunications and Computers, 3(1), 11.

Oral & Poster Presentations:

- Shvachiy, L., Geraldes, V., Amaro-Leal, Â., Rocha, I., *Physiological changes induced by intermittent low-level lead exposure*, at Annual Meeting of Sociedade Portuguesa de Farmacologia: XLVIII Reunião da Sociedade Portuguesa de Farmacologia, XXXVI Reunião de Farmacologia Clínica, e XVII Reunião de Toxicologia, held at Faculty of Medicine of University of Lisbon, Lisbon, Portugal, 5-7 February 2018.
- Presentation of SynaNet Short Term Scientific Mission Project Report: "*Immunohistochemistry on freely floating fixed tissue sections"* at the Second SynaNet Scientific Meeting, held at Instituto de Medicina Molecular, Lisbon, Portugal, 17-18 January 2018.
- Shvachiy, L., Geraldes, V., Amaro-Leal, Â., Rocha, I., *Behaviour and cardiorespiratory changes in an intermittent low-level lead exposure animal model*. at Third Mind-Brain College Meeting, University of Lisbon, Lisbon, Portugal at 19-20 October 2017. Abstract book page 7. Poster *P10*.
- Shvachiy, L., Geraldes, V., Amaro-Leal, Â., Rocha, I., *Intermittent low-level lead exposure causes anxiety and cardiorespiratory impairment*. at Twelfth YES - Young European Scientist - Meeting, from the 14th to the 17th September 2017, held at CIM- Centro de Investigação Médica, FMUP - Faculdade de Medicina da Universidade do Porto, Portugal. Abstract published in Porto, Lisbon in Porto Biomedical Journal Volume 2, Issue 5, September–October 2017, Pages 229–230. Poster *217.*

- Shvachiy, L., Geraldes, V., Amaro-Leal, Â., Rocha, I., *Cardiorespiratory impairment in an intermittent low-level lead exposure animal model.* at YRLS - Young Researchers in Life Sciences Conference, held at Imagine Institute, Paris, France at 15-17 May 2017. Abstract book page 184. Poster *29*, Session *3*.
- Presentation of SynaNet Short Term Scientific Mission Project: *"Immunohistochemistry on freely floating fixed tissue sections"* at SynaNet First Scientific Meeting, held at Instituto de Medicina Molecular, Lisbon, Portugal, 26-27 January 2017.
- Presentation of Master Thesis Project: *"Comparative study on the physiological dynamics evoked by different profiles of lead exposure"* at Cardiovascular Centre of University of Lisbon (CCUL) monthly meeting, on 11th January 2017, Faculdade de Medicina da Universidade de Lisboa, Lisbon, Portugal.
- Shvachiy, L., Geraldes, V., Carvalho, M., Rocha, I., *Autonomic Function Evaluation in an Intermittent Lead Exposure Animal Model.*, at CETC 2016, Conference on Electronics, Telecommunications and Computers, Instituto Superior de Engenharia de Lisboa, Lisbon, Portugal at 6-7 December 2016.

Prizes

Experimental Pathology section 1st Degree Diploma for oral presentation

'Physiological changes induced by different low-level lead exposure profiles through lifetime' at V International Medical and pharmaceutical Congress of students and young scientists, from the 4th to the 6th April 2018, held at Higher state educational establishment of Ukraine Bukovinian State Medical University, Chernivtsi, Ukraine

Physiology & Immunology Poster Presentation First Prize

'Intermittent Low-level Lead exposure causes anxiety and cardiorespiratory impairment' at the Twelfth YES - Young European Scientist - Meeting, from the 14th to the 17th September 2017, held at CIM- Centro de Investigação Médica, FMUP - Faculdade de Medicina da Universidade do Porto, Portugal.

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1 INTRODUCTION

Lead [1] (Pb, from the Latin *plumbum*) is a post-transitional metal which compounds are mostly found in the $^{+2}$-oxidation state.

The low melting point and ductility, the high density and the inertness to oxidation, combined with the relative abundance, easiness of extraction and low cost[2] justifies its leading position in human usage for more than 8000 years. In 2014, ten million tonnes of lead have been handled, over half of it in recycling processes[3]. Lead is heavily used in batteries production, pewters, construction, bullets and shotguns, plumbing, ceramics, weights, solders, fusible alloys, radiation shielding and leaded gasoline[3] but also in jewellery, pigments, stained glass, lead crystal glassware, toys, cosmetics, pesticides and traditional medicine, all of which have resulted in substantial introductions of free lead into the environment forming leaded dust and soils, contaminating water and air[1,3,4]and, overall, generating significant environmental pollution together with human and public health issues in many parts of the world[5,6] (***Figure 1***). In fact, and regarding human and animal health, the particle size and the route of exposure are the impact factors of lead absorption that is inversely proportional to these features. Nevertheless, the nutritional status (fat and caloric intakes; phosphorus, copper, zinc and especially iron and calcium levels, all affecting lead absorption), fasting/fed status (fasting humans or animals absorb much larger fractions than their fed counterparts), health, and age of the individual also account for lead negative biological effects[1,5,7].

There are also intraspecies differences regarding lead toxicology. For human subjects, children absorb more lead into the blood when compared to adults[1,8] In fact, adults, typically absorb up to 20% of ingested inorganic lead after a meal and up to 60-80% after a big period of hunger while children absorb about 50% of ingested lead after a meal and up to 100% on an empty stomach[1]. Exposure to lead dust (by the respiratory route) may elicit an increased level of absorption, when compared to the lead that has been ingested, usually, in larger sizes (digestive route), like lead chips in leaded paint.

Despite the main important routes of human exposure are inhalation or ingestion, the dust and soil that contain lead can also be absorbed by the skin. However, it is unlikely to happen at the present, since lead gasoline has been banned, which was the main source of lead to be absorbed by the skin[1,3,4,8]. Moreover, only a little amount of the absorbed lead can pass through skin to the blood. Although, if hands are contaminated, lead can be ingested whilst eating, drinking, smoking, or applying cosmetics[9].

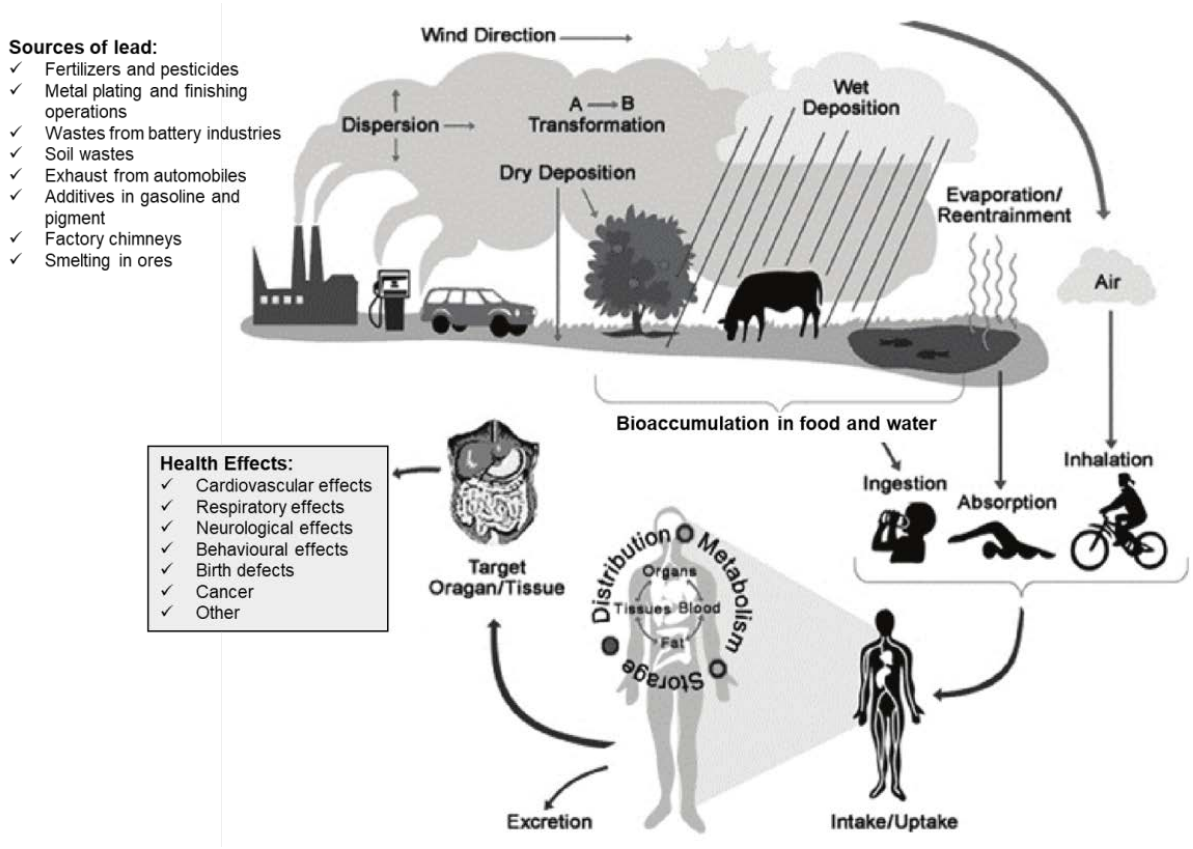

Figure 1 - *Environmental lead exposure cycle and its biological effects*
(extracted and adapted from Wani et.al, 2015[10])

Occupational lead exposure can be defined as high levels of exposure to lead during a short period of time, targeting a specific group of subjects, usually at their working premises[11]. Environmental lead exposure[5] involves a long-lasting exposure of large populations to lower levels of lead, of various sources, present in their living environment. Thus, the first can be considered a synonym of acute lead exposure, being of rare prevalence in developed countries where regulatory and safety procedures have been implemented, whereas the second, may represent a chronic contact to lead, being a public health issue in non-developed countries with high levels of lead emissions and the usage of old working methods in the industry and agriculture[1,2,5,8].

During an exposure, after entering the body and being absorbed, lead is primarily distributed among blood, mineralizing tissue, and soft tissues and organs (which include the liver, muscles, lungs, brain, kidneys spleen, and heart)[1,12]. Lead is not changed in the body and the metabolization of inorganic lead does not happen in the liver[1]. Approximately 99% of lead taken into the body in adult subjects will be excreted within a couple of weeks in the urine as the half-life of lead in adult human blood has been estimated as 28 days to 36 days. The fraction which is not absorbed will be excreted, via the bile, in the faeces[1,4,5]. Excretion of lead through sweat is of minor importance. However, only

about 32% of the lead taken into the body of an infant is excreted. Blood lead levels (BLL; µg/dL) and urine lead levels (not as usual) are measures used for diagnosis and treatment of lead toxicity[13,14]. A continuous exposure usually results in accumulation of lead in bone regions undergoing the most active calcification at the time of exposure. It is known that adults store about 94% of lead in bones and teeth whereas, in children, the amount is of around 73%[1]. It can stay there for decades, but can also re-enter the body circulation under certain circumstances (e.g., when a bone is broken, pregnancy and breast feeding or in osteoporosis cases in advancing age)[1,10]. In this condition, bone and kidney lead levels are usually used as greater measures of lead in the body[15,16]. ***Table 1*** shows the differences between chronic and acute poisoning.

The US Centre for Disease Control[17], Prevention Centre and the World Health Organization (WHO)[5,6,18] guidelines indicate that BBL´s ≥10 µg/dL are a cause for concern; however, lead may impair development and have harmful health effects even at lower levels[19,20] as 5 µg/dL suggesting that all lead levels may represent a danger to human health.

***Table 1** –Acute and chronic lead intoxication features.*
Main differences between acute and chronic exposure to lead regarding BBL's, health effects and associated clinical symptoms.

	Exposure	Type of exposure	Blood Lead levels	Health effects	Clinical symptoms
Acute poisoning	Intense exposure of short duration	Occupational (workspace)	100-120 µg/dL	- Loss of appetite - Headache - Hypertension - Arthritis - Hallucinations - Vertigo - Haemolytic anaemia	- Muscle pain - Fatigue - Abdominal pain - Headache - Vomiting - Seizure - Coma
Chronic poisoning	Low-level lead exposure of long duration	Environmental and domestic	10-60 µg/dL	- Mental retardation - Birth defects - Psychosis - Autism - Allergies - Dyslexia - Weight loss - Hyperactivity - Paralysis - Muscular weakness - Brain damage - Kidney damage - Frank anaemia - Hypertension - Cardiovascular disease	- Persistent vomiting - Encephalopathy - Lethargy - Delirium - Convulsions - Coma

PREVALENCE AND EPIDEMIOLOGY OF LEAD TOXICITY

Lead poisoning accounts for about 0.6% of the global burden of disease. The highest burden was estimated in low- and middle-income countries. Based on 2015 data, the Institute for Health Metrics and Evaluation (IHME) has estimated that lead exposure has accounted for 494 550 deaths and loss of 9.3 million disability-adjusted life years (DALYs) regarding the long-term effects on health. IHME also estimated that lead exposure accounted for 12.4% of the global burden of idiopathic developmental intellectual disability, 2.5% of the global burden of ischaemic heart disease and 2.4% of the global burden of stroke[4].

In USA, the Centre for Disease Control[21], identified, in 2010, that approximately 24,000 children aged <6 years presented blood lead levels (BLLs) ≥10 µg/dL and approximately 243,000 children aged <6 years had BLLs ≥5 µg/dL. Regarding European countries, the prevalence of lead poisoning was addressed in the 2004 WHO Report[22]. Data from 24 studies and population surveys conducted between 1996 and 2000 in children between 0 and 4 years allowed to divide European country into 3 categories:

- EUR A: prevalence of 0.1%, comprising Croatia, the Czech Republic, Finland, France, Germany, Greece, Italy, Portugal, Spain, Sweden and the United Kingdom.
- EUR B: prevalence of 2%, consisting of Armenia, Bulgaria, Poland, Turkey and Yugoslavia.
- EUR C: prevalence of 17%, consisting of Russia and Hungary.

In 2014, the European Environmental Agency (EEA) has performed a surveillance study on lead in the environmental air of EU countries. The results showed that ~97% of the surveillance stations detected levels of lead under 0.25 µg/m^3 in the analysed air without stations sensing values of 0.5 µg/m^3. However, the area of extent of the exceedances of critical loads was more that 12% of the European Union ecosystem area[23].

The World Health Organization (WHO) and the International Programme on Chemical Safety have been concerned about the adverse health effects of environmental lead exposures for more than 35 years. They convened working groups to evaluate human health risks and health-based guidance values of lead in water, workplace and air [4,5,8,24].

Lead toxicity is one of the most common and well-recognized childhood diseases from environmental toxins and children around the world, nowadays, are at risk of exposure to lead from multiple sources [4,24]. Therefore, some countries developed robust programmes for monitoring levels of lead in blood and in the environment, as well as strong programmes for primary and secondary prevention of developmental lead toxicity. These countries have imposed bans on certain uses of lead, have set environmental standards and have arranged screening programmes[4,5,8,24]. In the other

countries where lead poisoning has not yet been recognized, there are no screening or surveillance programmes and, as a result, public health authorities have little or no knowledge of the existence of a childhood lead-poisoning problem. Thus, the contribution of lead poisoning to the global burden of disease and its effects on the global economy and human development are still underestimated[1,5,7].

MECHANISMS OF LEAD TOXICITY

The mechanism of lead toxicity is not yet fully understood. Nevertheless, studies carried out reported various cellular, intracellular and molecular mechanisms behind toxicological manifestations resulting from lead exposure.

Oxidative stress

Oxidative stress is characterized as an imbalance between free radical production and the systems' ability to detoxicate from reactive intermediates. This has been reported as the main mechanism of lead toxicity[2,25,26].

Two pathways are simultaneously activated at the onset of oxidative stress provoked by lead exposure. One is production of reactive oxygen species (ROS) occurs leading to a depletion of the antioxidant reserves headed by glutathione. Glutathione is the primary antioxidant in cells and it exists in both reduced (90%) and oxidized (10%) forms [25] which are interchangeable according to the cell environmental condition. The other pathway is related to lead's ability to share electrons through covalent bonds between lead moiety and the sulfhydryl groups of the antioxidant enzymes which are lead main target. These enzymes may be inactivated by lead that binds to their sulfhydryl groups eliciting the synthesis of glutathione and forces the replacement of the enzyme zinc ions[26]. Lead has also the ability to inactivate other enzymes, which further reduce the glutathione levels[26,27] such as super oxide dismutase and catalase. In addition, lead also promotes lipid peroxidation, haemoglobin oxidation, which directly causes haemolysis[25,27,28]. All these mechanisms make the cell extremely vulnerable to oxidative stress and may lead to cell death. ***Figure 2*** schematically illustrates the oxidative stress mechanism due to lead toxicity.

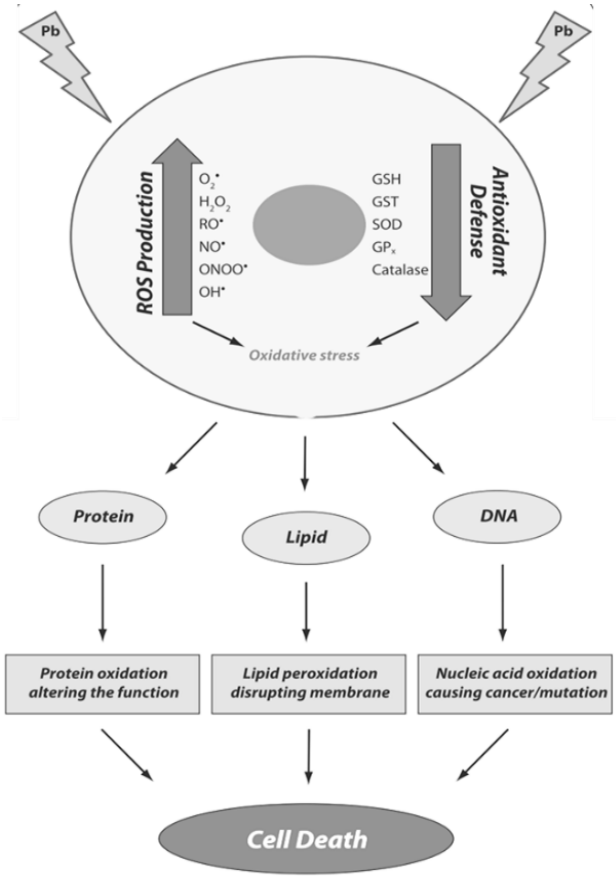

Figure 2 – *Lead-induced oxidative stress and subsequent mechanisms leading to cell death*

(extracted and adapted from Flora, G., 2012 [2])

Ionic changes

Lead also replaces various bivalent cations (calcium - Ca^{2+}, magnesium - Mg^{2+}, iron - Fe^{2+}) and monovalent cations (such as sodium - Na^{+}) by itself, which defines the ionic mechanisms of action of lead toxicity. These ionic changes may affect fundamental organic processes of the body[29] impairing both intra and extra cellular processes such as intra and intercellular signalling, protein folding and maturation, cell adhesion, enzyme regulation, apoptosis, release of neurotransmitters and ionic transportation[30].

The ionic mechanisms of lead toxicity are the main contributor to lead neurological impairment as, after its replacement by calcium ions, lead has the ability to cross the blood-brain barrier and accumulate in astrocytes. Therefore, toxic effects of lead are more prominent in the developing nervous system, comprising immature astroglial cells that lack lead binding proteins. Lead effortlessly damages the immature glial cells and blocks the formation of myelin sheath, both factors involved in BBB development[30–32]. Even at picomolar concentration, lead replaces calcium, consequently affecting key neurotransmitters, like protein kinase C, which regulates long term neural excitation and memory storage. It also affects the concentration of sodium ions, which plays a major role in several vital

biological activities, like generation of action potentials in the excitatory tissues for cell to cell communication, retention of calcium by synaptosomes, uptake and regulation of uptake of neurotransmitters. Therefore, this interaction between lead and sodium extremely impairs the normal functioning of the sodium dependent processes [30–33].

Activation of brain inflammatory pathways

Microglia and astrocytes are the two main types of glial cells involved in the regulation of the immune response to pathological processes in the brain. Activation of both microglia and astrocytes result in neuroinflammation, associated with infection, autoimmunity, and pathogenesis of neurodegenerative diseases. Lead exposure leads to the insufficient supply of energy from astrocytes to neurons. Also, following lead exposure, astrocytes secrete various inflammatory cytokines, such as, TNF-α, IL-6, and IL-10 into surrounding tissues. These cytokines further mediate the immune response, including activation of microglia and macrophages, and induce other adverse reactions, which might eventually result in the destruction of BBB tight junctions. Overexpression of inflammatory stimuli in the brain neurovascular unit may start a response to clear antigenic material, leading to destruction and increase of BBB permeability together with neuronal damage[34–37]. In summary, the mechanisms related to microglia and astrocytic activation are schematically presented in *Figure 3*.

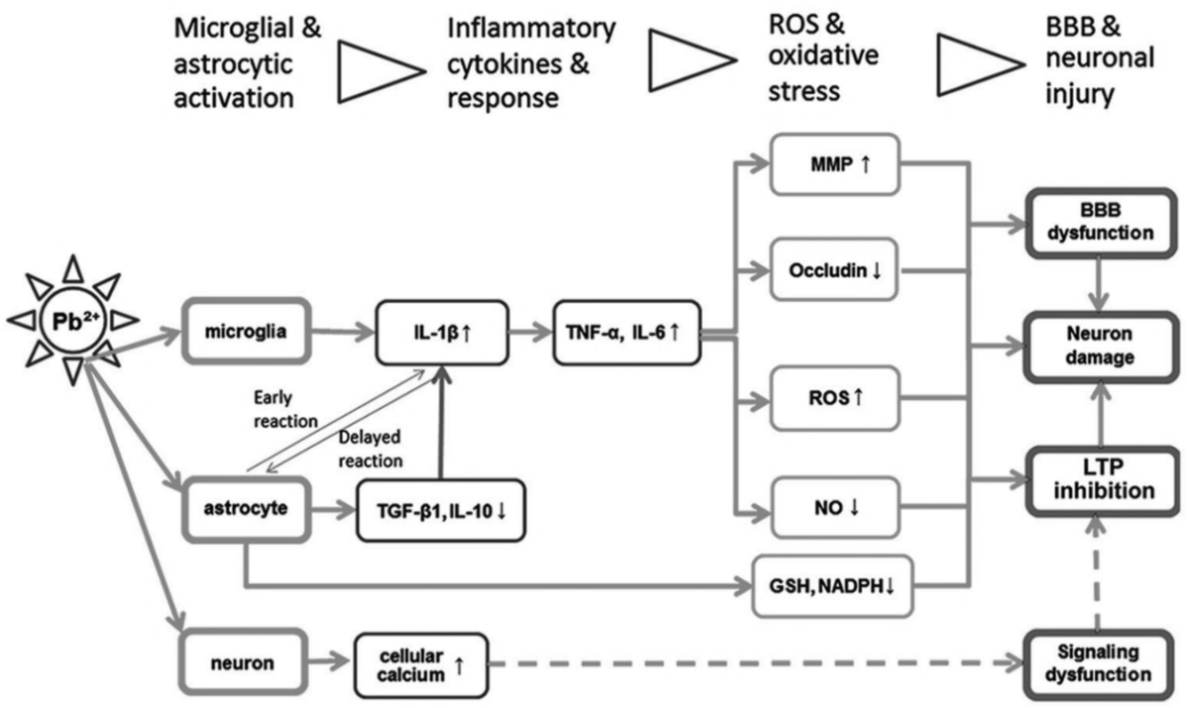

Figure 3 - *Neuroinflammation as lead induced toxicity mechanism*
Microglial and astroglial cells function in the generation of inflammatory cytokines and neuronal injury. Lead may contact and trigger microglial and astrocytic activation, enhancing inflammatory cytokines and their response, increasing ROS and oxidative stress, resulting in BBB dysfunction, long-term potentiation inhibition and inflammatory neuronal injury. (extracted from Liu, J., 2015 [34])

LEAD EFFECTS ON HUMAN HEALTH

Lead toxicity has the potential to cause irreversible health effects interfering with a wide number of body functions.

Lead and the cardiovascular system

Population studies have demonstrated that lead toxicology is associated with cardiovascular disease[38–41]. Studies from the early 1920s showed that long-term and high dose lead exposure correlates with an increased incidence of hypertension and brain stroke. More recently, several epidemiological studies evidenced that increased lead absorption, even at relatively low levels, is also consequent with significant elevation in blood pressure across general populations with no occupational exposure to lead [40,42]. Other studies in human subjects and in experimental animal models also identified a dose-dependent relationship between lead exposure and higher blood pressure [40,42]. Beyond hypertension, few studies in general populations have identified a positive association between exposure to lead and clinical cardiovascular events (mortality due to cardiovascular disease, coronary heart disease and stroke and peripheral arterial disease), some of them observed at blood lead levels ≤5 μg/dL. [40,43].

Molecular studies have shown that chronic lead exposure promotes oxidative stress, limits nitric oxide availability impairing nitric oxide signalling, increases adrenergic activity, raises vasoconstrictor prostaglandins lowering the vasodilator ones, promotes inflammation, increases endothelin production, alters renin-angiotensin signalling, disturbs calcium signalling in smooth muscle vessels, diminishes endothelium-dependent vessels relaxation and modifies the vascular response to vasoactive agonists causing HTN [25,42,44]. Additionally, it was shown that lead causes endothelial injury, prevents endothelial repair, inhibits angiogenesis, supresses proteoglycan production, stimulates vascular smooth muscle cell proliferation and phenotypic transformation, reduces plasminogen activator and raises plasminogen activator inhibitor-1 production. All these actions, and others not yet known, caused by lead exposure, are the key mechanisms that cause HTN and promote atherosclerosis, thrombosis, arteriosclerosis and cardiovascular disease[40,42,44].

Lead and the autonomic nervous system

Autonomic function is not known to be primarily affected by chronic lead exposure. However, the parasympathetic nervous system has been described as being affected by Pb-poisoning causing autonomic dysfunction[45]. Additionally, it was shown that lead increases the co-inhibition of sympathetic and parasympathetic activation during psychological stress and, also, reduces the baroreflex sensitivity,

vagal parasympathetic tone and increases sympathetic activity by mechanisms that include impairment of dopamine and acetylcholine transmission and oxidative stress[43].

Baroreflex hyposensitivity, sympathetic overexcitation and decreased parasympathetic tone are associated to several pathologies, such as, hypertension, acute cardiac ischemia or even heart failure. The mechanisms underlying those pathological effects have not yet been fully clarified, but it is thought, that there is an initial protective reaction that turns into deleterious sympathoexcitation with the passing time[45]. Studies have shown that chronic lead toxicity produces an increase in sympathetic activity that underlies alterations, namely high blood pressure, tachypnoea, decrease in baroreflex function and increased chemoreflex sensitivity [43]. Until last year, there were no evidences of chemoreflex function changes, but a recent work has shown that there is an increase in chemoreceptor reflex sensitivity, suggesting that chemoreceptor reflex could be involved in oxygen homeostasis maintenance in a similar way to hypertensive patients that exhibit an augmented ventilatory response to hypoxia and an increased sympathetic nerve activity[43,45].

Apparently, lead toxicity interferes with brainstem cardiorespiratory network function which, in turn, could account for the higher sympathetic tone[5,43,45]. Data shown in a recent study strongly indicates that both carotid body reflex, a protective sympathoexcitatory reflex, and the central autonomic network are involved in the augmented chemoreflex response and, this involvement, may contribute to the increased baseline sympathetic activity that was observed[45]. The mechanisms underlying those changes are yet unknown, but it has been hypothesised that those linking the hypothalamus with lower brainstem nuclei, especially, the PVN-NTS axis may be involved in the observed functional changes[46]. In addition to those mechanisms, the baroreflex impairment that has been described in lead poisoning and in other pathologies is one of the major consequences of persistent increase of sympathetic tone and arterial blood pressure [43,45,46].

The most recent studies (from 2017) of low-level and sub-chronic lead exposure in rodents [20,47] have shown that arterial hypertension in animals was accompanied by an increase in sympathetic tone and decrease in vagal tone, baroreflex impairment without changes on cardiopulmonary reflex. The changes went together with an increase in the renin-angiotensin system mediated by the AT_1receptor activation and decrease in nitric oxide (NO) bioavailability. As a compensatory mechanism of the changes found in the sympathetic tone, a downregulation of β1- adrenoceptors in the heart was described. These studies have shown that levels of lead below BLL cut-off for lead toxicity promote alterations in autonomic and cardiovascular system and should be considered as a risk factor for cardiovascular disease.

Neurological effects of lead

Brain is considered to be the most sensitive organ to lead exposure. Research in the past decades for characterizing lead toxicology has shown lead as a potent neurotoxicant, especially during nervous system development[37,48]. Various deleterious effects have been described, including cognitive impairment[49–52] in children that persists in adults, even with low BBLs (<5μg/dL). Also, a positive correlation between lead exposure and the increase of neuropsychiatric disorders, such as attention deficit hyperactivity disorder and antisocial behaviour has been described. Other researchers reported that violent crimes in adulthood are correlated to lead exposure in prenatal and early childhood periods[19,53,54]. Due to the ability of lead to bypass BBB by replacing calcium ions and being taken up by calcium-ATPase pumps, lead can interfere with synapse formation (in particularly in children), production of neurotransmitters and organization of ion channels[30].

Novel findings in this area of research also include advances in understanding the mechanisms and cellular specificity for Pb. Studies have shown that stress alters Pb effects that are mediated by modifications in glucocorticoids, a brain mesocorticolimbic dopamine system which is involved in several pathologies. Lead-induced cognitive impairments have been studied in cellular models of learning and memory by examining the long-term potentiation in rodent hippocampus by increasing threshold, decreasing magnitude and shortening retention times of synaptic plasticity[37,50]. Hippocampal modifications may be the main reason for lead interference with learning, particularly in children, at molecular and morphofunctional levels of neurons and glial cells[52,55,56]. Lead exposure also impairs structural plasticity in adult neurogenesis in the hippocampus, causing perturbations in synaptic plasticity by acting on glutamate release, NMDA receptor function and structural plasticity and thus, contributing to learning impairments [30,54,57] .

In vitro models also evidenced that lead binds to 78-kDa molecular chaperone glucose-regulated protein (GRP78), inducing its aggregation and, consequently, blocking IL-6 secretion in astroglial cells. In the long term, chaperone deficiency could trigger protein conformational diseases, such as, Alzheimer's Disease (AD) and Parkinson's Disease (PD)[37,54,58]. These results are in contradiction with others from studies on the mechanisms of lead toxic effects where IL6 levels are increased. Furthermore, lead exposure in early life has been implicated in subsequent progression of amyloidogenesis in elder rodents. This exposure resulted in an increase in proteins associated with AD pathology, beta-amyloid precursor protein (β-APP), and beta-amyloid (Aβ)[59].

PREVENTION, TREATMENT AND REDUCTION OF LEAD TOXICITY

The first step in treating lead poisoning is to remove the source of the contamination. For children and adults with relatively low lead levels, simply avoiding exposure to lead might be enough to reduce blood lead levels. However, when levels are high, oral therapy with chelating agents (dimercaprol and succimer[60]) is proposed to promote lead urinary excretion. This therapeutic algorithm is essentially recommended for children with circulating levels ≥45 µg/dL or adults with symptoms of lead poisoning. The use of injectable EDTA as a chelating agent is particularly used in adults with serious lead levels (≥45 µg/dL) and for children who do not tolerate oral chelating agents. Antioxidants have been also described as promising agents for lead poisoning treatment and removing its related compounds mainly through nanoencapsulation[2].

Although, the availability of various treatments, they only ameliorate the deleterious effects already caused by lead poisoning. Considering the toxic effects of lead, preventive measures are preferred over the treatment regimens as, once lead enters the body, it is almost impossible its complete removal and the reversal of its damaging effects. A preliminary preventive approach towards lead toxicity is defined as a three-way plan which includes individual intervention, preventive medicine strategy and public health strategy[61]. To each individual at risk it is recommended to frequently wash their hands, to discourage putting contaminated hands in the mouth and to increase the intake of calcium and iron, other mineral elements, flavonoids and vitamins [1,2,10,62,63]. Preventive medicine strategy aims mainly at screening children whom are at risk of lead exposure[61]. Various preventive strategies have been suggested by the public health services for controlling lead[4,18,64]. The most important of them include the prohibition of setting up industries dealing with lead close to habitable areas and the complete banning of lead usage whenever appropriate like as happened with the leaded gasoline and leaded paint banning.

TRANSLATION FROM ANIMAL TO HUMAN STUDIES

Choosing the animal specie

Various population studies have been carried out since lead has been a concern for the human health. However, due to the vast variability of health effects that has been reported, mainly because of the diversity of human exposures, environmental confounders and genetic backgrounds of the populations, animal and molecular studies were performed over the years to study specific health effects that were observed in the human populations and mechanisms underlying those effects.

Studies in rodents and nonhuman primates have demonstrated the same cardiovascular[8,42,45], haematological[41,65,66], neurodevelopmental[31,48,55,58,67–69], and renal lead adverse effects[66,70] , that have been observed in humans, providing insights into possible mechanisms underlying the health changes that low-level lead exposure provokes. These studies also provided the support for the concept of blood lead concentration as a metric of internal dose for use in dose-response assessments in humans.

Rodents are the most used animals in different types of research. Lead exposure health effects have been vastly studied in rodents as well[33,65,68,71–74]. However, comparing rats and mice, rats are a better animal model comparing to mice for numerous reasons. First, the rat is genetically, physiologically and morphologically closer to humans than mice[75,76]. Its large body and brain size facilitates drug administration, in vivo electrophysiology, as well as neurosurgical/stereotaxic and neuroimaging approaches. Second, the mouse represents a less complex behavioural repertoire and much less flexibility in dealing with novel situations. Moreover, the rat appears to be more advantageous in its use in neurobehavioral research, presenting a higher level of behavioural functioning complexity, when compared to mice. These behavioural variations may be accounted since rats, like humans, and opposed to mice, have a post-natal brain development that might result in an extra wide variety of synapses[76]. Therefore, the rat, as an animal model of disease and toxicology, including low-level lead exposure animal models allow a more state-of-the-art characterization of behavioural and physiological changes in the body, permitting a better translation of the alterations to humans.

Experimental protocols to mimic human chronic low-level lead exposure

Due to the importance of animal models in toxicological studies, chronic low-level lead exposure animal models have been developed and used for over 40 years. In 1976, Kostas and her collegues[77] exposed *Long-Evans* dams and pups to different concentrations of lead (5%; 0,5%; 0,05% - by weight), mixing lead acetate in the chow that was fed to animals. At 21 days of age of the pups, the concentration was reduced (25, 2.5 and 0.25 ppm). At 45 days after birth of pups, animals underwent behavioural evaluation for memory and locomotor activity assessment. This type of lead exposure is representative of an exposure to lead by ingestion in children from foetal period and until early ages of life. In the same year, other authors[78] exposed *Sprague-Dawley* pregnant female rats to high lead acetate solution and low lead acetate solution diluted in water. The pups, after birth, underwent the same exposure until 30 days of age. After exposure, animals were tested with open field test for locomotor and exploratory activities. The authors of both studies, although using different routes of lead exposure with different concentrations, only assessed the general behavioural alterations in the animals, without performing other analysis, for a more complete evaluation of toxicological effects of lead.

In 1980, Grant. and collegues[72] also performed a study in *Sprague-Dawley* animals. Females were exposed to increasing concentrations of lead diluted in water and divided in two groups (Group A - 0, 0.5, 5.0, 50, and 250 ppm and Group B - 0, 5, 25, and 50 ppm) 6-7 weeks before mating with non-exposed males. Pups were exposed to the same lead solution and were evaluated at different timepoints from day 1 after birth until 9 months. General health observations, physical development landmarks, pre and post weaning behavioural evaluations were performed in this study. These authors performed a more complex study than the previous ones, testing different physiological and behavioural parameters and various concentrations of lead exposure through a long period of time. Cory-Slechta, in 1985[79], exposed *Long-Evans* hooded rats to 25 ppm lead acetate dissolved in distilled water from 20 days after birth until 50 days. At 50 days of age, authors performed behavioural evaluation using operant chambers. This was a simple study with a short exposure to low-levels of lead in animals during development.

Boscolo and Carmingani , in 1988[44], performed a more complex study in *Sprague-Dawley* rats that received 0, 15, 30, and 60 ug/mL of lead acetate dissolved in deionized drinking water for 18 months. Authors evaluated various physiological parameters in anaesthetized animals, assessing cardiac inotropism, pressor, inotropic and chronotropic responses. Blood and organ lead levels were determined by atomic absorption spectrophotometry. Some years later, in 1993, Altmann and colleagues[56] completed a study in *Wistar* animals. Female *Wistar* rats were fed diets containing 0 and 750 ppm lead acetate, for 50 days prior to mating, during gestational period and until 16 days after

birth. Animals were tested for active avoidance learning and LTP was measured in hippocampal slices and brain and blood lead levels were determined. Such a study is an example of a complex neurotoxicological assessment of lead exposure which includes behavioural and electrophysiological evaluation, comparing the effects to lead levels.

In 1996, Bielarczyk[80] published a new animal model of chronic low level lead exposure, in which the authors exposed pregnant *Sprague-Dawley* animals (7 days before parturition) with 0.2% lead acetate solution. After birth, pups were exposed until weaning at 28 weeks of age, after which animals were maintained at normal drinking diet. Unilateral superior cervical ganglionectomy and partial bilateral transection of fimbria-fornix were performed and cholinacetyltransferase and tyrosine hydroxylase activities and blood lead levels were assessed. In the same year, Lasley and Gilbert[81] exposed *Long-Evans* dams at 14-15 days of gestation to 0.2% lead solution and the pups from day 1 to 135 after birth. Blood lead levels were determined, intracerebral dialysis, chromatography for GABA and glutamate quantification and electrophysiological evaluations were performed. Zawia and Harry[71] in the same year, used *Long-Evans* animals and exposed pups to 0.2% lead solution from postnatal day 1 until 20 days through dams. Total RNA isolation and estimation, Northern analysis and specific mRNA quantification were performed. All these authors established the developmental lead exposure animal models, however, Bielarczyk[80] characterized lead health effects during foetal and developmental periods.

One year after, in 1997, three studies were published. Kuhlmann[57] exposed male *Long-Evans* rats animals to a pelleted Pb-containing diet (750ppm) during different stages of development for reference memory (Morris Water Maze) and blood lead levels assessment at 100 days of age. Jett et al.[82] published a different kind of lead exposure protocol. Ten days prior to breeding and through gestation and lactation, female *Long-Evans* rats were fed diets containing 250 ppm of Pb acetate. Pups were exposed from day 1 until day 91. Behavioural evaluation with Morris Water Maze and Pb levels in the hippocampus were assessed in this study. These studies are complementary, being published by the same research group, evaluating the memory deficits in an animal model that mimics lead exposure through contaminated food ingestion by pregnant females and their children. However, only the behavioural alterations were considered on both these studies, without any neuropathological evaluations. The third study was published by Bourjeily and Suszkiw[83]. These authors exposed timed-pregnant *Sprague-Dawley* rats with 0.2% lead acetate solution diluted in water from gestational day 16 until weaning pups at post-natal day 21 (P21). Rats (females' pups) were sacrificed at P1, P7, P21, P81, P112 and P200 for biochemical and morphological measurements. This study presents a similar lead exposure animal model to that described before by Bielarczyk[80] but without developmental post-weaning exposure and with an evaluation of the animals made after a long non-exposure period (8, 12

and 24 weeks). Even though this is a complex study, only molecular changes of lead exposure were considered, without describing any physiological or behavioural changes. The same protocol of exposure was published some years after (in 2007) by Han et al[84], to study the protective effects of ascorbic acid in lead toxicity. This type of studies is crucial as an attempt of finding a treatment or prevention of lead toxicity in humans by performing animal experimentation.

Moreira[85] and others published, in 2001, a wide-ranging behavioural study on lead-exposed animals. For that, researchers exposed pregnant *Wistar rats* with 500pm Pb acetate solution during pregnancy and lactation. Pups, at 23 and 70 days of age, were assessed by elevated plus maze and open field tests, holeboard and shuttle-avoidance tasks, social interaction test and rotarod for a broad behavioural characterization upon lead exposure during development. Also, Pb levels in brain and blood were determined in both dams and pups. Such a study is very important for a wide characterization of an animal model, however, only the behavioural changes during development of the brain in foetal period and during lactation were considered, without any molecular or physiological evaluation. In 2003, Yang[86] developed, using *Wistar* rats, a gestational lead exposure animal model, without taking into account the developmental phase and evaluated only the cognitive impairment induced by lead exposure by Morris Water Maze test.

In 2005, Virgolini[87] and colleagues published a long-term study of chronic low-level lead exposure (50 and 500ppm drinking lead solution), starting at day 21 after birth and until 9 months of age of male *Long-Evans* rats. Animals were assessed for behavioural changes by fixed interval performance test, stress challenges and locomotor activity chambers and catecholamine, corticosterone and glucocorticoid receptor levels were determined, as well as blood lead levels, through lifetime. This study is an example of an overtime research for toxicological lead effects upon behaviour of the animals and, therefore important for results translation humans, although not taking into account the foetal period of exposure and only evaluating the changes in male animals. Also, the molecular changes that underlie the behavioural changes observed were considered and assessed for a wider examination of lead effects.

A group of scientists from Poland, led by Struzynska, in 2007[88] described a completely new way of lead exposure in animals. Wistar rats of both sexes were injected daily with lead acetate (15 mg/kg, ip) for 2 weeks starting at day 15 after birth. Animals were sacrificed 24 hours after the last injection and brains removed for a broad inflammatory study of glial and neuronal cells. Blood and brain lead levels were also estimated. Although this a very complex study of mechanisms of lead toxicity in the brain, the type of exposure to lead is not well translated to human environmental exposure. Even though, this type of studies is important to characterize the molecular mechanisms underlying lead neurotoxicity. Other studies with injectional lead exposure animal model were published after the

previously described. In 2014, Silveira et al[89] published a study in which authors exposed male rats to lead acetate (first dose 4mg/100g, subsequent doses 0.05mg/100g,im) for 30 days, in animals that weighted between 250–300g. Vascular reactivity experiments in thoracic aortas were performed, indirect systolic blood pressure was measured weekly using tail-cuff plethysmography and molecular studies for oxidative stress assessment, as well as blood lead levels were determined. Another study, of 2016[90], was performed in two months old female *Wistar* rats that were exposed to lead acetate 0.5 mg/kg by intraperitoneal injection for 60 days. The authors performed some histopathological and immunohistochemistry analysis for lead effects assessment on molecular level. Also, in 2017, Simões[47] and collaborators published a very broad study of physiological and autonomic dysfunctions in *Wistar* rats, exposing adult male Wistar rats to lead acetate by intramuscular injection (first dose of 4 μg/100 g body weight and subsequent doses of 0.05 μg/100 g/day for 30 days). Haemodynamic recordings in conscious rats were performed for BP changes assessment and systolic arterial pressure and pulse interval variability calculated for autonomic evaluation using Fast Fourier Transform (by LF, HF and LF/HF calculations), baroreceptor and Bezold–Jarisch Cardiopulmonary Reflexes were also determined. This is a very wide-raging study of cardiorespiratory and autonomic changes in animal model that could be translated to children that are exposed to lead in early stages of life, however, the type of exposure that have been chosen by the authors is not the type of exposure that happens in the normal conditions with humans.

Some studies have been focused in the relationship of lead exposure and neurodegenerative diseases, such as Alzheimer's disease. New animal models have been developed to investigate the association. Bihaqi[69]et al in 2014, described the first double lead exposure animal model, exposing C57BL/6 mice to 0.2% Pb acetate through drinking water from day 1 to 20 after birth and between 9 and 9 months of age. Brains were removed in different timepoints of the study (post-natal days 20, 180, 270, 540, and 700). Behavioural assessments were performed by Morris Water Maze and elevated plus maze, western blotting and real-time PCR to trace Alzheimer's biomarkers. The same group of researchers, published another study in 2016[91] in which only the developmental lead exposure profile was evaluated (C57BL/6 mice exposed to 0.2% Pb-acetate from PND 1 to PND 20 through the drinking water of the dam) with similar testing protocol of the previous study. Both these studies, and others that have been performed, have shown a strong relationship between lead exposure in early stages of life and the development of Alzheimer's and other neurodegenerative diseases in elder stages of life, although, using a simpler animal species for evaluation.

In the most recent years, other studies with animal models have been developed. Sobin et al[68] published, in 2017, their work that was performed in female and male C57BL/6J mice exposed to low (30ppm) and high (430ppm) lead acetate solution from day 0 to day 28 after birth. Behavioural

evaluation by OIP task (object-in place) for spatial and visual object memory retrieval in preadolescence mice. No molecular or physiological assessments were performed. Although, the study mimics the human exposure in the early stages of life, no broad evaluation of the lead effects has been performed. Another study was performed in 2017 by Toscano and collaborators[20], focused in the physiological alterations provoked by lead, in which two months old male *Wistar* rats were exposed to lead acetate (100ppm) for 30 days. Systolic blood pressure (SBP) was assessed weekly by tail plethysmography and, after 30 days of lead exposure, haemodynamic measurements were taken (sBP, dBP and HR), blood pressure reactivity, baroreflex stimulations were performed and heart was withdrawn for western blot analysis of β1 adrenergic receptor expression. This is a more complex study with a broader evaluation of lead exposure effects during developmental stages, in which, not only the physiological parameters have been assessed, but also, the neural regulation system dysfunction that underlies the physiological changes evaluated and described.

2 AIM OF THE WORK

Lead exposure can be highly variable in both intensity and frequency inducing, during developmental stages and through lifetime, adverse health effects including behavioural changes, cognitive impairment, tachypnoea, hypertension and autonomic dysfunction. Most of these adverse effects have been described in the course of a continuous chronic exposure to lead in both humans and animals. In fact, various animal models have been described for continuous low-level lead exposure and health effects assessed. Yet, there are situations eg, migrations, implementation of school exchange programs and/or residential change, where intermittent lead exposure occurs and may leading to functional damage. However, the available studies in human subjects and standardized exposure protocols mimicking human intermittent exposure to lead are scarce.

Therefore, the overall purpose of this Master thesis is to evaluate lead effects on mammal's physiology along different profiles of lead exposure, including a new protocol of intermittent low-level lead exposure.

For that,

- three different protocols of lead exposure were developed;
- leading functional parameters, critical for cardio-respiratory and autonomic homeostasis, were characterized;
- behavioural changes and cognitive impairment produced by lead exposure were assessed;
- morphofunctional parameters as neuronal degeneration, gliosis and synaptic alterations in the hippocampus (dentate gyrus), upon different low-level lead exposure protocols, were evaluated.

3 MATERIALS AND METHODS

3.1 ETHICS STATEMENT

All described procedures were carried out in agreement with the European Community legislation on animal experimentation (Directive 2010/63/ EU) and were approved by the Ethical Committee of the Academic Medical Center of Lisbon (CAML).

3.2 DEVELOPMENT OF LEAD EXPOSURE PROTOCOLS

By taking into account that ingestion is one of the three main intake routes for body lead absorption, animal models of lead poisoning were developed as described previously [16,45]. Briefly, seven days pregnant *Wistar* rats (n=8; Charles River Laboratories, France) were divided into Pb-treated and control groups. In Pb-treated group, the tap drinking water was replaced by 0.2% (p/v) lead acetate (II) solution dissolved in deionized water (Acros Organics, New Jersey, USA). After weaning at 21 days, rat pups of both sexes were divided into two groups following the previous exposure: Pb-exposed pups **(Pb)** and tap water for control pups **(Ctl-rats)**.

These two groups were themselves sub-divided into the following ones (***Figure 4***):

- ***Long-term Exposure (PbP)*** → *Wistar* rats (n=30) of both sexes were exposed to lead *permanently* and evaluated at 12, 20 and 28 weeks of age;
- ***Short-term Exposure (PbS)*** → *Wistar* rats (n=30), of both sexes, exposed to lead solution until 12 weeks of age without any adult lead exposure, evaluated at 12, 20 and 28 weeks of age;
- ***Intermittent Exposure (PbI)*** → *Wistar* rats (n=30), of both sexes, exposed to *intermittent* lead intake (two periods of exposure): until 12 weeks of age and between 20 to 28 weeks. Animals underwent a period of lead abstinence for 8 weeks, between 12 and 20 weeks of age and were evaluated at 12, 20 and 28 weeks of age;
- ***Without exposure to Pb (Control - CTL)*** → *Wistar* healthy rats (n=30), of both sexes, not exposed to lead were evaluated at three different time-points: 12 week, 20 weeks and 28 weeks.

All animals were subjected to the same experimental protocol at three distinct time-points (T= 12, 20 and 28 weeks of age) for a broad functional and morphological characterization of lead exposure profiles.

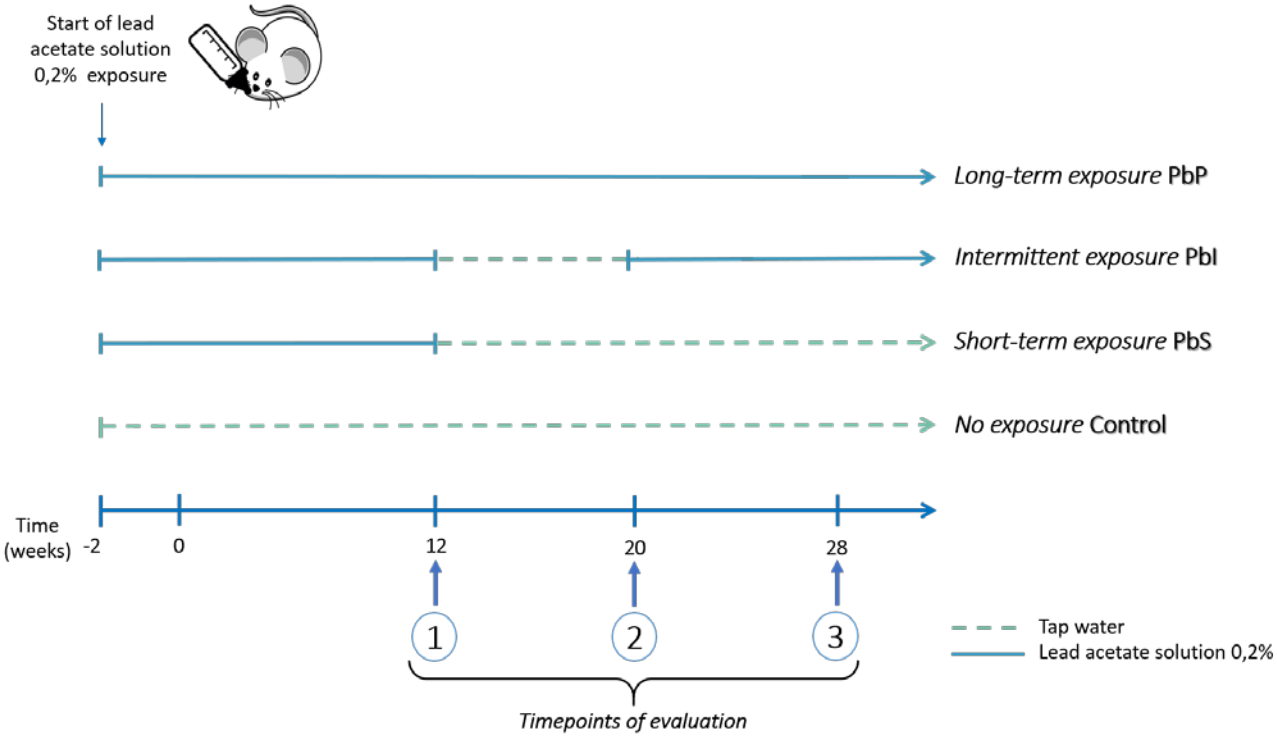

Figure 4 - *Timeline of the study, discriminating the development of different low-level lead exposure protocols (PbP, PbI and PbS) and the matching control (Ctl).*

Numbers represent different evaluations performed at relevant time-points of the study: **1** - Behavioural tests and Functional evaluation; **2** - Behavioural tests, Functional evaluation and Immunofluorescence studies; **3** - Behavioural tests, Functional evaluation and Immunofluorescence studies.

All rats were housed in the animal facility of the Faculty of Medicine of the University of Lisbon, in a maximum number of 4 animals per cage and divided by sexes (after weaning, at 21 days of age), with controlled temperature (22 ± 1°C) and humidity (50 ± 5%) and synchronized for a 12/12h light/dark cycle (lights on between 7 am and 7 pm). Food (Mucedola, Italy) and tap water (Epal, Portugal) were provided *ad libitum*. All procedures (functional and behavioural evaluation) were performed during the light period of the day.

3.3 Behavioural Testing Procedures

Two weeks before functional evaluation, animals underwent a set of standard behavioural tests to access: *I)* anxiety and stress levels[92] (Elevated Plus Maze test); *II)* spontaneous locomotor activity and exploratory behaviour[93] (Open Field Test); *III)* spatial working memory[94] (Y-Maze test); and, *IV)* episodic long-term memory[95] (Novel Object Recognition test).

At the experimental days, animals were taken into the behaviour testing room, for at least, 1h prior to the start of the testing session. All behavioural studies were performed between 9am and 6pm, in a quiet room with dim light and all animals underwent a prior handling period for testing room and researcher habituation[96]. All behaviour apparatus used were cleaned with 70% ethanol between animals, so that any residual smell of the disinfectant was experienced equally by every animal. After placing the animal in the behavioural apparatus, the investigator immediately left the room, to avoid introducing an unintentional bias into the study or serving as a cue for the animal. All experiments were video-recorded by an UV camera (Chacon, Belgium) and all videos were posteriorly analysed by ANY-maze© software (Stoelting Co, Ireland).

I. Elevated Plus-Maze

The elevated plus-maze (EPM) is one of the most used tests of anxiety behavior evaluation in rodents[92,97]. The apparatus consists in an elevated maze with four arms (two open arms – 50 x 10cm - perpendicular to two enclosed arms 50 x 10 x 30cm height) that form a plus shape, elevated 50cm from de ground (***Figure 5)***.

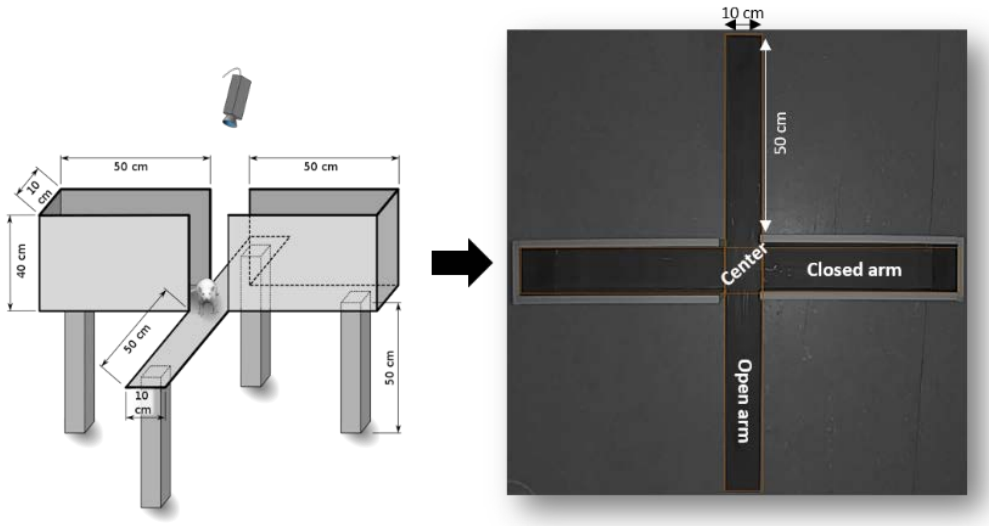

Figure 5 - *Elevated plus test apparatus.*
The apparatus consists in an elevated maze with four arms (two open arms – 50 x 10cm - perpendicular to two enclosed arms 50 x 10 x 30cm) that form a plus shape, elevated 50cm from de ground.

Each animal was left at the centre of the maze to freely explore the maze during 5 minutes without prior habituation to the maze. Usually, a trial of 5 min is sufficient to capture the critical components of anxiety behaviour in animals[98].

The parameters evaluated from the EPM test were: the number of entries in each zone (all four paws on open or enclosed arms); the percentage of time spent in open and closed arms using the following ratio: *[time spent in open or closed arms/total time] x 100*. Normal exploratory behavior in rodents is in favor of the closed arms [99]; thus, animal exposure to a novel maze area evokes an avoidance conflict which is stronger in open arm compared to the enclosed one, being a lower ratio an indication of anxiety[92,100].

II. Open Field Exploration test

The Open Field Test (OFT) provides a unique opportunity to systematically assess novel environment exploration, general locomotor activity, and allows an initial screening for anxiety-related behaviour in rodents[101]. This apparatus consists in a square black box (measures of 67 x 67 x 57cm height) "virtually" divided in three concentric squares: 1- peripheral zone (near the walls), 2- intermediate zone and 3- centre, as shown, in *Figure 6*.

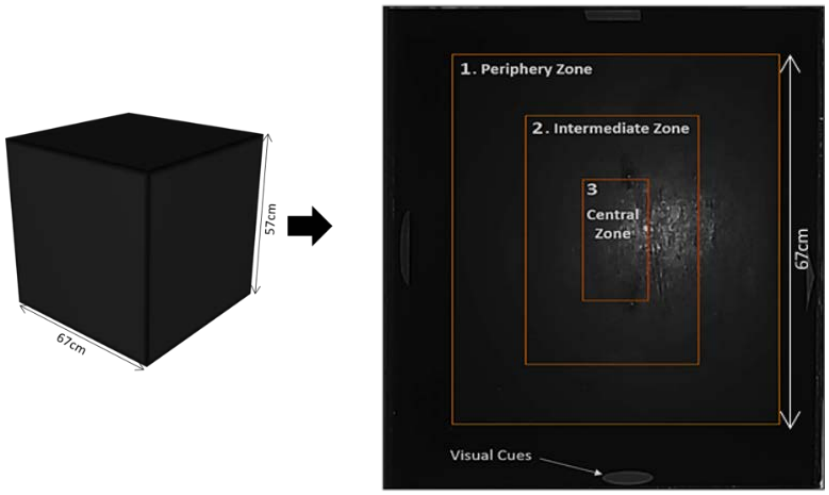

***Figure 6 -** Open field test apparatus*.
The OFT apparatus consists in a square black box (measures of 67 x 67 x 57cm height) with three virtual zones discrimination: 1. Periphery zone; 2. Intermediate zone; 3. Central zone.

The locomotor behaviour has been tested without previous habituation to the box. Usually, a 5-min test session is sufficient to evaluate the general exploratory locomotion. It is known that rats typically spend an appreciably greater amount of time exploring the periphery of the arena, usually in contact with the walls (i.e., thigmotaxis)[102–104], than in the unprotected centre area[105].

The parameters evaluated during this test were: the percentage of time spent exploring the centre, the total amount of entering times into the three virtual zones, the total travelled distance and the average velocity of the animal during the test[93]. The total amount of entries in the virtual zones and

the presence time in the central zone are measures of exploratory behaviour and anxiety. A high frequency/duration of these behaviours indicates high exploratory behaviour and low anxiety levels, and, in the contrary, a low frequency/duration of these behaviours is indicative of poor exploration and high anxiety levels[93]. Total travelled distance of the animal and average velocity are measures of the general locomotor activity[85,93,106]. High values of both these parameters are indicative of hyperactivity behaviour of animals, and, in the contrary, decreased values of these parameters indicate a locomotor impairment[93,107].

III. Y-Maze

Y-Maze Spontaneous Alternation[94] is a behavioural test taking advantage of the willingness of rodents to explore new environments[92,101,108]. The Spontaneous Alternation Behaviour measured in this test mirrors the process of spatial working memory - hippocampal dependent process[94]. It is a simple memory test, reason why it has been widely used by behaviour researchers and already used for lead neurotoxicity studies[49].

The apparatus for Y-Maze test consists of an Y-shaped labyrinth with a black interior, with three identical arms at angles of 120 ° (arm dimensions of 35cm length x 10cm width x 20cm height) was used. Visual hints were placed on the walls to mark a previous visit. (*Figure 7*). The detailed protocol was described elsewhere[49]. Briefly, each rat was placed at the end of one arm (the chosen arm) and allowed to move freely through the maze during 8 min, without prior habituation.

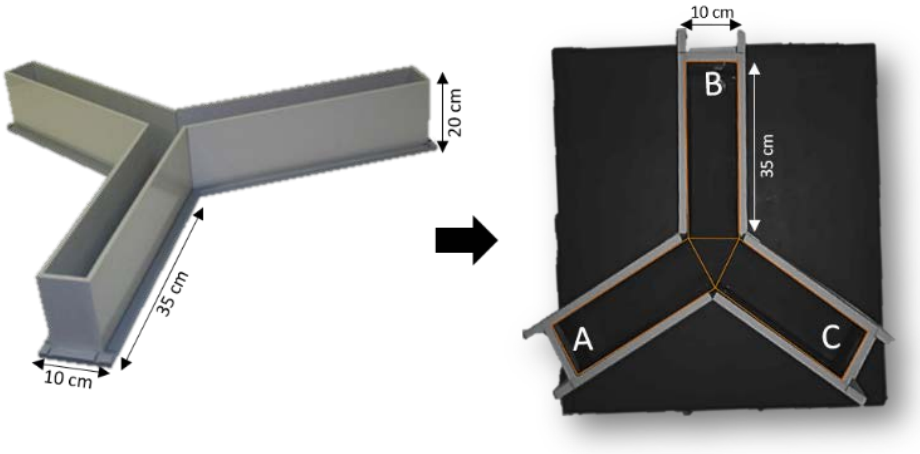

Figure 7 - *Y-Maze apparatus.*
A wooden Y-shaped labyrinth, with the interior in black, with three identical arms at *an angle of 120 ° of the other.* *T*he arm dimensions*:* 35cm x 10cm x 20cm*.*

The series of arm entries were calculated. An entry occurred when all four limbs were within the arm and an alternation was defined as entries in all three arms on consecutive occasions. The number of maximum possible alternations for each animal was therefore the total number of arm entries minus two. The percentage of spontaneous alternation behaviour (SAB) was calculated as following: ***[actual alternations/maximum alternations] x 100***. In addition, and for each animal, the total number of arm entries was used as a measure of exploratory activity [94].

IV. Novel Object Recognition Test

The Novel Object Recognition (NOR) Test is very useful to evaluate different types of memory through manipulation of the retention interval (RI) that resembles the amount of time that animals must retain the information of the sample objects presented during the training phase, before the test phase.[95] Since the aim of this test was to study the long-term memory changes of the animals, in our protocol, a retention interval of 24h was chosen[95].

To perform this test, the open field (OFT) arena (square black box with measures of 67 x 67 x 57cm height) was used. The objects used, were transparent and brown glass shapes proportional to the animal size. There were various examples of every object (n=2), and they were randomized and used interchangeably between trials. The role of the object, namely sample or novel, was randomized between object types. Also, their position relatively to the other object was permuted with the aim of using every object as a familiarity or novelty. The object position was frequently changed so that the object exploration of the animal was independent of that specific object preference by the animal[95,109]. The objects were attached to the bottom of the arena with a round piece of Velcro that could not be seen or touched by the animals. The objects were placed in symmetric and opposed corners of the arena[95].

The testing protocol, schematically described in ***Figure 8***, consists of three phases: habituation, training and the testing phase. In the habituation phase (3 consecutive days), each animal could explore freely the open field (OFT) arena for 15 min in the absence of objects. In the initial 5 min of the first day of habituation, animal behaviour was quantified as a measure of locomotor activity (OFT) throughout the ANY-maze® software. During the training phase, in the fourth day, the animal was presented with the two objects to-be-familiarized, named as sample objects (S and S' objects) for 5 min. Following sample-objects exposure, the animal was put back to the home cage for 24h. During the test phase, the animal was exposed to two objects: one previously experienced (sample object -S) and a novel object (N), for 5 min [95].

Between every trial, the arena and the objects were carefully cleaned with a 70% ethanol solution to erase any olfactory clues. Training and testing days were recorded and analysed by 3-point analysis (head, torso and tail of the animal) using ANY-maze® software, and only the data from the head point analysis was relevant for exploration of the objects. Exploratory behaviour was quantified as the amount of time animals spent around each object in both, training and testing phases. The number of approaches that included sniffing the object, rearing towards the object or touching the object, were counted. Sitting backwards to the object or crossing in front of the object without pointing the snout in the object direction was not considered as exploration[95]. Exploration was quantified as following:

ET (%)= [time exploring the object/overall exploring time] x 100.

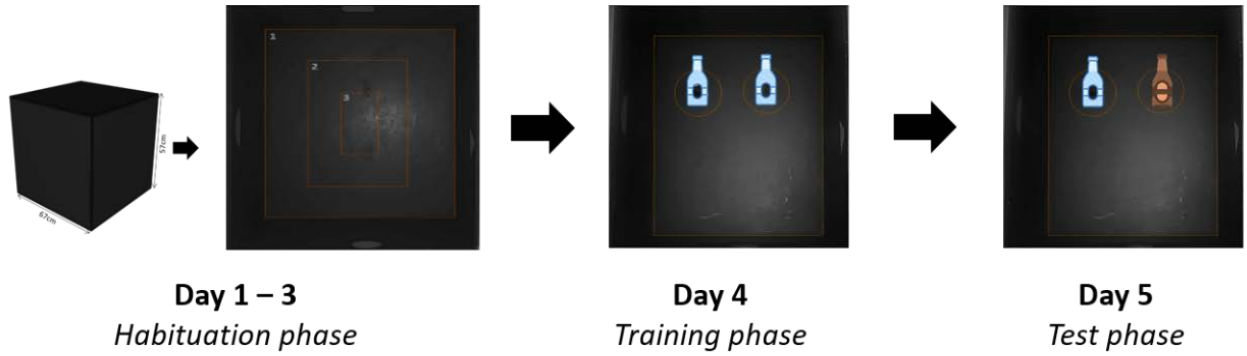

Figure 8 - *Novel object recognition test schematic representation.*

Novelty index was calculated from the data obtained in the NOR testing day, as: *[ET% Novel – ET% Sample] / [ET% Novel + ET% Sample]*. This index ranges from -1, to 1, where negative values to 0 represent absence of discrimination between novel and familiar objects, i.e. more time exploring sample object, equal time exploring both objects, and 1 corresponds to exploration of the novel object only. [109]

3.4 FUNCTIONAL EVALUATION

Metabolic evaluation

Animals were housed in a metabolic cage for 24h for food, water intake and urine and faeces production quantification. Animals were weighted before and after being placed in the metabolic cage.

Cardiorespiratory and autonomic evaluation

Anaesthesia, surgical and experimental protocols

Wistar rats, both sexes, aged > 12weeks, were anesthetized with sodium pentobarbital (60 mg/kg, ip). The levels of anaesthesia were maintained, when necessary, with a 20% solution (v/v) of the same anaesthetic, after testing the withdrawal reflex. The trachea was cannulated below the larynx for recording of the tracheal pressure and artificial ventilation (if necessary) with O_2-enriched air through a positive pressure ventilator. Femoral artery and vein were cannulated for blood pressure monitoring and injection of saline and drugs, respectively. Rectal temperature was maintained between 37.5-39ºC through a homoeothermic blanket connected to a rectal probe (Harvard Apparatus). The electrocardiogram (ECG) was recorded from subcutaneous electrodes placed into three of four limbs; heart rate has been derived from the ECG recording (Neurolog, Digitimer). The right carotid artery bifurcation was identified, and the tip of a catheter was placed within the right carotid sinus by retrograde cannulation of the external carotid artery. Carotid body receptors were stimulated by the injection of lobeline[45] (0.2ml, 25 µg/ml, Sigma) through this indwelling cannula. Baroreceptors were stimulated by an intravenous injection of phenylephrine[45] (0.2ml, 25 µg/ml, Sigma). As a control, the same volume of saline was also injected at the beginning of the experiment and was shown not to evoke any change in the recorded variables.

At the beginning of the experimental protocol, and upon parameters stabilization, a recording of 5min was taken for further autonomic evaluation. There was an interval of, at least, 3 min between each provocation to allow the recovery to basal values. Throughout the experiment, blood pressure (BP), ECG, heart rate (HR), tracheal pressure (TP) and respiratory frequency (RF) were continuously monitored and recorded (PowerLab, ADInstruments).

At the end of the experimental protocol, blood was collected from the femoral artery for BLL by atomic absorption spectroscopy (n=2). The guidelines for BLL monitoring in human children consider a value superior to 5 µg/dL to be harmful to human health[5,6,18].

The animal was, then, sacrificed with an overdose of anaesthetic. The brain was carefully removed and maintained 4% paraformaldehyde in phosphate buffer (PBS) (pH 7.4) at 4°C overnight, after which, was immersed in increasing concentrations of sucrose, of 15% and 30%. Subsequently, the brain was embedded in gelatine (7.5% gelatine in 15% sucrose solution) and frozen with liquid nitrogen and 2-metilbutane (Sigma-Aldrich, UK) for further histological procedures.

Data acquisition and analysis

All the recorded variables were acquired at 1 kHz, amplified and filtered (Neurolog, Digitimer; Powerlab, ADInstruments). For the recorded variables, the baseline values were taken immediately before the beginning of each type of stimulation.

I. Baro- and chemoreceptor reflex analysis

To evaluate the baroreceptor reflex function, the baroreceptor reflex gain (BRG) has been quantified, calculating the variation of HR in relation to mean BP variation: $\Delta HR \,/\, \Delta BP$ upon phenylephrine provocation.

The evaluation of the chemoreceptor response elicited by intra-carotid injection of lobeline was calculated through basal respiratory frequency (RF, in cpm) before [average of 30sec] and during lobeline stimulation, or, $\Delta_{\,chemoreflex\,(lob)} = RF_{\,stimulation} - RF_{basal}$.

II. Cardiovascular variability

Heart rate and blood pressure variabilities are indirect methods for non-invasive evaluation of autonomic nervous system. Systolic blood pressure and R-R intervals were analysed, in periods of 3min, through discrete wavelet transform[110–112] using an in-house software FisioSinal[113], to calculate Low Frequencies (LF) and High Frequencies (HF). Low frequencies (LF; [0.15-0.6] Hz) obtained from systolic BP are a marker of sympathetic activity and high frequencies (HF; [0.6-2.0] Hz) obtained from R-R interval represent both parasympathetic and respiratory variations. The ratio $LF_{sBP} \,/\, HF_{RR}$ represents the autonomic balance to the cardiovascular system.

3.5 IMMUNOFLUORESCENCE STUDIES

Sample processing

To address neuroinflammation and neurodegeneration, the hippocampus was identified (B=-2.92 to -5.04), and the brain was sectioned around that region on a cryostat (Leica CM 3050S, Germany) and coronal slices (25µm) collected in a cryoprotectant solution (PBS, ethylene glycol and Glycerine) and stored at -20°C. For immunohistochemical studies, the slices were washed with TBS (3x/5min; Bio-Rad, USA), placed in citrate buffer (Sigma-Aldrich, UK) at 90°C for 30min for antigen retrieval[114]. After cooling down to room temperature, the samples were washed with TBS (3x/5min) and treated with 0.3% Triton X-100 (Sigma-Aldrich, UK) for 15 min. Sample blocking was then performed by 5% Goat Serum (BioWest, France) and 1% Bovine serum (VWR, USA) blocking solution for 1h, after which the incubation with primary antibodies over-night has been performed (concentrations and markers are shown in *Table 2*).

Table 2 - *List of primary antibodies used in immunohistochemistry protocol.*

Marker	*Antigen*	*Antibody (Dilution)*	*Host*	*Supplier*
Neuronal marker	Neuronal nuclei	NeuN (1:500)	Rabbit polyclonal	Abcam® (UK)
Astrocytic marker	Glial fibrillary acidic protein	GFAP (1:500)		
Microglial marker	Ionized Ca binding adaptor molecule 1	Iba-1 (1:250)		
Synaptic marker	Synaptophysin	Syn (1:200)		

At the second day of staining protocol, tissue slices were washed with TBS (3x/10min) and incubated with a secondary antibody, in concentrations presented in *Table 3*, for 1 hour at room temperature, after which, were washed (TBS; 3x/10min) and mounted in salinized SuperFrost® Microscope Slides treated with ProLong Gold Antifade with DAPI (Sigma-Adrich, UK), dried and stored at -20ºC until further visualisation and analysis of the dentate gyrus (DG).

Table 3 *- Secondary antibody used in the immunohistochemistry protocol.*

Secondary antibody	*Conjugate (Dilution)*	*Supplier*
Goat anti-Rabbit IgG (H + L) Secondary antibody	Alexa Fluor® 594 (1:1000)	Life Technologies - ThermoFisher Scientific (USA)
	Alexa Fluor® 488 (1:1000)	

Image acquisition

Image acquisition was performed under a confocal point-scanning microscope (Zeiss LSM 880 with Airyscan) (***Table 4***). All images where taken at DG level because it is known that this area receive the most number of input pathways to the hippocampus executing three main functions: (i) accumulating encoding of multiple sensory inputs, (ii) spatial pattern separation, and (iii) facilitation of encoding of spatial information based on its outputs to CA_3[48].

***Table 4** – Image acquisition and quantification.*
Lasers, objectives and ZEN Software (Carl Zeiss, Germany) used for high-resolution fluorescence images of DG

Marker	*Primary Ab*	*Secondary Ab Conjugate*	*Laser*	*Objective*	*Type of images*
Neuronal marker	NeuN	594	**HeNe594** (594 nm)	20x, dry	Snap optimal
Astrocytic marker	GFAP	488	**Argon** (458nm) (488nm) (514 nm)		Z-stack (compilation of images in Z axis)
Microglial marker	Iba-1				
Synaptic marker	Syn			63x, oil	Snap optimal

Image analysis

Subsequently the appropriate image acquisition, fluorescent images of GFAP, Iba-1 and Synaptophysin were analysed and quantified using Fiji[115] open source software by specific features. Morphological categorization of GFAP and Iba-1 stained cells into various types of glial cells, form reactive to resting state cells was performed by comparison of the cells to the ones described in different sources[55,116–118]. Also, for GFAP and Iba-1, a manual quantification of positive cells was performed, using Z-stack images by Cell Counter plugin in the software to mark cells.

Synaptophysin fluorescence intensity staining quantification was completed using ROI manager tool, choosing 5 regions of interest (ROIs), equal in size and shape and then normalized to a negative control image (sample obtained by incubation of secondary antibody without primary antibody for non-specific binding quantification).

Number of neurons in Neu-N stained tissue slides were quantified using an in-house software developed by Bioimaging facility of Instituto de Medicina Molecular João Lobo Antunes named Multichannel Cell Counter RGB. Succinctly, single-cell nuclei were identified via DAPI staining thresholding and particle analysis, and dilated regions of interest (ROIs), based on a user-defined radius. For each channel and ROI, a staining was considered positive if a minimum number of pixels (usually 5, above a given threshold), and a filter for cell counting was defined based on staining.

3.6 STATISTICAL ANALYSIS

Data are expressed as mean ± SEM and plotted as the composite of the mean values of all subjects, unless otherwise specified. Normality distribution of the continuous variables was analysed with the Kolmogorov-Smirnov test (Lilliefors' correction) and Levene's test was used for assessment of homogeneity of variance. For assessing data within the same group or between Pb group and the Control group at a specific timepoint (12, 20 and 28 weeks), Student's t-Test for paired or unpaired observations, respectively, was used.

Comparisons between groups (PbS vs. PbP vs PbI) were performed using a "repeated measures" analysis of variance (ANOVA) with Tukey's multiple comparison.

A value of $p < 0.05$ was considered statistically significant. Data were analysed using GraphPad Prism 6 (GraphPad Software Inc, USA).

4 RESULTS

4.1 LONG - TERM LEAD EXPOSURE PROTOCOL (PBP)

The animal model of long-term lead exposure evaluates the behavioural, overall functional and hippocampal histological consequences of a continuous exposure of animals to lead since the foetal period until 28 weeks after birth. Animals were divided into three groups in accordance to the time of evaluation: 12, 20 and 28 weeks.

Effect on metabolic parameters and BLL

The weight of the animals, evaluated at three different time-points, did not change significantly in animals under long-term lead exposure protocol. Animals exposed to lead until 12 weeks ingest more water and less food, without alterations in the urine and faeces excretion. At 20 weeks of age no significant differences were observed, however in the animals exposed to lead until 28 weeks, a small increase in the water intake was detected, without an increase in urine production (*Table 5*).

All the metabolic parameters evaluated in CTL groups, at three different time-points, did not change significantly.

Regarding the blood lead levels, Pb animals presented a high lead concentration at 20 and 28 weeks of age, without BLL evaluation at 12 weeks (see *Table 5* for values).

Table 5 – *Values of metabolic parameters and BLL in CTL and PbP groups at 12, 20 and 28 weeks of age.*
Values are presented as mean ± SEM. n=10/group; *p < 0.05; **p < 0.01.

Group	Age	BBL (ug/dL)	Weight (g)	Food intake (g)	Water intake (ml)	Urine (ml)	Faeces (g)
CTL	12 weeks	na	358 ± 34	25 ± 1	24 ± 3	19 ± 1	12 ± 1
Pb		na	345 ± 39	22 ± 1*	32 ± 3*	15 ± 1	9 ± 1
CTL	20 weeks	< 0.1	386 ± 41	23 ± 3	39 ± 3	17 ± 2	12 ± 1
PbP		28 ± 2.3	390 ± 42	24 ± 2	33 ± 2	20 ± 3	10 ± 1
CTL	28 weeks	< 0.1	368 ± 36	24 ± 2	24 ± 2	11 ± 1	8 ± 1
PbP		21 ± 10.7	434 ± 50	24 ± 3	30 ± 2*	16 ± 3	8 ± 1

Effect on blood pressure, heart rate and respiratory frequency

Long-term lead exposure significantly increased mean blood pressure values, mainly due to the continuous increase of systolic and diastolic blood pressure through time. These hypertensive values were maintained between 20 and 28 weeks. Heart rate apparently did not account for those changes, since they did not change significantly during the continuously lead exposure. The changes of respiratory frequency follow the inverse profile of blood pressure (***Figure 9;*** see ***Table 6*** for values).

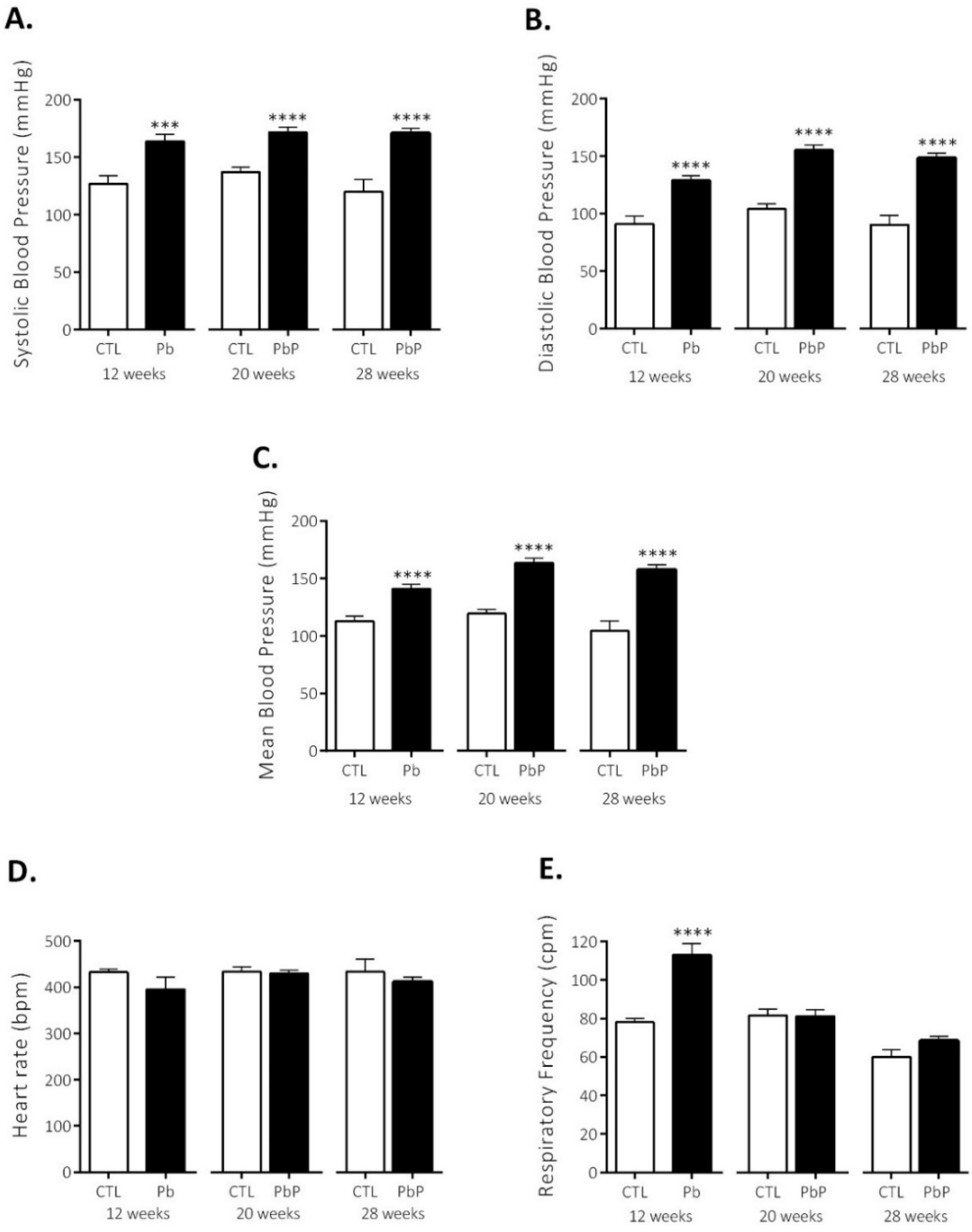

Figure 9 – *Basal physiological parameters evaluation of PbP and CTL protocols at 12, 20 and 28 weeks*
Systolic (A), diastolic (B) and mean (C) blood pressures, heart rate (D) and respiratory frequency (E). Values are mean ± SEM. ***p < 0.001; ****p < 0.0001.

Table 6 – *Values of basal physiological parameters of PbP and CTL groups at 12, 20 and 28 weeks.*
Values are presented as mean ± SEM. n=10/group; ***p < 0.001; §p < 0.0001.

Group	Age	Blood pressure (mmHg)			Heart rate (bpm)	Respiratory frequency (cpm)
		Systolic	Diastolic	Mean		
CTL	12 weeks	125 ± 7	91 ± 7	113 ± 4	432 ± 7	78 ± 2
Pb		164 ± 6***	129 ± 4§	141 ± 4§	395 ± 27	113 ± 6§
CTL	20 weeks	137 ± 4	104 ± 4	119 ± 3	433 ± 10	81 ± 3
PbP		171 ± 4§	155 ± 4§	163 ± 4§	429 ± 6	81 ± 3
CTL	28 weeks	120.1 ± 10.76	90 ± 7	104 ± 8	433 ± 27	60 ± 3
PbP		171 ± 4§	148 ± 4§	157 ± 4§	411 ± 9	68 ± 2

Effect on baroreceptor and chemoreceptor reflexes

Baroreceptor function seems to have undergone a remodelling process along time. In fact, the baroreflex gain decreased until 20 weeks, from which was kept without significant changes, suggesting that the putative remodelling process that was ongoing since early life to accommodate the increases in blood pressure has terminated.

Long-term exposure to lead was responsible for an augmentation of the carotid chemoreflex sensitivity at 12 weeks that has been aggravated until 20 weeks of age and then maintained with the same profile until the end of exposure (28 weeks).

The temporal evolution of both baroreceptor and chemoreceptor reflexes changes due to prolonged lead exposure is represented in *Figure 10* and values in *Table 7*.

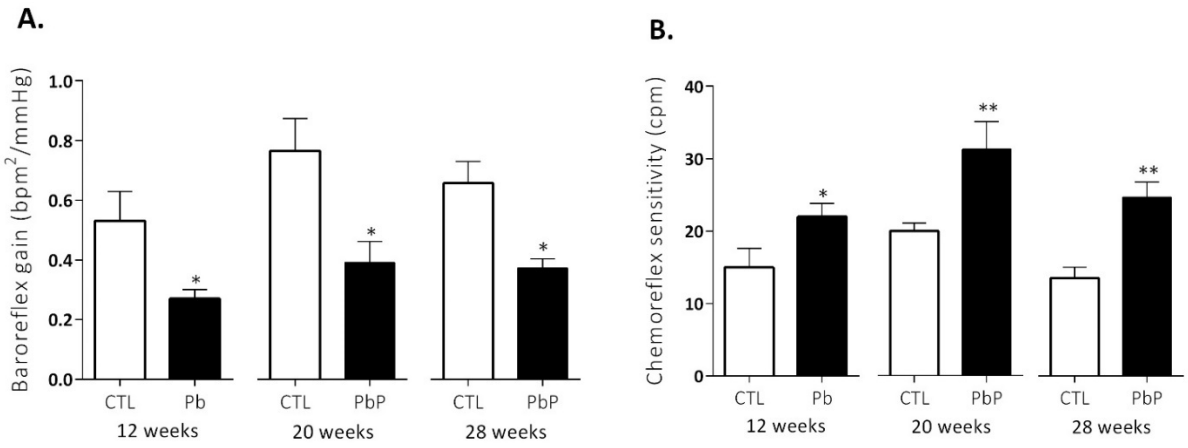

Figure 10 – *Baroreceptor and chemoreceptor reflex evaluation of PbP and CTL protocols at 12, 20 and 28 weeks*.
Baroreflex gain (A) and chemoreflex sensitivity (B). Values are mean ± SEM; *p < 0.05; **p < 0.01.

Table 7 – *Values of baroreflex gain and chemoreflex sensitivity of PbP and CTL groups at 12, 20 and 28 weeks.*
Values are presented as mean ± SEM n=10/group; *p < 0.05; **p < 0.01.

Group	Age	Baroreflex gain ($bpm^2/mmHg$)	Chemoreflex sensitivity (cpm)
CTL	12 weeks	0.53 ± 0.10	15 ± 2.6
Pb		0.27 ± 0.03*	22 ± 1.8*
CTL	20 weeks	0.76 ± 0.11	20 ± 1.1
PbP		0.39 ± 0.07*	31 ± 3.8**
CTL	28 weeks	0.65 ± 0.07	14 ± 1.5
PbP		0.37 ± 0.03**	25 ± 2.1**

Effect on autonomic output measured indirectly

Autonomic function is impaired since earlier life. This impairment, expressed mainly in the sympathetic tone increase (LF band), is observed at 12 and 28 weeks, which contributed to the autonomic balance (LF/HF ratio) increase at 12 weeks. Interestingly, in opposition to other clinical conditions, in which the autonomic system is provoked for further adaptation leading to sympathetic overactivation independently of the parasympathetic tone (HF band), in this case, the activity of both peripheral branches follow the same incremental tendency changes resulting in a decrease of autonomic balance seen at 20 and 28 weeks of age.

The time evolution of the LF, HF and LF/HF parameters for the 3 time-points of evaluation are presented in *Figure 11* and values in *Table 8*.

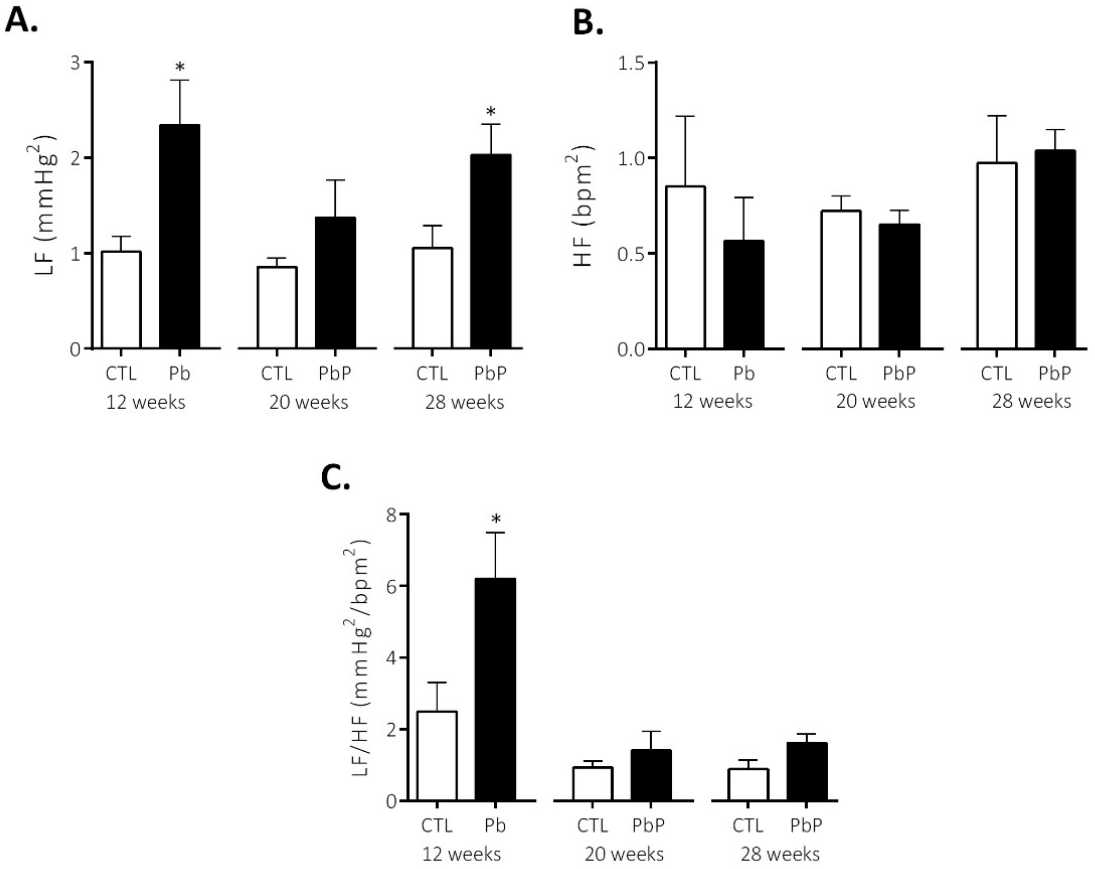

Figure 11 – *Autonomic function evaluation of PbP and CTL protocols at 12, 20 and 28 weeks*
Low frequency band– LF (A), high frequency band - HF (B) and LF/HF index (C) are presented.
Values are mean ± SEM; *p < 0.05.

Table 8 – *Values of autonomic output measure indirectly of PbP and CTL groups at 12, 20 and 28 weeks*
Values are presented as mean ± SEM. n=10/group; *p < 0.05.

Group	Age	LF_{sBP} ($mmHg^2$)	HF_{HR} (bpm^2)	LF_{sBP}/ HF_{HR} ($mmHg^2/bpm^2$)
CTL	12 weeks	1.015 ± 0.16	0.85 ± 0.37	2.49 ± 0.82
Pb		2.34 ± 0.47*	0.56 ± 0.23	6.20 ± 1.29*
CTL	20 weeks	0.85 ± 0.09	0.72 ± 0.08	0.93 ± 0.19
PbP		1.37 ± 0.39	0.65 ± 0.076	1.41 ± 0.52
CTL	28 weeks	1.05 ± 0.23	0.97 ± 0.25	0.88 ± 0.25
PbP		2.02 ± 0.32*	1.04 ± 0.11	1.61 ± 0.25

Effect on behavioural parameters

Anxiety, locomotion and exploratory activity

Long-term exposure to lead provokes irreversible anxiety behaviour in the animals through life that aggravates with the passing time. However, the initial time of exposure was the most relevant for the behavioural change. This behaviour was inferred by the reduction of the presence time in the open arms of the EPM apparatus thus, increasing the presence time in the closed arms of the maze. Also, variations in the number of entries that were observed are coherent with the presence time percentage changes, characteristic of an anxiety behaviour due to long-term lead exposure (see ***Figure 12***; and ***Table 9*** for values).

Long-term lead exposure also induced some significant changes in the exploratory behaviour of the animals that was evaluated by OFT, leading to poor exploration and increasing the anxiety levels of the subjects since earlier life. These exploratory behaviour modifications were recovered through life (animals do not show changes in the presence time percentage in the central zone of the arena at 28 weeks of age). Also, the exposure to lead in the early stages of life is a cause of a hyperactive behaviour in the animals that was also recovered at 28 weeks. This change was inferred by the augmented values of the average velocity parameter in the Pb-exposed animals at 12 and 20 weeks of age thus increasing the total travelled distance of the animals in the arena (see ***Figure 13***; and ***Table 10*** for values).

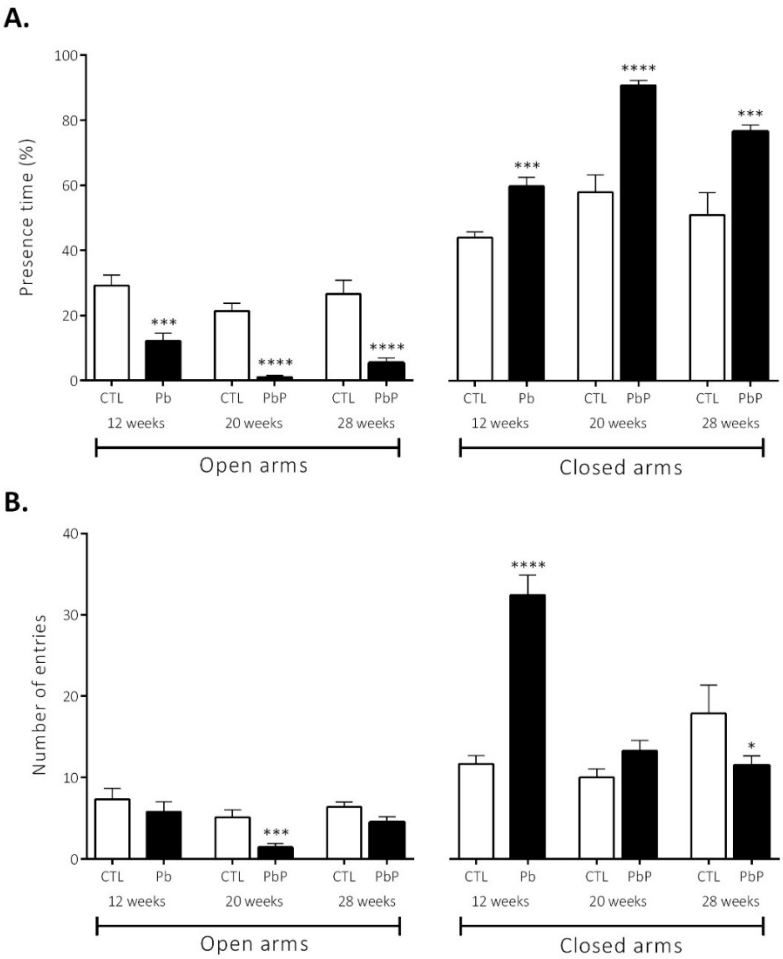

Figure 12 – *Anxiety behaviour assessment by EPM of PbP and CTL protocols at 12, 20 and 28 weeks*
Presence time percentage (A) and number of entries (B) in open and closed arms of the maze are presented. Values are mean ± SEM; *p < 0.05; ***p < 0.001; ****p < 0.0001.

Table 9 – *Values from anxiety behaviour assessment of PbP and CTL groups at 12, 20 and 28 weeks*
Values are presented as mean ± SEM. n=10/group; *p < 0.05; ***p < 0.001; §p < 0.0001.

Group	Age	Presence Time (%)		Number of entries	
		Open Arms	Closed arms	Open Arms	Closed arms
CTL	12 weeks	29 ± 3.2	44 ± 1.8	7 ± 1.3	12 ± 1.0
Pb		12 ± 2.3***	60 ± 2.7***	6 ± 1.2	33 ± 2.4§
CTL	20 weeks	21 ± 2.5	58 ± 5.4	5 ± 0.9	10 ± 1.1
PbP		1 ± 0.4§	91 ± 1.4§	1 ± 0.4***	13 ± 1.3
CTL	28 weeks	27 ± 4.3	51 ± 6.8	6 ± 0.6	18 ± 3.5
PbP		5 ± 1.4§	77 ± 2.0***	5 ± 0.7	12 ± 1.2*

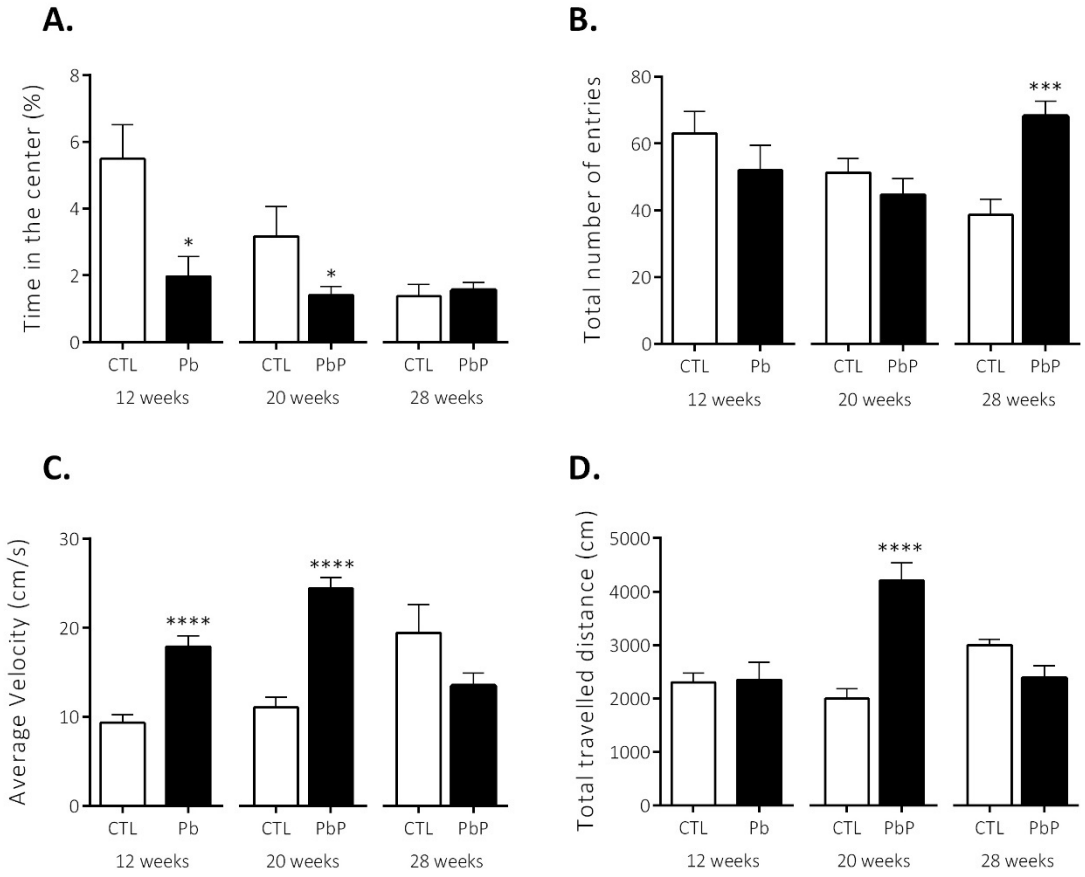

Figure 13 – *Locomotor and exploratory behaviour assessment by OFT of PbP and CTL protocols at 12, 20 and 28 weeks*
Presence time percentage in the centre (A), total number of entries (B), average velocity (C) and total travelled distance (D) are represented. Values are mean ± SEM; *p < 0.05; ***p < 0.001; ****p < 0.0001.

Table 10 – *Values of locomotor and exploratory activity assessment by OFT of PbP and CTL protocols at 12, 20 and 28 weeks*
Values are mean ± SEM. n=10/group; *p < 0.05; ***p < 0.001; §p < 0.0001.

Group	Age	Time in the centre (%)	Total number of entries	Average velocity (cm/s)	Total travelled distance (cm)
CTL	12 weeks	6 ± 1.0	63 ± 6.6	9.3 ± 0.9	2302 ± 171
Pb		2 ± 0.6*	52 ± 7.5	17.9 ± 1.2§	2350 ± 330
CTL	20 weeks	3 ± 0.9	51 ± 4.3	11.0 ± 1.2	1993 ± 192
PbP		1 ± 0.3*	45 ± 4.8	24.4 ± 1.2§	4204 ± 331§
CTL	28 weeks	1 ± 0.4	39 ± 4.6	19.4 ± 3.2	2998 ± 105
PbP		2 ± 0.2	68 ± 4.5***	13.5 ± 1.4	2380 ± 232

Effect on memory

Spatial working memory

The spatial working memory seems not to be primary affected by the persistent lead exposure since foetal period and through lifetime, which was inferred from the lack of alterations in the spontaneous alternation behaviour in the animals that were exposed to lead. Also, no changes were observed in the exploration behaviour while performing the Y-Maze test, which could be a confounding factor for data interpretation.

The time evolution of the spontaneous alterations percentage and the total number of entries parameters of the 3 periods of evaluation are presented in *Figure 14* and values in *Table 11*.

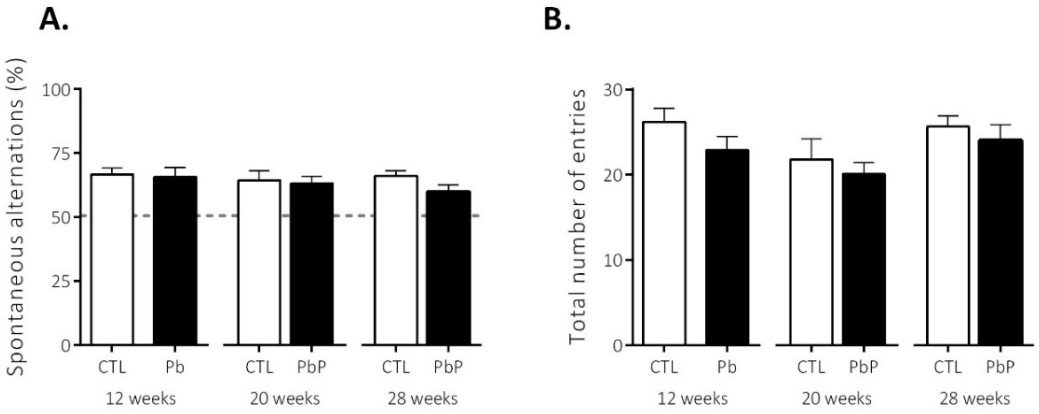

Figure 14 – *Spatial working memory evaluation by Y-Maze test of PbP and CTL protocols at 12, 20 and 28 weeks*
Spontaneous alternations percentage (A) and the total number of entries in the arms (B) are presented. Values are mean ± SEM.

Table 11 – *Values from spatial working memory evaluation by Y-Maze test of PbP and CTL groups at 12, 20 and 28 weeks*
Values are presented as mean ± SEM. n=10/group.

Group	Age	Spontaneous alternations (%)	Total number of entries
CTL	12 weeks	67 ± 2.5	26 ± 1.6
Pb		66 ± 3.7	23 ± 1.6
CTL	20 weeks	64 ± 3.8	22 ± 2.4
PbP		63 ± 2.6	20 ± 1.3
CTL	28 weeks	66 ± 2.1	26 ± 1.3
PbP		60 ± 2.7	24 ± 1.9

Episodic long-term memory

As it is seen in the ***Figure 15*** and ***Figure 16*** and in the ***Table 12***, PbP group shows no novel object recognition (data of exploration time percentage), as the animals explore the two objects (sample and Novel) similarly in the testing day, in the contrary to the CTL group, even though, showing no significant differences between the novelty recognition indexes at 20 and 28 weeks of age, a more refined and sensitive to variability parameter.

Therefore, exposure to lead in the early stages causes a long-term memory impairment through life, even though, some recovery seems to be happening over time, which could be indicative of remodelling and adjustment processes to the lead presence within the regions responsible for long-term memory-evoking.

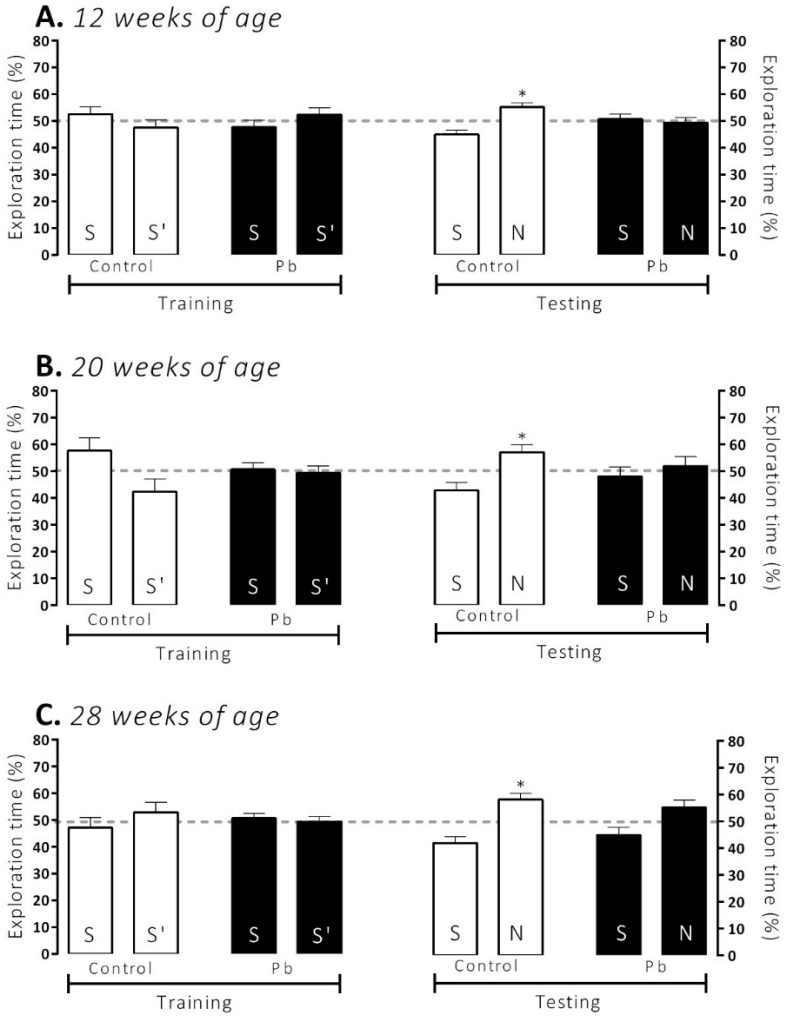

Figure 15 – *Episodic long-term memory evaluation by NOR test of PbP and CTL protocols at 12, 20 and 28 weeks*
Training and testing exploration time percentage data from 12 weeks of age (A), 20 weeks of age (B) and 28 weeks of age (C) are presented. Values are mean ± SEM; *p < 0.05 (paired).

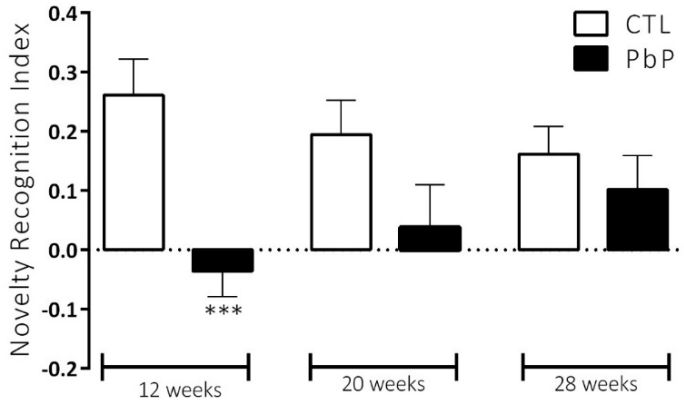

Figure 16 – *Novelty recognition index data of PbP and CTL protocols at 12, 20 and 28 weeks*
Values are mean ± SEM; ***p < 0.001 (unpaired).

Table 12 – *Values of episodic long-term memory evaluation by NOR test of PbP and CTL groups at 12, 20 and 28 weeks*
Values are presented as mean ± SEM. n=10/group; $p < 0.05, (paired); ***p < 0.001 (unpaired).

Group	Age	Exploratory time %				Novelty Recognition Index
		Training		Testing		
		S	S'	S	N	
CTL	12 weeks	53 ± 2.8	47 ± 2.8	45 ± 1.7	55 ± 1.7$	0.26 ± 0.06
Pb		48 ± 2.5	52 ± 2.5	51 ± 1.9	49 ± 1.9	-0.04 ± 0.04***
CTL	20 weeks	58 ± 4.8	42 ± 4.8	43 ± 2.9	57 ±2.9$	0.19 ± 0.06
PbP		51 ± 2.5	49 ± 2.5	48 ± 3.6	52 ± 3.6	0.04 ± 0.07
CTL	28 weeks	47 ± 3.7	53 ± 3.7	42 ± 2.3	58 ± 2.3$	0.16 ± 0.05
PbP		51 ± 1.8	49 ± 1.8	45 ± 2.9	55 ± 2.9	0.10 ± 0.06

Effect on morphofunctional processes in dentate gyrus subregion of the hippocampus

Neurons

Data from ***NeuN*** staining (see ***Figure 17*** and ***Table 13***) showed that long-term exposure to lead seem to provoke some tendency to augmentation of the neurodegenerative processes in dentate gyrus hippocampal region.

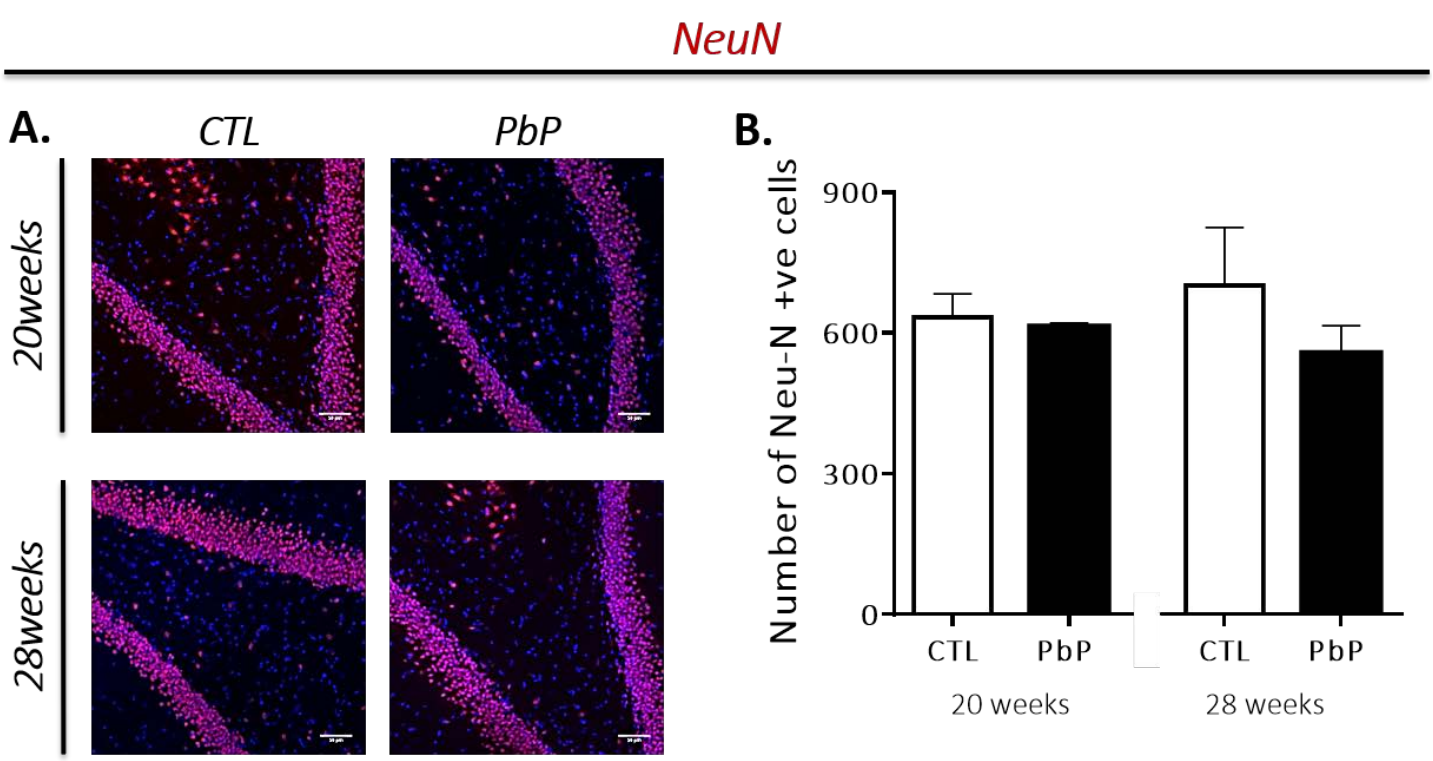

Figure 17 – *Detection and quantification of NeuN in DG hippocampal area of PbP and CTL protocols at 12, 20 and 28 weeks*
Confocal images (A) and quantitative analysis data (B) are represented.
Scale bar is 50µm for staining images. Values are mean ± SEM.

Synapses

Data from ***Syn*** staining (see ***Figure 18*** and ***Table 13***) showed that a continuous Pb exposure from the early stages of life leads to increased synaptic processes in the hippocampus at 20 weeks of age, that seem to undergo a slow recovery through time, even though, not complete at 28 weeks of age. This synaptic overexcitation could be a cause of some general behavioural changes that were observed in the animals permanently exposed to lead.

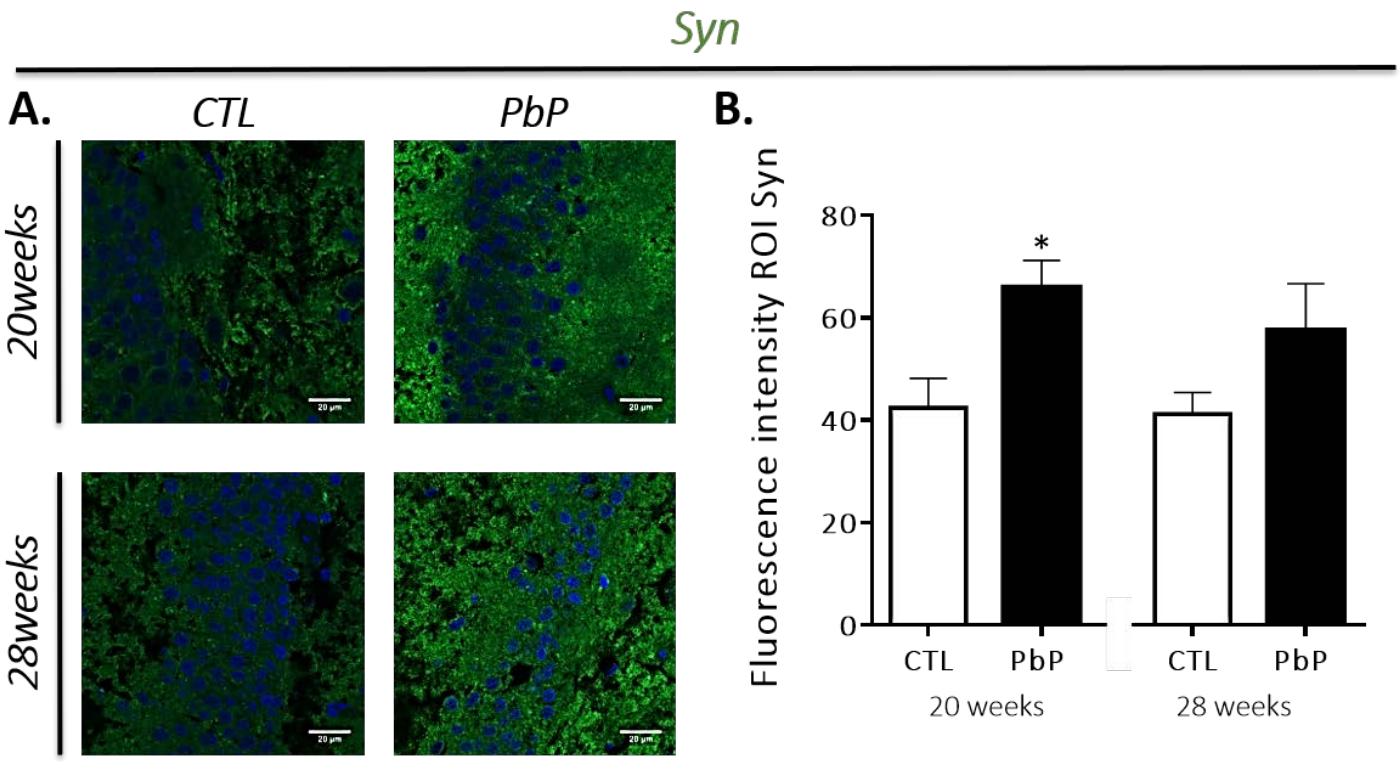

Figure 18 – *Detection and quantification of Syn in DG hippocampal area of PbP and CTL protocols at 12, 20 and 28 weeks*
Confocal images (A) and quantitative analysis data (B) are represented.
Scale bar is 20µm for staining images. Values are mean ± SEM; *p < 0.05.

Astrogliosis

Data from *GFAP* staining (see *Figure 19* and *Table 13*) showed that long-term exposure to lead drastically affects the astrocytic cells, changes that persist through the whole time of exposure to lead, both in morphological and functional levels. Reactive astrogliosis (characterized by astrocytes marked with GFAP staining that are denser and upregulated for the marker, showing hypertrophic branches), which is reminiscent of chronic neuroinflammatory mechanisms and alterations in the tripartite synaptic processes for neuronal communication presence in the hippocampus area.

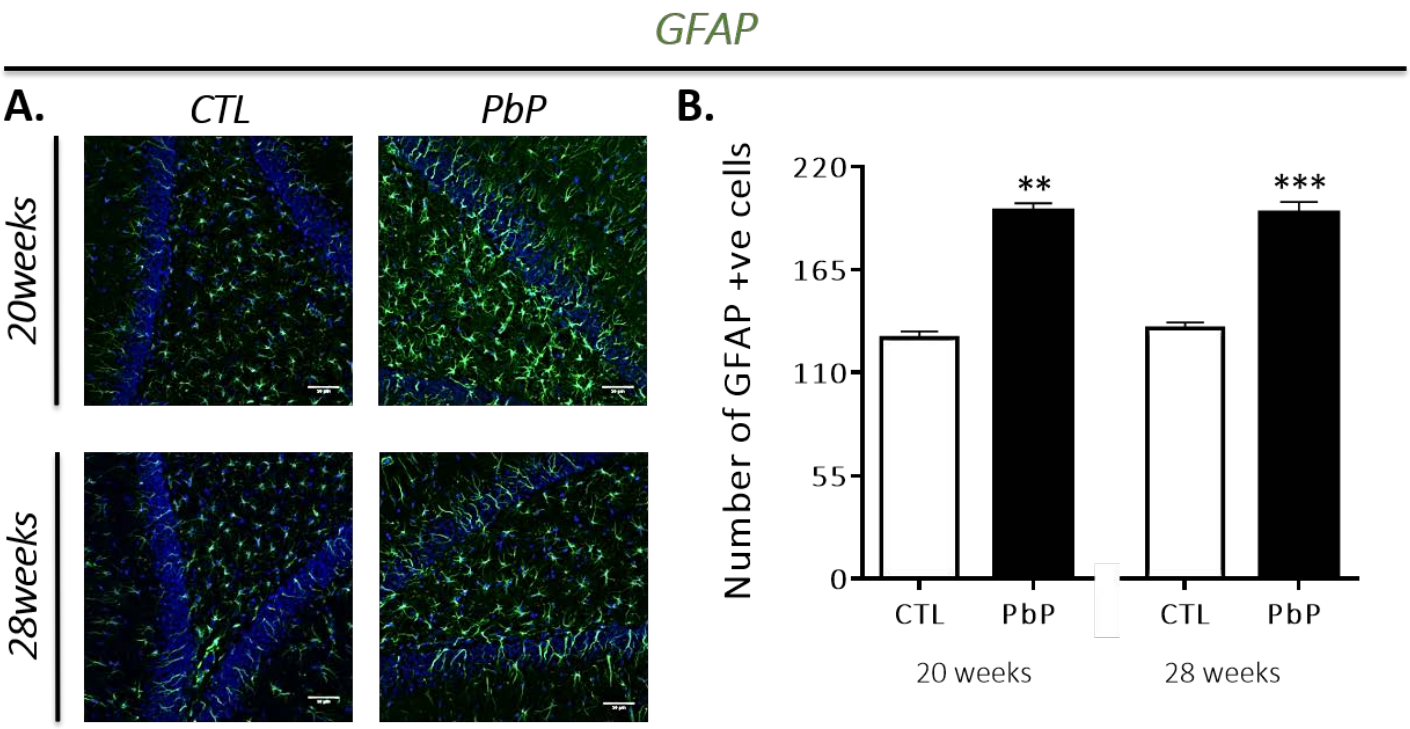

Figure 19 – *Detection and quantification of GFAP in DG hippocampal area of PbP and CTL protocols at 12, 20 and 28 weeks*
Confocal images (A) and quantitative analysis data (B) are represented. Scale bar is 50µm for staining images. Values are mean ± SEM; **p < 0.01; ***p < 0.001.

Microgliosis

No visible alterations at the morphological level of the microglial cells were observed in animals that were permanently exposed to lead. In fact, data from *Iba-1* staining (see *Figure 20* and *Table 13*) showed that microglia is ramified, with small cell bodies and numerous long branching processes. However, an increased number of these cells at both 20 and 28 weeks was observed, which is evocative of microglial activation promoting long-lasting pro-neuroinflammatory processes that contribute to central nervous system protection from the adverse effects of lead exposure.

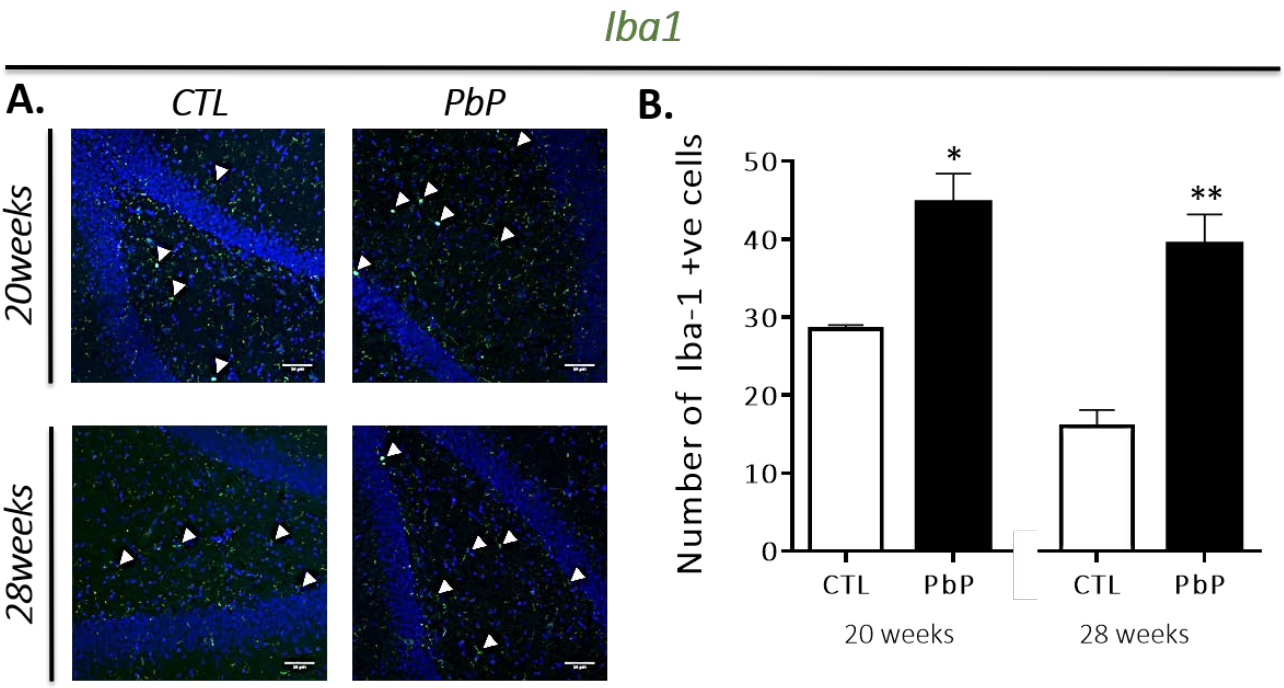

Figure 20 – *Detection and quantification of Iba-1 in DG hippocampal area of PbP and CTL protocols at 12, 20 and 28 weeks*
Confocal images (A) and quantitative analysis data (B) are represented. Scale bar is 50µm for staining images. Values are mean ± SEM; *p < 0.05; **p < 0.01.

Table 13 – *Values of NeuN, Syn, GFAP and Iba-1 stainings quantification in DG hippocampal area of PbP and CTL groups at 12, 20 and 28 weeks*
Values are presented as mean ± SEM. n=3/group; *p < 0.05; **p < 0.01; ***p < 0.001.

Group	Age	Number of *NeuN* positive cells	*Syn* staining fluorescence intensity	Number of *GFAP* positive cells	Number of *Iba-1* positive cells
CTL	20 weeks	634 ± 50	42 ± 5.8	129 ± 3.5	29 ± 0.5
PbP		613 ± 8.1	66 ± 5.3*	196 ± 4.6**	45 ± 3.8*
CTL	28 weeks	702 ± 122.8	41 ± 4.2	134 ± 3.2	16 ± 2.1
PbP		557 ± 58.7	58 ± 9.1	195 ± 6.3***	39 ± 3.8**

4.2 SHORT - TERM LEAD EXPOSURE PROTOCOL (PBS)

The animal model of short-term lead exposure evaluates the behavioural, overall functional and hippocampal morphofunctional alterations due to a short, single exposure of animals to lead since the foetal period until 12 weeks after birth, without being exposed during adulthood. Animals were divided into three groups in accordance to the time of evaluation: 12, 20 and 28 weeks.

Effect on metabolic parameters and BLL

Animals exposed to lead until 12 weeks of age and without any adult exposure do not present fluctuations in weight at different time-points of evaluations (at 12, 20 and 28 weeks of age) (*Table 14*).

Regarding other metabolic parameters, it was found that just after lead exposure, at 12 weeks of age, a little food and water intake increase were observed in these animals, even though, without changes in the urine and faeces production. Interestingly, at 20 weeks of age, a small decrease in faeces production was reported, without food intake alterations, and at 28 weeks, an increase in urine excretion. All the metabolic parameters evaluated in CTL groups, at three different time-points, did not change significantly (*Table 14*).

Regarding BLL, at 20 weeks, after an 8-week period lead abstinence, a low concentration of lead in the blood was observed in these animals. These levels are even lower after another 8 weeks without lead exposure, at 28 weeks of age. This can be explained by the increase in urine production, as it is one of the main routes of lead excretion (*Table 14*).

Table 14 – *Values of metabolic parameters and BLL of PbS and CTL groups at 12, 20 and 28 weeks of age.*
Values are presented as mean ± SEM. n=10/group; *p < 0.05; ***p < 0.001.

Group	Age	BBL (ug/dL)	Weight (g)	Food intake (g)	Water intake (ml)	Urine (ml)	Faeces (g)
CTL	12 weeks	na	358 ± 34	25 ± 1	24 ± 3	19 ± 1	12 ± 1
Pb		na	345 ± 39	22 ± 1*	32 ± 3*	15 ± 1	9 ± 1
CTL	20 weeks	< 0.1	386 ± 41	23 ± 3	39 ± 3	17 ± 2	12 ± 1
PbS		6 ± 0.7	390 ± 35	25 ± 1	34 ± 1	14 ± 1	8 ± 1*
CTL	28 weeks	< 0.1	368 ± 36	24 ± 2	24 ± 2	11 ± 1	8 ± 1
PbS		4 ± 0.4	445 ± 54	23 ± 2	31 ± 2	17 ± 1***	9 ± 1

Effect on blood pressure, heart rate and respiratory frequency

Short-term lead exposure since foetal period until 12 weeks of age significantly increases mean blood pressure values, due to the strong increase of both systolic and diastolic blood pressure values. A period of 8 weeks of lead abstinence was not sufficient for a decrease of blood pressure values, however, a longer period of 16 weeks without exposure to lead accounted for a small decrease of all blood pressure values, even though they maintained a hypertensive profile.

Heart rate did not change with the presence of lead in the system and after its abstinence. The tachypnoea is only observed at 12 weeks of age, when lead is still in high levels in the organism. After lead removal, a normal respiratory frequency was observed (*Figure 21; Table 15*).

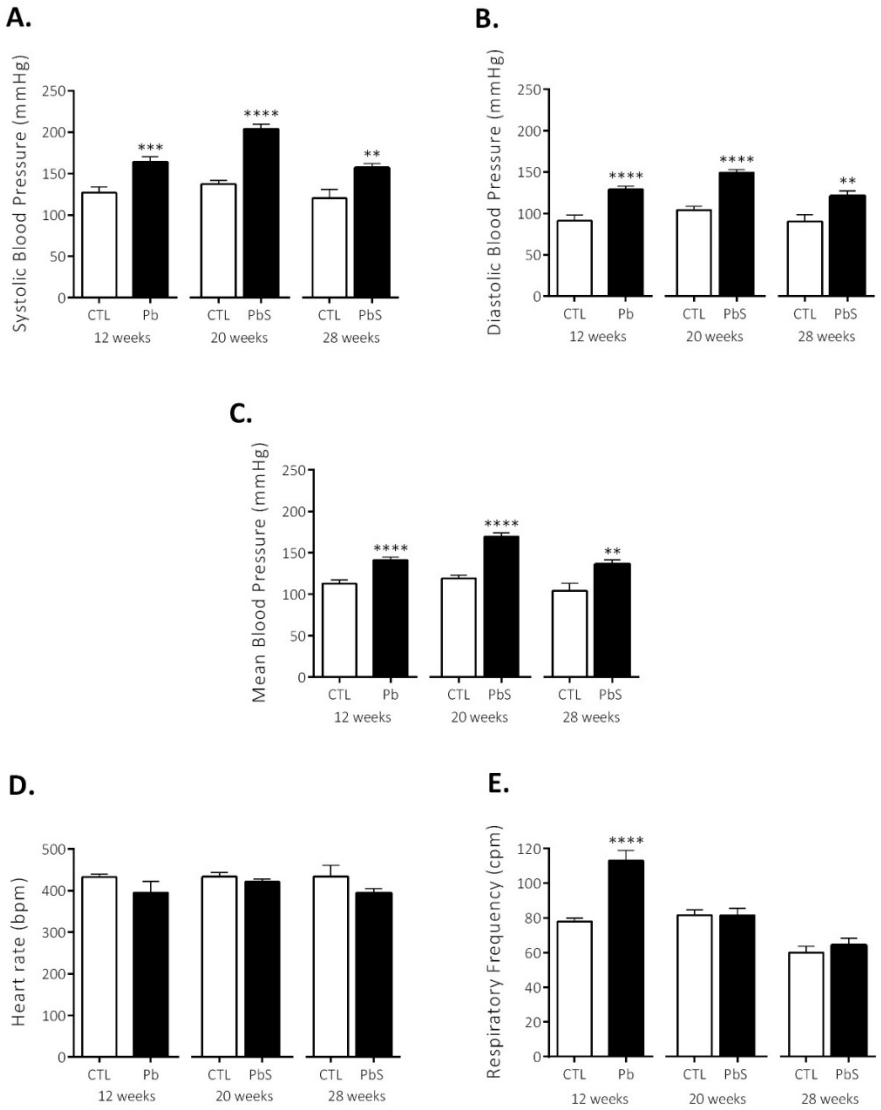

Figure 21 – *Basal physiological parameters evaluation of PbS and CTL protocols at 12, 20 and 28 weeks*
Systolic (A), diastolic (B) and mean (C) blood pressures, heart rate (D) and respiratory frequency (E). Values are mean ± SEM; **p < 0.01; ***p < 0.001; ****p < 0.0001.

Table 15 – *Values of basal physiological parameters of PbS and CTL groups at 12, 20 and 28 weeks*
Values are presented as mean ± SEM. n=10/group; **p < 0.01; ***p < 0.001; §p < 0.0001.

Group	Age	Blood pressure (mmHg)			Heart rate (bpm)	Respiratory frequency (cpm)
		Systolic	Diastolic	Mean		
CTL	12 weeks	125 ± 7	91 ± 7	113 ± 4	432 ± 7	78 ± 2
Pb		164 ± 6***	129 ± 4 §	141 ± 4 §	395 ± 27	113 ± 6 §
CTL	20 weeks	137 ± 4	104 ± 5	119 ± 4	433 ± 11	82 ± 3
PbS		204 ± 6 §	149 ± 4 §	169 ± 4 §	421 ± 7	81 ± 4
CTL	28 weeks	120± 11	90 ± 8	104 ± 9	434 ± 27	60 ± 3
PbS		157 ± 6**	121 ± 6**	136 ± 5**	394 ± 11	64 ± 4

Effect on baroreceptor and chemoreceptor reflexes

Baroreceptor reflex seems to be primarily affected by the lead exposure and rapidly recovers after lead removal. Actually, baroreflex gain suffered a huge decrease until 12 weeks (when animals were exposed to lead), slowly increasing after lead exposure was cessed, reaching normal values. Though, a period of 8 weeks was not sufficient for the baroreflex gain recovery to normal levels. A longer period of time was necessary for this process to occur and, only at 28 weeks of age, animals presented normal baroreflex gain values.

In the contrary, the carotid chemoreceptor reflex, being affected by the lead exposure, did not undergo the remodelling and recovery processes. The increase of chemoreflex sensitivity that was found in the lead exposed animals at 12 weeks persisted through life, even after 16 weeks of lead abstinence.

A temporal evolution of both baroreceptor and chemoreceptor reflexes changes due to a single, short-term exposure are presented in *Figure 22* and values in *Table 16*.

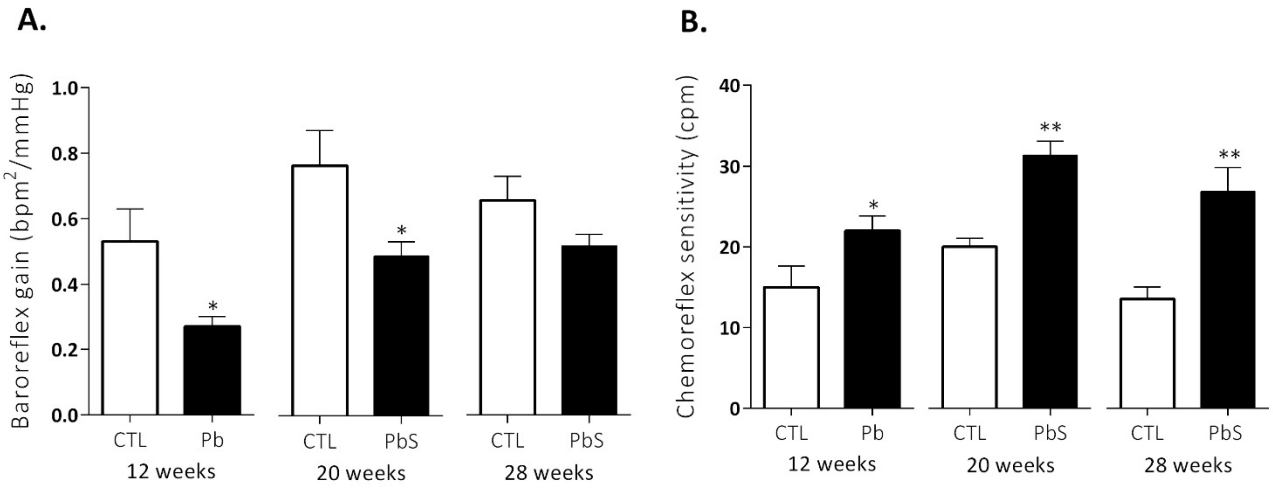

Figure 22 – *Baroreceptor and chemoreceptor reflex evaluation of PbS and CTL protocols at 12, 20 and 28 weeks*
Baroreflex gain (A) and chemoreflex sensitivity (B).
Values are mean ± SEM; *p < 0.05; **p < 0.01.

Table 16 – *Values of baroreflex gain and chemoreflex sensitivity of PbS and CTL groups at 12, 20 and 28 weeks*
Values are presented as mean ± SEM. n=10/group; *p < 0.05; **p < 0.01.

Group	Age	Baroreflex gain ($bpm^2/mmHg$)	Chemoreflex sensitivity (cpm)
CTL	12 weeks	0.53 ± 0.10	15 ± 2.6
Pb		0.27 ± 0.03*	22 ± 1.8*
CTL	20 weeks	0.77 ± 0.11	20 ± 1.1
PbS		0.49 ± 0.04*	31 ± 1.8**
CTL	28 weeks	0.66 ± 0.07	14 ± 1.5
PbS		0.52 ± 0.04	27 ± 2.9**

Effect on autonomic output measured indirectly

In the animals that were exposed to lead until 12 weeks, the sympathetic autonomic function output (evaluated by the LF band) follows the same pattern of the baroreflex gain changes. The maximum increase of the LF band was observed shortly after lead exposure and it was slowly recovering through time without exposure to lead, reversing into values within normal range after a 16-week absence of lead (at 28 weeks). The HF band that accounts for the parasympathetic nervous system was not changed at the evaluated time-points.

Due to the changes in the LF band, the LF/HF index (representative of the autonomic output) suffered an increase during lead exposure that was reversed after lead was removed from the diet, becoming normal after a long period of lead abstinence (i.e., > 16 weeks).

The time evolution of the LF, HF and LF/HF parameters of the 3 periods of evaluation are presented in *Figure 23* and the values in *Table 17*.

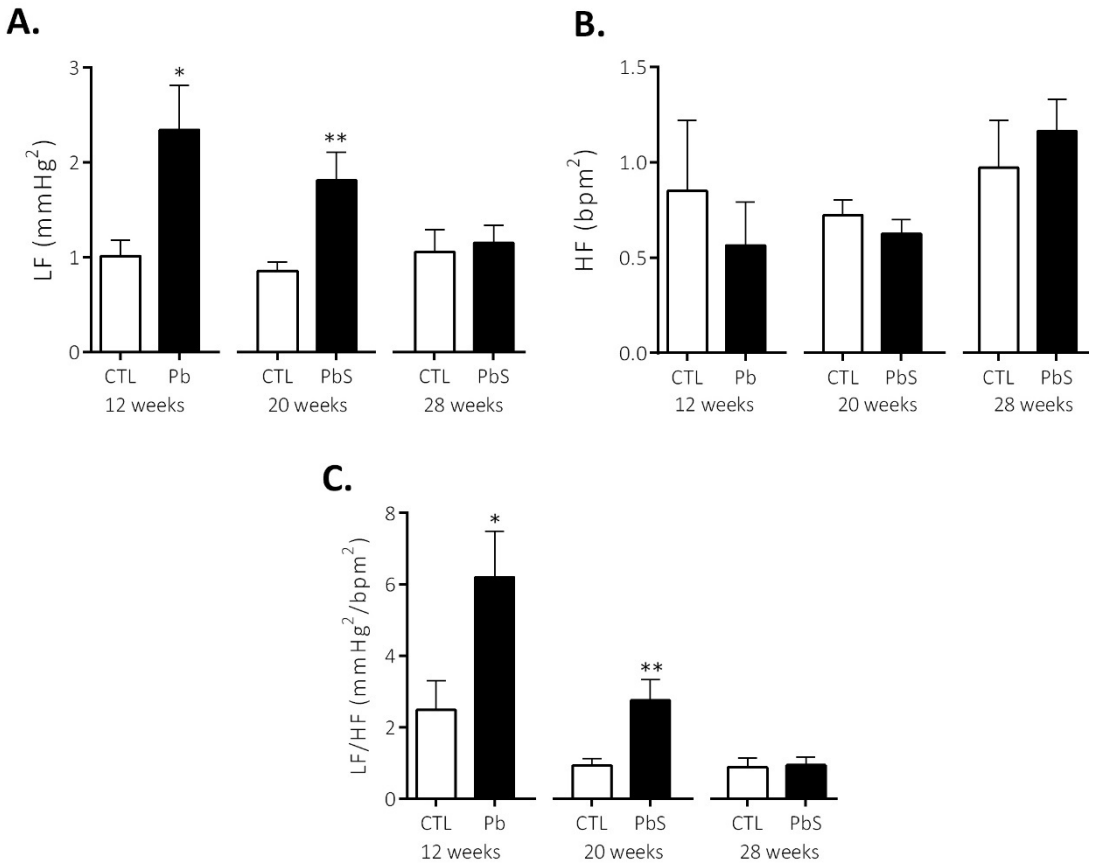

Figure 23 – *Autonomic function evaluation of PbS and CTL protocols at 12, 20 and 28 weeks*
Low frequency band– LF (A), high frequency band - HF (B) and LF/HF index (C) are presented. Values are mean ± SEM; *p < 0.05, **p < 0.01.

Table 17 – *Values of autonomic output measure indirectly of PbS and CTL groups at 12, 20 and 28 weeks*
Values are presented as mean ± SEM. n=10/group; *p < 0.05; **p < 0.01.

Group	Age	LF_{sBP} ($mmHg^2$)	HF_{HR} (bpm^2)	LF_{sBP}/ HF_{HR} ($mmHg^2/bpm^2$)
CTL	12 weeks	1.02 ± 0.16	0.85 ± 0.37	2.49 ± 0.82
Pb		2.34 ± 0.47*	0.56 ± 0.23	6.20 ± 1.29*
CTL	20 weeks	0.86 ± 0.09	0.72 ± 0.08	0.93 ± 0.19
PbS		1.81 ± 0.29**	0.62 ± 0.07	2.76 ± 0.57**
CTL	28 weeks	1.05 ± 0.23	0.97 ± 0.25	0.88 ± 0.25
PbS		1.15 ± 0.18	1.16 ± 0.17	0.94 ± 0.22

Effect on behavioural parameters

Anxiety, locomotion and exploratory activity

Anxiety levels were increased due to the sort-term lead exposure for 12 weeks and through life course, even after lead was absent for a long period of time (16 weeks). Thus, the initial time of exposure was of significant relevance for this behavioural alteration. This behaviour was inferred by the reduction of the presence time in the open arms of the EPM apparatus thus, increasing the presence time in the closed arms of the maze. Though, the number of entries in the open arms were not significantly different of those of the controls, the number of entries in the closed arms were increased at 12 weeks of age which shows that animals were moving in the maze, without choosing the open arms. In the contrary, at 28 weeks, animals preferred to stay in the closed arms, without challenging themselves to move to the open arms (see ***Figure 24***; and ***Table 18*** for values).

Lead exposure induced some significant changes in the exploratory behaviour of the animals that was evaluated by OFT, leading to poor exploration and increasing the anxiety levels of the subjects just after the terminus of exposure (data at 12 weeks). These alterations were recovered through life when lead was removed from the drinking diet (animals do not show changes in the presence time percentage in the central zone of the arena). Also, the exposure to lead in the early stages of life is a cause of a hyperactive behaviour in the animals that was also recovered in the life course, after lead removal (increased values of the average velocity parameter in the Pb-exposed animals at 12 weeks of age). Though, lead does not cause alterations in the locomotor activity (total travelled distance is similar to controls) (see ***Figure 25*** and ***Table 19*** from values from OFT evaluation).

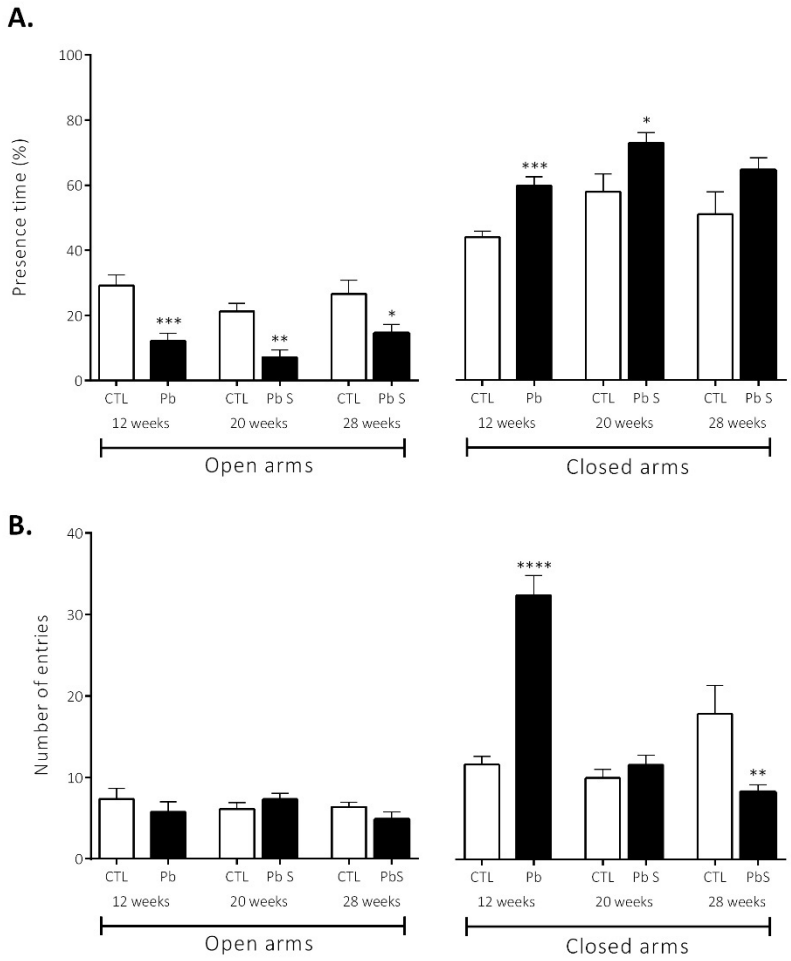

Figure 24 – *Anxiety behaviour assessment by EPM of PbS and CTL protocols at 12, 20 and 28 weeks*
Presence time percentage (A) and number of entries (B) in open and closed arms of the maze are presented. Values are mean ± SEM; *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001.

Table 18 – *Values from anxiety behaviour assessment of PbS and CTL groups at 12, 20 and 28 weeks*
Values are presented as mean ± SEM. n=10/group; *p < 0.05; **p < 0.01; ***p < 0.001; §p < 0.0001.

Group	Age	Presence Time (%)		Number of entries	
		Open Arms	Closed arms	Open Arms	Closed arms
CTL	12 weeks	29 ± 3.2	44 ± 1.8	7 ± 1.3	12 ± 1.0
Pb		12 ± 2.3***	60 ± 2.7***	6 ± 1.2	33 ± 2.4§
CTL	20 weeks	21 ± 2.5	58 ± 5.3	5 ± 0.9	10 ± 1.0
PbS		7 ± 2.2**	73 ± 3.2*	7 ± 0.7	12 ± 1.2
CTL	28 weeks	27 ± 4.3	51 ± 6.8	6 ± 0.6	18 ± 3.5
PbS		15 ± 2.7*	65 ± 3.8	5 ± 0.9	8 ± 0.9**

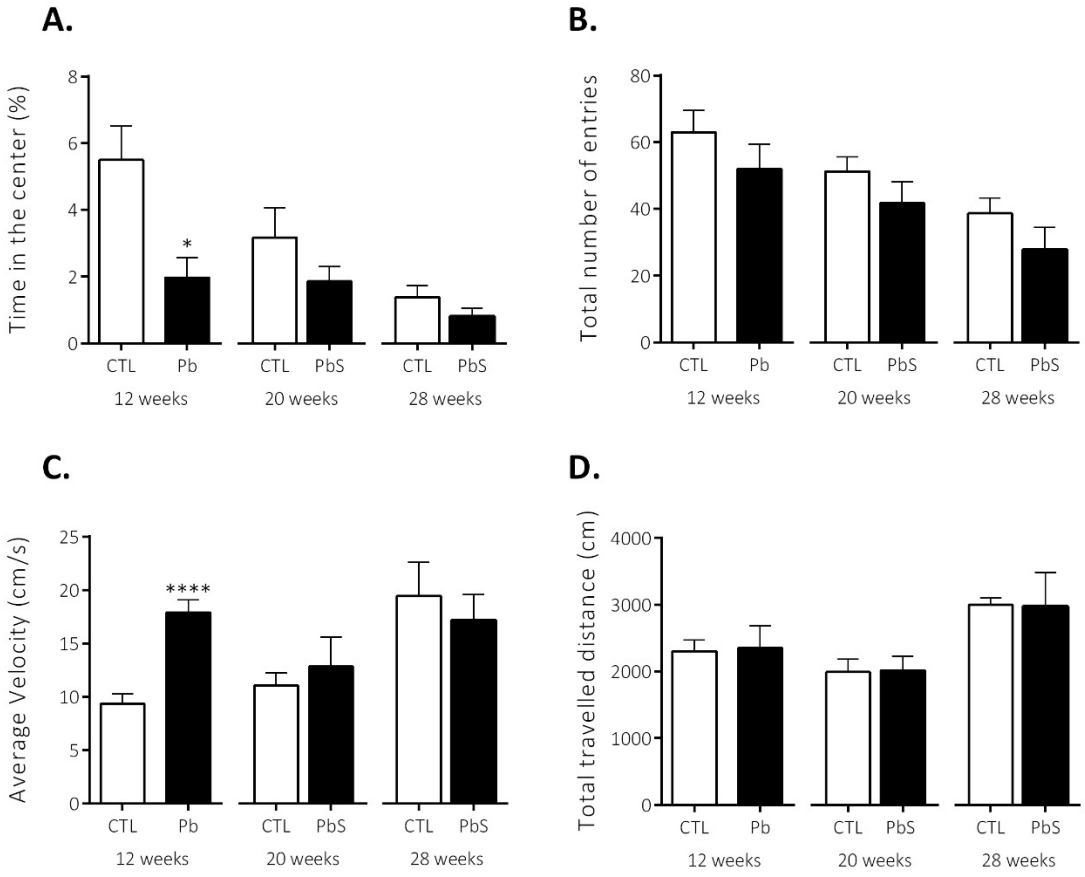

Figure 25 – *Locomotor and exploratory behaviour assessment by OFT of PbS and CTL protocols at 12, 20 and 28 weeks*
Presence time percentage in the centre (A), Total number of entries (B), average velocity (C) and total travelled distance (D) are represented. Values are mean ± SEM; *p < 0.05; ****p < 0.0001.

Table 19 – *Values from locomotor and exploratory activity assessment by OFT of PbS and CTL protocols at 12, 20 and 28 weeks*
Values are mean ± SEM. n=10/group; *p < 0.05; §p < 0.0001.

Group	Age	Time in the centre (%)	Total number of entries	Average velocity (cm/s)	Total travelled distance (cm)
CTL	12 weeks	6 ± 1.0	63 ± 6.6	9 ± 0.9	2302 ± 171
Pb		2 ± 0.6*	52 ± 7.5	18 ± 1.2§	2350 ± 330
CTL	20 weeks	3 ± 0.9	51 ± 4.3	11 ± 1.2	1993 ± 192
PbS		2 ± 0.4	42 ± 6.4	13 ± 2.8	2013 ± 214
CTL	28 weeks	1 ± 0.4	39 ± 4.6	19 ± 3.2	2998 ± 105
PbS		1 ± 0.2	28 ± 6.7	17 ± 2.5	2976 ± 507

Effect on memory

Spatial working memory

A lack of alterations in the spontaneous alternation behaviour in the animals that were exposed to lead until 12 weeks of age is indicative of no alterations in the spatial working memory in these animals. Interestingly, at 28 weeks of age, PbS group present a reduction of the total number of entries, and no changes in the spontaneous alternations behaviour, which favours the hypotheses that lead does not primarily affect the spatial working memory. Even though, its removal is favourable for a memory improvement (see ***Figure 26***, and ***Table 20*** for values of the 3 time-points of evaluation).

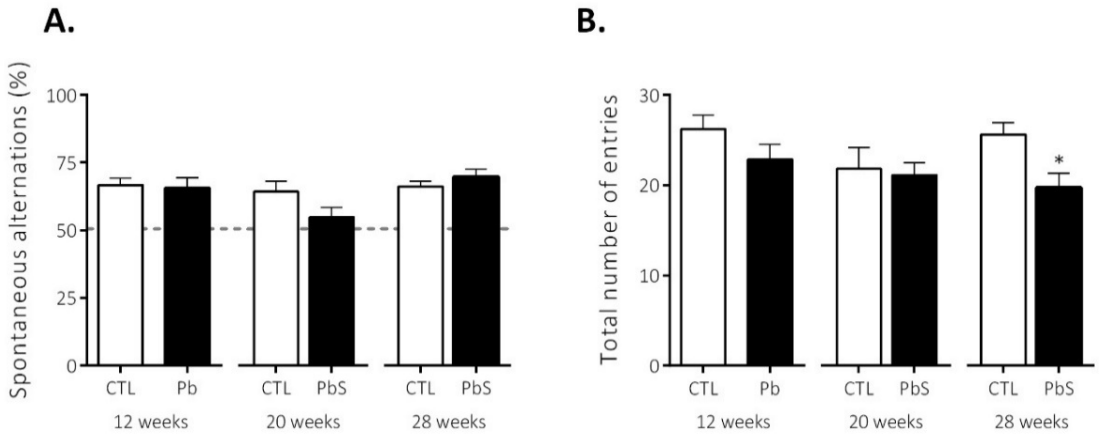

Figure 26 – *Spatial working memory evaluation by Y-Maze test of PbS and CTL protocols at 12, 20 and 28 weeks*
Spontaneous alternations percentage (A) and the total number of entries in the arms (B) are presented. Values are mean ± SEM; *p < 0.05.

Table 20 – *Values from spatial working memory evaluation by Y-Maze test of PbS and CTL groups at 12, 20 and 28 weeks*
Values are presented as mean ± SEM. n=10/group; *p < 0.05.

Group	Age	Spontaneous alternations (%)	Total number of entries
CTL	12 weeks	67 ± 2.5	26 ± 1.6
Pb		66 ± 3.7	23 ± 1.6
CTL	20 weeks	64 ± 3.8	22 ± 2.4
PbS		55 ± 3.6	21 ± 1.3
CTL	28 weeks	66 ± 2.1	26 ± 1.3
PbS		70 ± 2.8	20 ± 1.6*

Episodic long-term memory

The episodic long-term memory is strongly affected by the lead exposition at the early stages (at 12 weeks). No recovery of these alterations has been observed after a period of 8 weeks without lead exposure, with lead continuing its nefarious effects in memory. However, a longer period of time without exposure seem to culminate in some recovery of this alterations to normality, as seen by the novelty recognition data, even though animals do not recognise the novel object at the testing day when exploration time percentage is calculated, contrary to controls that explore the novelty object for more time.

Data of the exploration time percentage at 12, 20 and 28 weeks is shown in ***Figure 27,*** of the novel recognition index in ***Figure 28*** and values of both parameters at the 3 time-points of evaluation are represented in ***Table 21.***

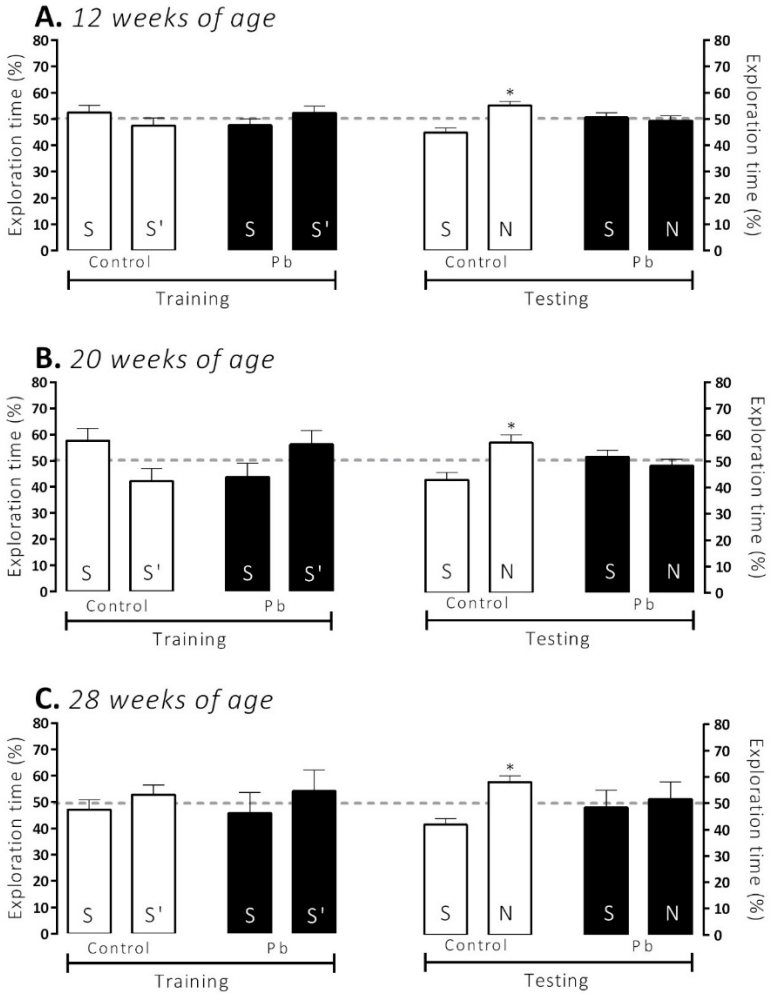

Figure 27 – *Episodic long-term memory evaluation by NOR test of PbS and CTL protocols at 12, 20 and 28 weeks*
Training and testing exploration time percentage data from 12 weeks of age (A), 20 weeks of age (B) and 28 weeks of age (C) are presented. Values are mean ± SEM; *p < 0.05 (paired).

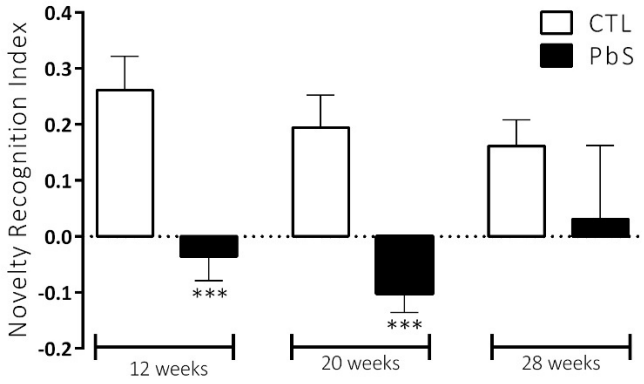

Figure 28 – *Novelty recognition index data of PbS and CTL protocols at 12, 20 and 28 weeks*
Values are mean ± SEM; ***p < 0.001 (unpaired).

Table 21 – *Values of episodic long-term memory evaluation by NOR test of PbS and CTL groups at 12, 20 and 28 weeks*
Values are presented as mean ± SEM. n=10/group; $p < 0.05 (paired); ***p < 0.01 (unpaired).

Group	Age	Exploratory time %				Novelty Recognition Index
		Training		Testing		
		S	S'	S	N	
CTL	12 weeks	53 ± 2.8	47 ± 2.8	45 ± 1.7	55 ± 1.7$	0.26 ± 0.06
Pb		48 ± 2.5	52 ± 2.5	51 ± 1.9	49 ± 1.9	-0.04 ± 0.04***
CTL	20 weeks	58 ± 4.8	42 ± 4.8	43 ± 2.9	57 ±2.9$	0.19 ± 0.06
PbS		44 ± 5.3	56 ± 5.3	52 ± 2.5	48 ± 2.5	-0.10 ± 0.03***
CTL	28 weeks	47 ± 3.7	53 ± 3.7	42 ± 2.3	58 ± 2.3$	0.16 ± 0.05
PbS		46 ± 7.9	54 ± 7.9	48 ± 6.6	52 ± 6.6	0.03 ± 0.13

Effect on morphofunctional processes in dentate gyrus subregion of the hippocampus

Neurons

Data from ***NeuN*** staining (see ***Figure 29*** and ***Table 22*** for values) showed that short-term exposure to lead seem not be affecting neurodegenerative processes in dentate gyrus hippocampal region, even after long periods of lead abstinence (namely, after 8 e 16 weeks).

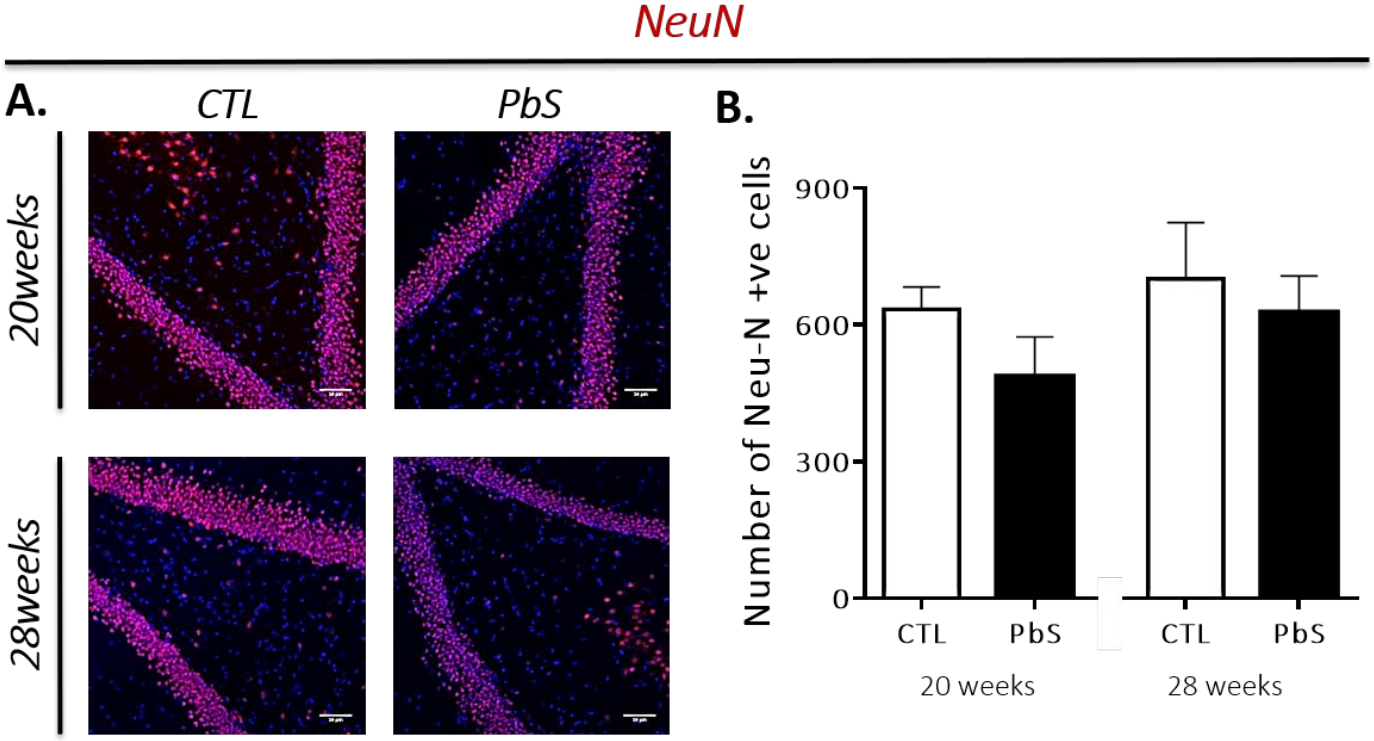

Figure 29 – *Detection and quantification of NeuN in DG hippocampal area of PbS and CTL protocols at 12, 20 and 28 weeks*
Confocal images (A) and quantitative analysis data (B) are represented.
Scale bar is 50µm for staining images. Values are mean ± SEM.

Synapses

Data from ***Syn*** staining (see ***Figure 30*** and ***Table 22*** for values) showed that, at 20 weeks of age, animals that underwent an exposure to lead until 12 weeks and were withdrawn from that drinking solution for 8 weeks have a reduction in the synaptic transmission. Though, a longer period of lead abstinence (for 16 weeks) permitted the recovery of synaptic transmission similar to controls (at 28 weeks of age).

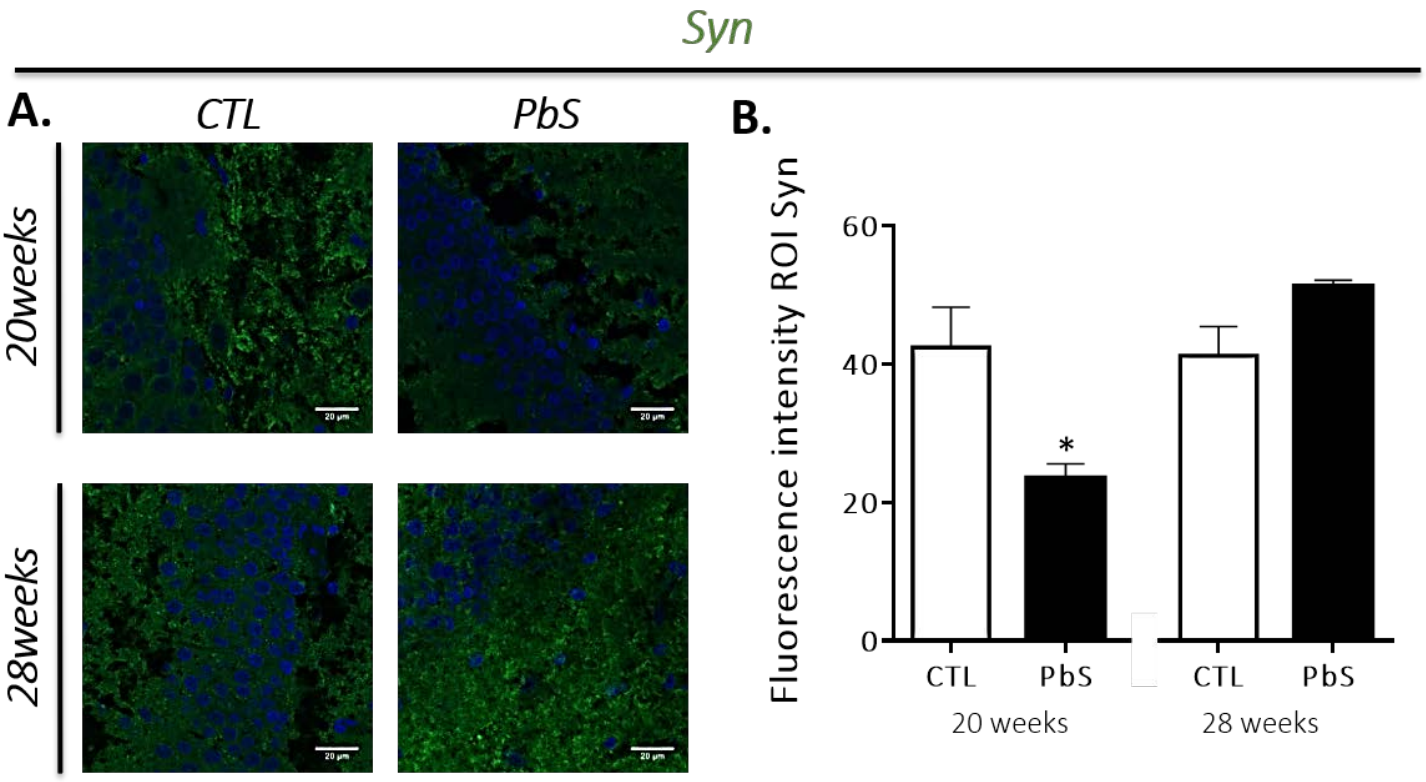

Figure 30 – *Detection and quantification of Syn in DG hippocampal area of PbS and CTL protocols at 12, 20 and 28 weeks*
Confocal images (A) and quantitative analysis data (B) are represented.
Scale bar is 20µm for staining images. Values are mean ± SEM; *p < 0.05.

Astrogliosis

Data from *GFAP* staining (see *Figure 31* and *Table 22* for values) showed that an exposure to lead in the early stages of life drastically affects the astrocytic cells, at morphological and functional levels, changes that persist through life, even after a prolonged period of lead abstinence.

In PbS group, the astrocytic cells are in the activated state within the hippocampus (astrocytes marked with GFAP staining are dense and upregulated, with hypertrophic branches), that is reminiscent of chronic neuroinflammatory mechanisms and alterations in the tripartite synaptic processes for neuronal communication, even though lead was removed from the drinking water of the animals for 8 or 16 weeks.

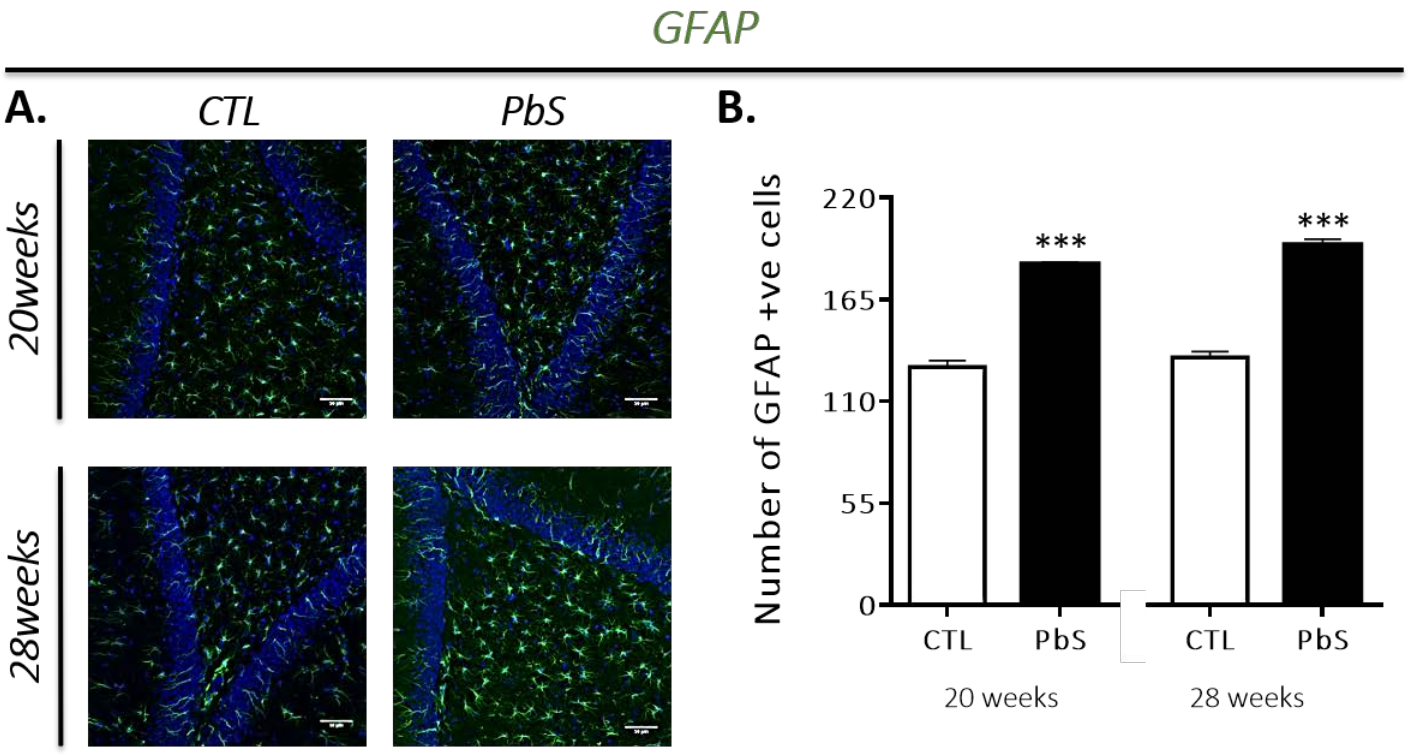

Figure 31 – *Detection and quantification of GFAP in DG hippocampal area of PbS and CTL protocols at 12, 20 and 28 weeks*
Confocal images (A) and quantitative analysis data (B) are represented. Scale bar is 50µm for staining images.
Values are mean ± SEM; ***p < 0.001.

Microgliosis

Data from *Iba-1* staining (see *Figure 32* and *Table 22* for values) showed no visible alterations at the morphological level of the microglial cells, with microglia being in ramified state, small cell bodies and numerous long branching processes.

However, even after a long period of time without lead exposure, an increased number of these cells were observed at both 20 and 28 weeks, which is evocative of the microglial activation, which becomes reactive, leading to long-lasting pro-neuroinflammatory mechanisms activation that acts to protect the central nervous system.

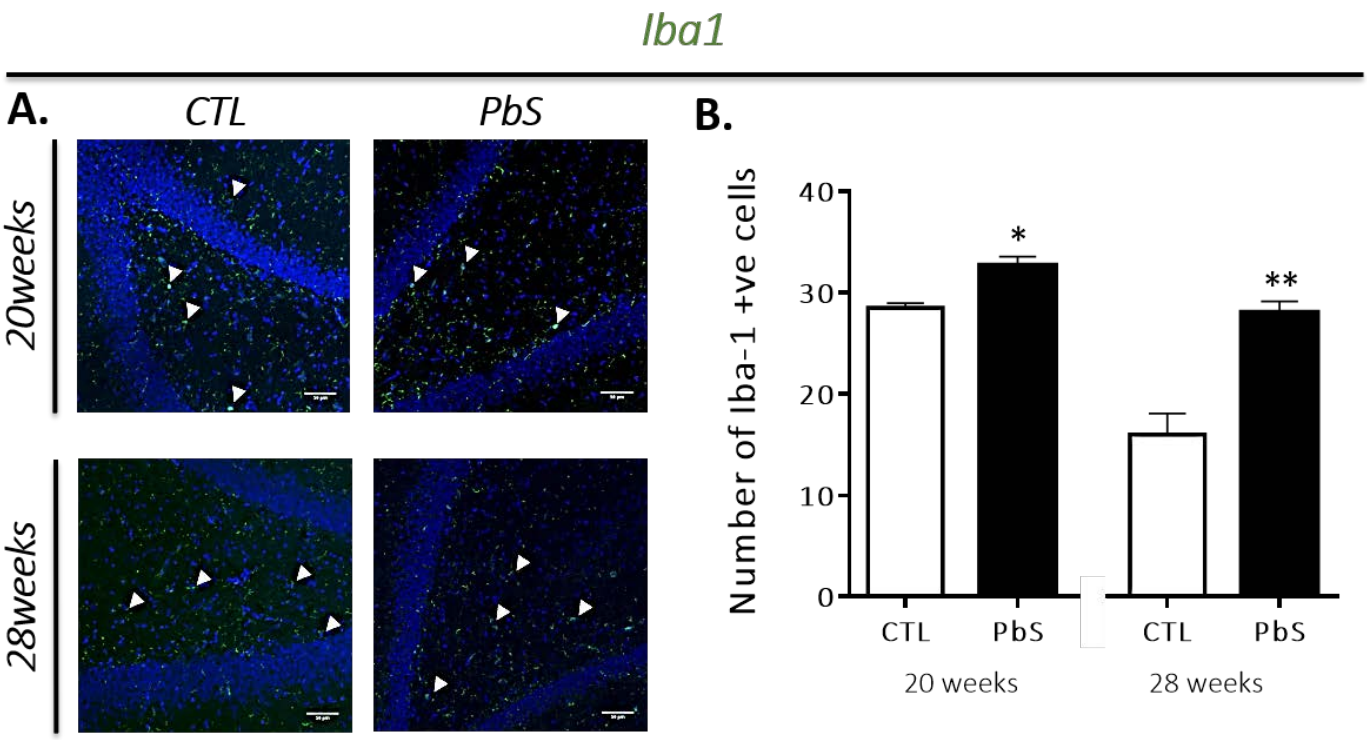

Figure 32 – *Detection and quantification of Iba-1 in DG hippocampal area of PbS and CTL protocols at 12, 20 and 28 weeks*
Confocal images (A) and quantitative analysis data (B) are represented. Scale bar is 50µm for staining images.
Values are mean ± SEM; *p < 0.05; **p < 0.01.

Table 22 – *Values of NeuN, Syn, GFAP and Iba-1 stainings quantification in DG hippocampal area of PbS and CTL groups at 12, 20 and 28 weeks*
Values are presented as mean ± SEM. n=3/group; *p < 0.05; **p < 0.01; ***p < 0.001.

Group	Age	Number of *Neu N* positive cells	*Syn* staining fluorescence intensity	Number of *GFAP* positive cells	Number of *Iba-1* positive cells
CTL	20 weeks	634 ± 49.7	42 ± 5.8	129 ± 3.5	29 ± 0.5
PbS		486 ± 87.8	23 ± 2.1*	184 ± 1.2***	33 ± 0.9*
CTL	28 weeks	702 ± 122.8	41 ± 4.2	134 ± 3.2	16 ± 2.1
PbS		627 ± 80.3	51 ± 0.9	194 ± 3.3***	28 ± 1.2**

4.3 INTERMITTENT LEAD EXPOSURE PROTOCOL (PBI)

The intermittent lead exposure protocol evaluates the behavioural, overall functional and hippocampal morphofunctional alterations from a double exposure to lead, first exposure since the foetal period until 12 weeks after birth, and a second from 20 to 28 weeks of age, with a lead-free period in between (from 12 to 20 weeks). Animals were divided into three groups in accordance to the time of evaluation: 12, 20 and 28 weeks.

Effect on metabolic parameters and BLL

The weight of the animals, evaluated at three different time-points, did not change significantly in animals under intermittent lead exposure protocol. At 12 weeks of age, a little food and water intake increase were observed in these animals, even though, without changes in the urine and faeces production. At 20 weeks, only a small decrease in the faeces excretion was observed, that could also be one of the signs of some stress related to the metabolic cages. All the metabolic parameters evaluated in CTL groups, at three different time-points, and in PbI group evaluated at 28 weeks did not change significantly (*Table 23*).

The levels of lead in the blood are decreased after an 8-week period of lead abstinence and strongly increase after a second exposure for 8 weeks (*Table 23*).

***Table 23 -** Metabolic parameters of PbI and CTL groups at 12, 20 and 28 weeks of age.*
Values are presented as mean ± SEM. n=10/group; *p < 0.05.

Group	Age	BBL (ug/dL)	Weight (g)	Food intake (g)	Water intake (ml)	Urine (ml)	Faeces (g)
CTL	12 weeks	na	358 ± 34	25 ± 1	24 ± 3	19 ± 1	12 ± 1
Pb		na	345 ± 39	22 ± 1*	32 ± 3*	15 ± 1	9 ± 1
CTL	20 weeks	< 0.1	386 ± 41	23 ± 3	39 ± 3	17 ± 2	12 ± 1
PbI		6 ± 0.7	390 ± 35	25 ± 1	34 ± 1	14 ± 1	8 ± 1*
CTL	28 weeks	< 0.1	368 ± 36	24 ± 2	24 ± 2	11 ± 1	8 ± 1
PbI		21 ± 2.7	428 ± 50	24± 2	25 ± 2	11 ± 0.4	9 ± 2

Effect on blood pressure, heart rate and respiratory frequency

Blood pressure is highly increased after the first exposure to lead from foetal period until 12 weeks, without recovering to normotensive values at 20 weeks, after an 8-week period of lead abstinence. Nevertheless, the effect of a second exposure to lead in the adulthood (from 20 to 28 weeks of age) is not as nefarious as the first exposure, mainly seen in the diastolic blood pressure that is reduced at 28 weeks, however not reaching the normotensive values (***Figure 33; Table 24***).

Heart rate did not change at the lead exposure and at lead abstinence periods. Respiratory frequency is only increased when high lead levels are present in the blood (at 12 weeks, after the first lead exposure and at 28 weeks, after the second exposure), fully recovering after lead removal. Also, the adult exposure to lead (second period of exposure) did not provoke a very strong increase in this parameter as seen in the first exposure (***Figure 33;*** see ***Table 24*** for values).

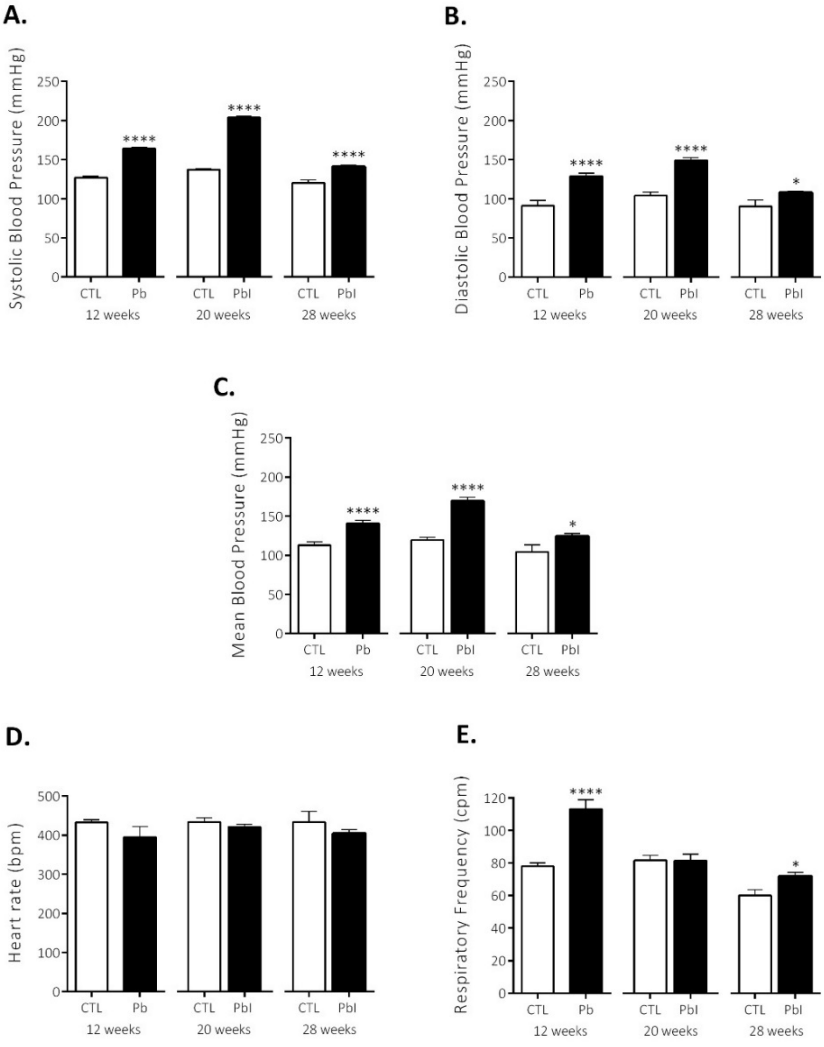

Figure 33 – *Basal physiological parameters evaluation of PbI and CTL protocols at 12, 20 and 28 weeks*
Systolic (A), diastolic (B) and mean (C) blood pressures, heart rate (D) and respiratory frequency (E). Values are mean ± SEM;*p < 0.05; ***p < 0.001; ****p < 0.0001.

Table 24 – *Values of basal physiological parameters of PbI and CTL groups at 12, 20 and 28 weeks*
Values are presented as mean ± SEM. n=10/group; *p < 0.05; ***p < 0.001; §p < 0.0001.

Group	Age	Blood pressure (mmHg)			Heart rate (bpm)	Respiratory frequency (cpm)
		Systolic	Diastolic	Mean		
CTL	12 weeks	125 ± 7	91 ± 7	113 ± 4	432 ± 7	78 ± 2
Pb		164 ± 6***	129 ± 4§	141 ± 4§	395 ± 27	113 ± 6§
CTL	20 weeks	137 ± 4	104 ± 5	119 ± 4	433± 11	82 ± 3
PbI		204 ± 6§	149 ± 4§	169 ± 4§	421 ± 7	81 ± 4
CTL	28 weeks	120 ± 11	90 ± 8	104 ± 9	433 ± 27	60 ± 4
PbI		141 ± 2§	108 ± 2*	124 ± 4*	404 ± 10	72 ± 2*

Effect on baroreceptor and chemoreceptor reflexes

Baroreceptor reflex is strongly affected by the lead presence in the system, suffering an impairment after the first lead exposure. The period of lead abstinence of 8 weeks was not sufficient for baroreflex gain to recover to its normal values and a second exposure to lead even potentiated more the impairment that was observed after the first exposure.

The chemoreflex sensitivity is strongly increased after the first exposure at the developmental period, without suffering a remodelling and recovery processes after lead was removed. A second, adult exposure to lead, potentiated the increase in this parameter even more that the first exposure (***Figure 34,*** see ***Table 25*** for values).

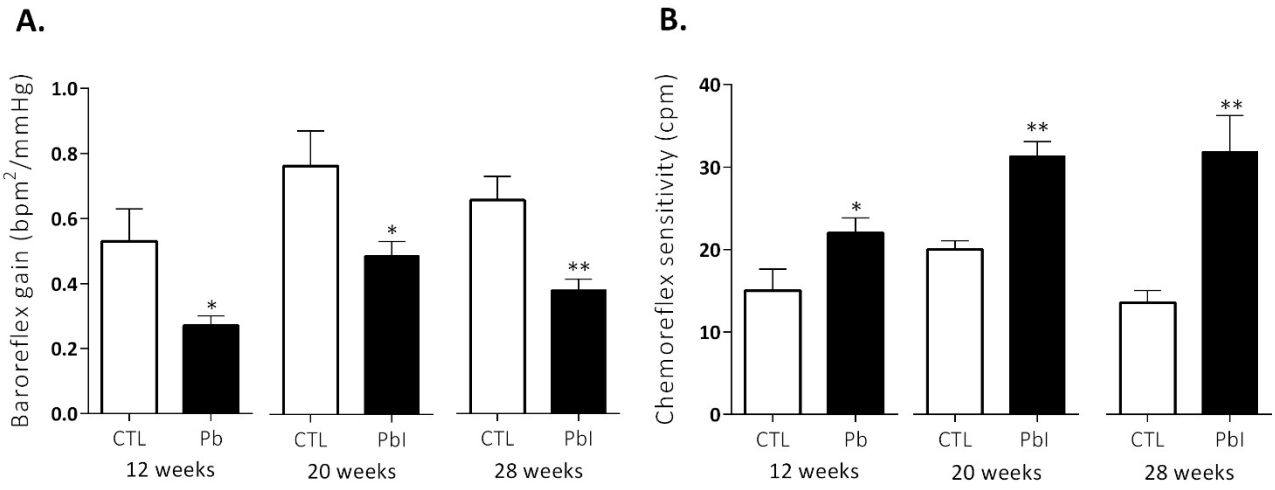

Figure 34 – *Baroreceptor and chemoreceptor reflex evaluation of PbI and CTL protocols at 12, 20 and 28 weeks*
Baroreflex gain (A) and chemoreflex sensitivity (B). Values are mean ± SEM; *p < 0.05; **p < 0.01.

Table 25 – *Values of baroreflex gain and chemoreflex sensitivity of PbI and CTL groups at 12, 20 and 28 weeks*
Values are presented as mean ± SEM. n=10/group; *p < 0.05; **p < 0.01.

Group	Age	Baroreflex gain ($bpm^2/mmHg$)	Chemoreflex sensitivity (cpm)
CTL	12 weeks	0.53 ± 0.10	15 ± 2.6
Pb		0.27 ± 0.03*	22 ± 1.8*
CTL	20 weeks	0.76 ± 0.11	20 ± 1.1
PbI		0.49 ± 0.04*	31 ± 1.8**
CTL	28 weeks	0.66 ± 0.07	14 ± 1.5
PbI		0.38 ± 0.03**	32 ± 4.4**

Effect on autonomic output measured indirectly

Autonomic function is impaired since the early exposure to lead. A sympathetic overexcitation (increase in the LF band) was reported after the first, developmental exposure to lead. This increase in the LF band persisted even after a period of 8 weeks without lead exposure. Although, an exposure in the adulthood (from 20 to 28 weeks of age) did not increase the LF band, being at its normal range values. The HF band, representative of the parasympathetic nervous system, did not suffer significant alterations through the whole exposure protocol.

By reason of the changes in the LF band, the LF/HF index (representative of the autonomic output) suffered an increase after first exposure and after the period of lead abstinence for 8 weeks, not suffering alterations after the second, adult exposure to lead.

Time evolution of the LF, HF and LF/HF parameters and the values for the 3 periods of evaluation are presented in *Figure 35* and *Table 26*.

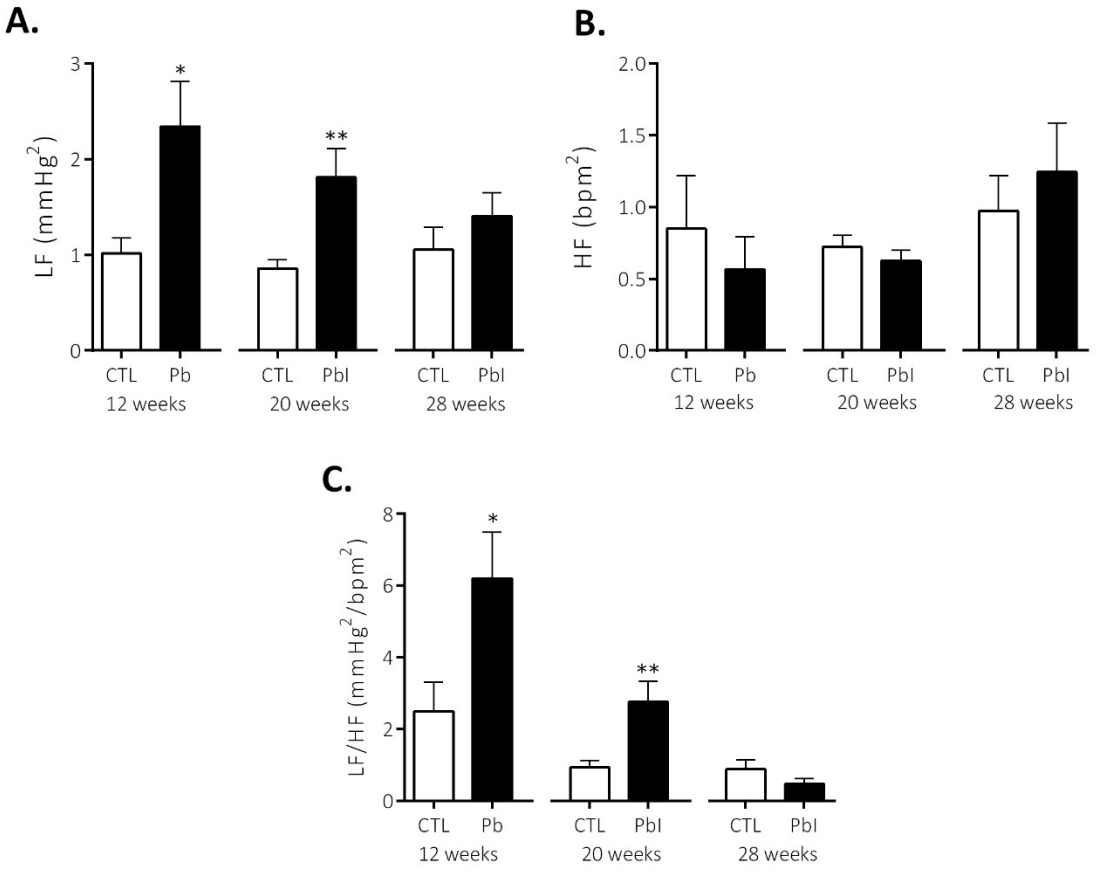

Figure 35 – *Autonomic function evaluation of PbI and CTL protocols at 12, 20 and 28 weeks*
Low frequency band– LF (A), high frequency band - HF (B) and LF/HF index (C) are presented. Values are mean ± SEM; *p < 0.05, **p < 0.01.

Table 26 – *Values of autonomic output measure indirectly of PbI and CTL groups at 12, 20 and 28 weeks*
Values are presented as mean ± SEM. n=10/group; *p < 0.05; **p < 0.01.

Group	Age	LF_{sBP} ($mmHg^2$)	HF_{HR} (bpm^2)	LF_{sBP}/ HF_{HR} ($mmHg^2/bpm^2$)
CTL	12 weeks	1.02 ± 0.16	0.85 ± 0.37	2.49 ± 0.82
Pb		2.34 ± 0.47*	0.56 ± 0.23	6.20 ± 1.29*
CTL	20 weeks	0.86 ± 0.09	0.72 ± 0.08	0.93 ± 0.19
PbI		1.81 ± 0.29**	0.62 ± 0.08	2.75 ± 0.57**
CTL	28 weeks	1.05 ± 0.23	0.97 ± 0.25	0.88 ± 0.25
PbI		1.40 ± 0.24	1.24 ± 0.34	0.47 ± 0.15

Effect on behavioural parameters

Anxiety, locomotion and exploratory activity

Anxiety levels seem to be primarily increased after the first, early-life lead exposure, without recover to normal levels after the period of lead abstinence. A second, adult lead exposure seems not to affect as strongly this behaviour, even though, the stress levels were increased through the whole experimental protocol in these animals. This behaviour was inferred by the reduction of the presence time in the open arms of the EPM apparatus thus, consequently increasing the presence time in the closed arms of the maze. Though, the number of entries in the open arms were significantly different of those of the controls, the number of entries in the closed arms were increased at 12 weeks of age, which shows that animals were moving in the maze, without choosing the open arms. In the contrary, at 28 weeks, animals preferred to stay in the closed arms, without challenging themselves to move to the open arms (see ***Figure 36***; and ***Table 27*** for values).

The developmental lead exposure (from foetal period until 12 weeks of age) seem to be the most nefarious for the exploratory behaviour alterations, leading to the anxiety and hyperactivity behaviours in the animals (decreased time in the centre and increased average velocity, respectively). However, both these alterations in the behaviour seem to be recovered when lead is removed from the diet and a second, adulthood exposure, not having a strong effect on these behaviours. The total number of entries and the total travelled distance of these animals did not change a lot through the whole experimental protocol (see ***Figure 37***; and ***Table 28*** for values).

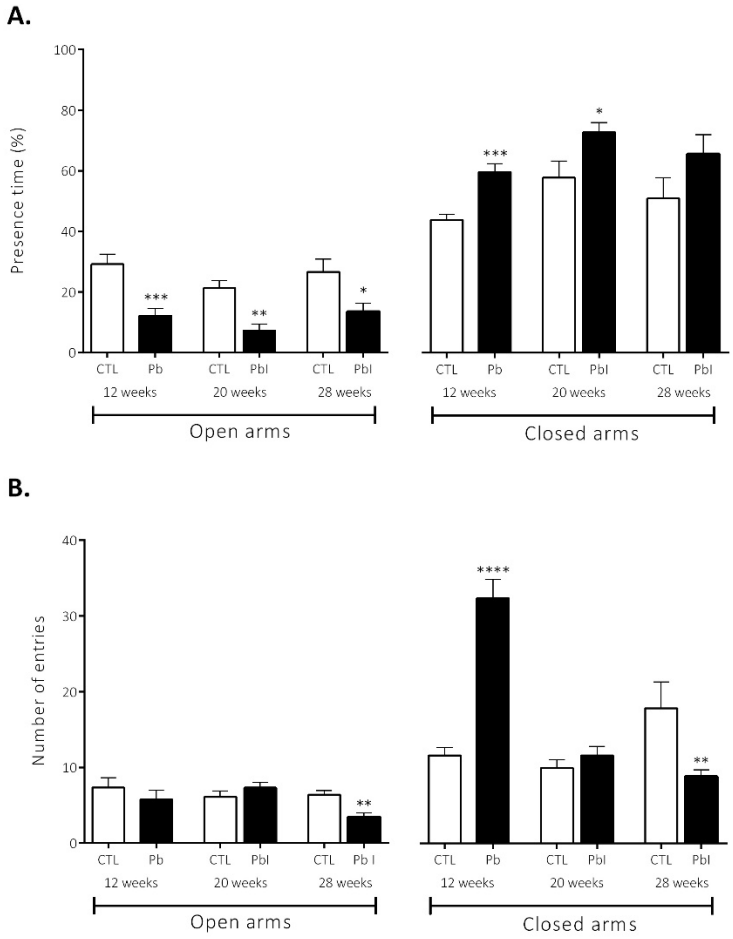

Figure 36 – *Anxiety behaviour assessment by EPM of PbI and CTL protocols at 12, 20 and 28 weeks*
Presence time percentage (A) and number of entries (B) in open and closed arms of the maze are presented. Values are mean ± SEM; *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001.

Table 27 – *Values from anxiety behaviour assessment of PbI and CTL groups at 12, 20 and 28 weeks*
Values are presented as mean ± SEM. n=10/group; *p < 0.05; **p < 0.01***p < 0.001; §p < 0.0001.

Group	Age	Presence Time (%)		Number of entries	
		Open Arms	Closed arms	Open Arms	Closed arms
CTL	12 weeks	29 ± 3.2	44 ± 1.8	7 ± 1.3	12 ± 1.0
Pb		12 ± 2.3***	60 ± 2.7***	6 ± 1.2	33 ± 2.4§
CTL	20 weeks	21 ± 2.5	58 ± 5.3	5 ± 0.9	10 ± 1.1
PbI		7 ± 2.2**	73 ± 3.2*	7 ± 0.7	12 ± 1.2
CTL	28 weeks	27 ± 4.2	51 ± 6.8	6 ± 0.6	18 ± 3.5
PbI		14 ± 2.8*	66 ± 6.4	3 ± 0.6**	9 ± 0.9**

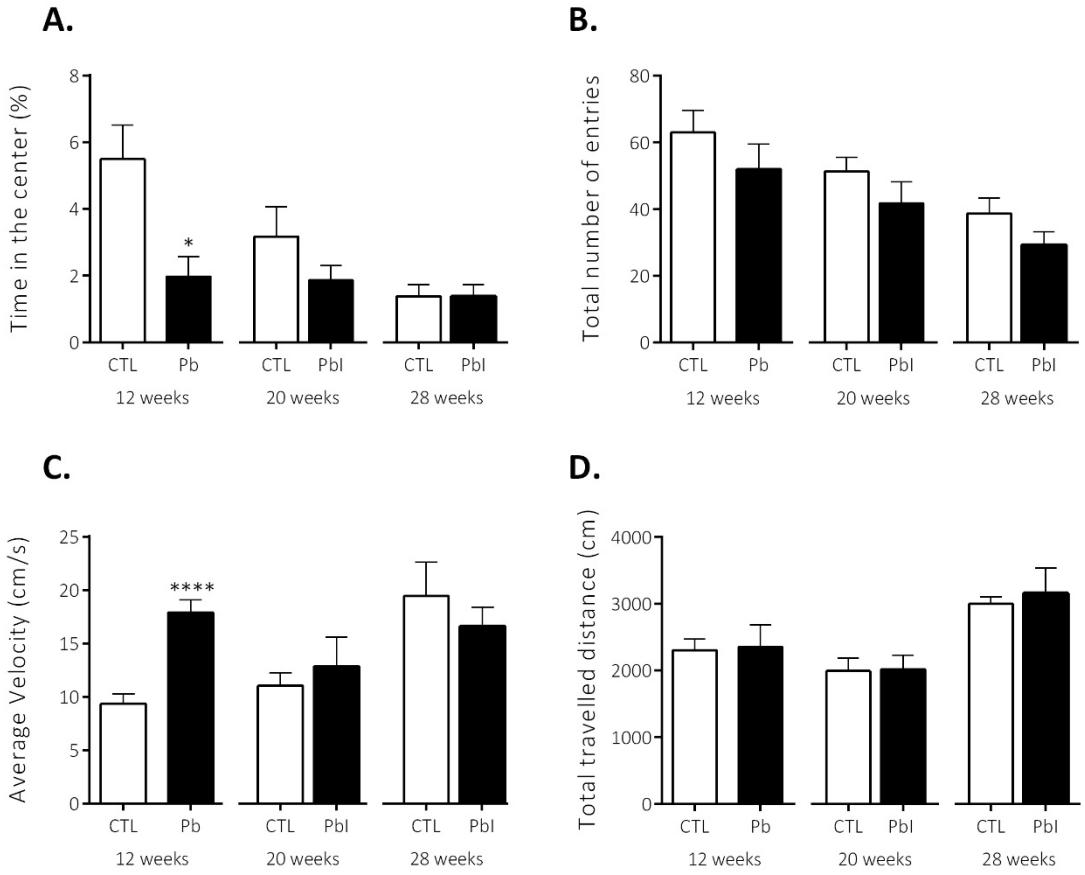

Figure 37 – *Locomotor and exploratory behaviour assessment by OFT of PbI and CTL protocols at 12, 20 and 28 weeks*
Presence time percentage in the centre (A), Total number of entries (B), average velocity (C) and total travelled distance, (D) are represented. Values are mean ± SEM; *p < 0.05; ****p < 0.0001.

Table 28 – *Values from locomotor and exploratory activity assessment by OFT of PbI and CTL protocols at 12, 20 and 28 weeks*
Values are mean ± SEM. n=10/group; *p < 0.05; §p < 0.0001.

Group	Age	Time in the centre (%)	Total number of entries	Average velocity (cm/s)	Total travelled distance (cm)
CTL	12 weeks	6 ± 1.0	63 ± 6.6	9 ± 0.9	2302 ± 171
Pb		2 ± 0.6*	52 ± 7.5	18 ± 1.2§	2350 ± 330
CTL	20 weeks	3 ± 0.9	51 ± 4.3	11 ± 1.2	1993 ± 192
PbI		2 ± 0.4	42 ± 6.4	13 ± 2.7	2013 ± 214
CTL	28 weeks	1 ± 0.3	39 ± 4.6	19 ± 3.2	2998 ± 105
PbI		1± 0.4	29 ± 3.9	17 ± 1.8	3152 ± 380

Effect on memory

Spatial working memory

A lack of alterations in the spontaneous alternation behaviour in the animals that were exposed to lead until 12 weeks of age, which is indicative of no alterations in the spatial working memory in these animals. Interestingly, at 28 weeks of age, animals that suffered an intermittent (double) lead exposure present a reduction of the total number of entries, and no changes in the spontaneous alternations behaviour, which favours the hypotheses that lead does not primarily affect the spatial working memory (see ***Figure 38***, and ***Table 29*** for values at the 3 evaluated time-points).

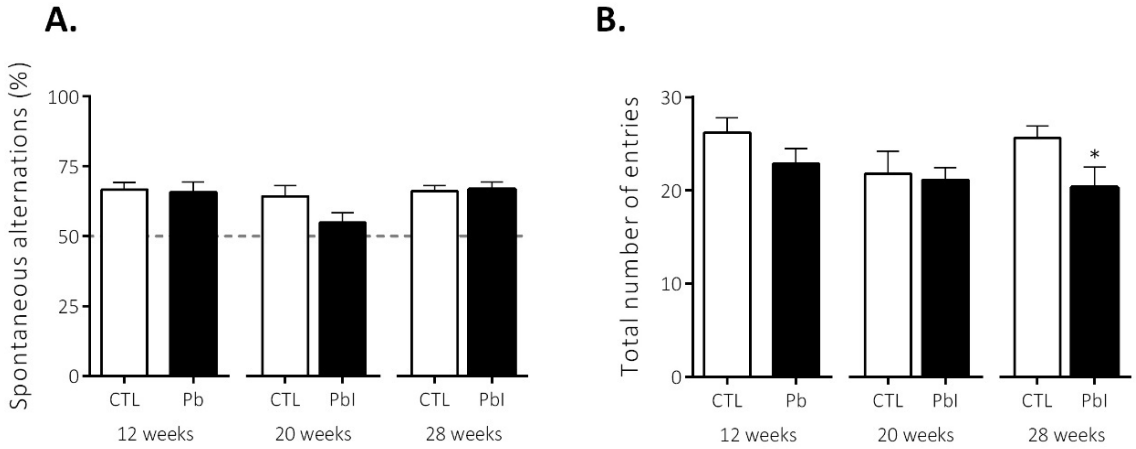

Figure 38 – *Spatial working memory evaluation by Y-Maze test of PbI and CTL protocols at 12, 20 and 28 weeks*
Spontaneous alternations percentage (A) and the total number of entries in the arms (B) are presented. Values are mean ± SEM; *p < 0.05.

Table 29 – *Values from spatial working memory evaluation by Y-Maze test of PbI and CTL groups at 12, 20 and 28 weeks*
Values are presented as mean ± SEM. n=10/group; *p < 0.05.

Group	Age	Spontaneous alternations (%)	Total number of entries
CTL	12 weeks	67± 2.5	26 ± 1.6
Pb		66 ± 3.7	23 ± 1.6
CTL	20 weeks	64 ± 3.8	22 ± 2.4
PbI		55 ± 3.6	21 ± 1.3
CTL	28 weeks	66 ± 2.1	26 ± 1.3
PbI		67 ± 2.7	20 ± 2.2*

Episodic long-term memory

The episodic long-term memory is strongly affected by the lead exposition at the early stages (exposure until 12 weeks). No recovery of these alterations has been observed after a period of 8 weeks without lead exposure, with lead continuing its nefarious effects in memory. However, an adult exposure to lead (from 20 to 28 weeks of age) seem not to affect the episodic long-term memory, as seen by the novelty recognition data, even though animals do not recognise the novel object at the testing day when exploration time percentage is calculated, contrary to controls that explore the novelty object for more time due to familiarization with the sample object.

Data of the exploration time percentage at 12, 20 and 28 weeks is shown in ***Figure 39,*** of the novel recognition index in ***Figure 40*** and values of both parameters at the 3 time-points of evaluation are represented in ***Table 30.***

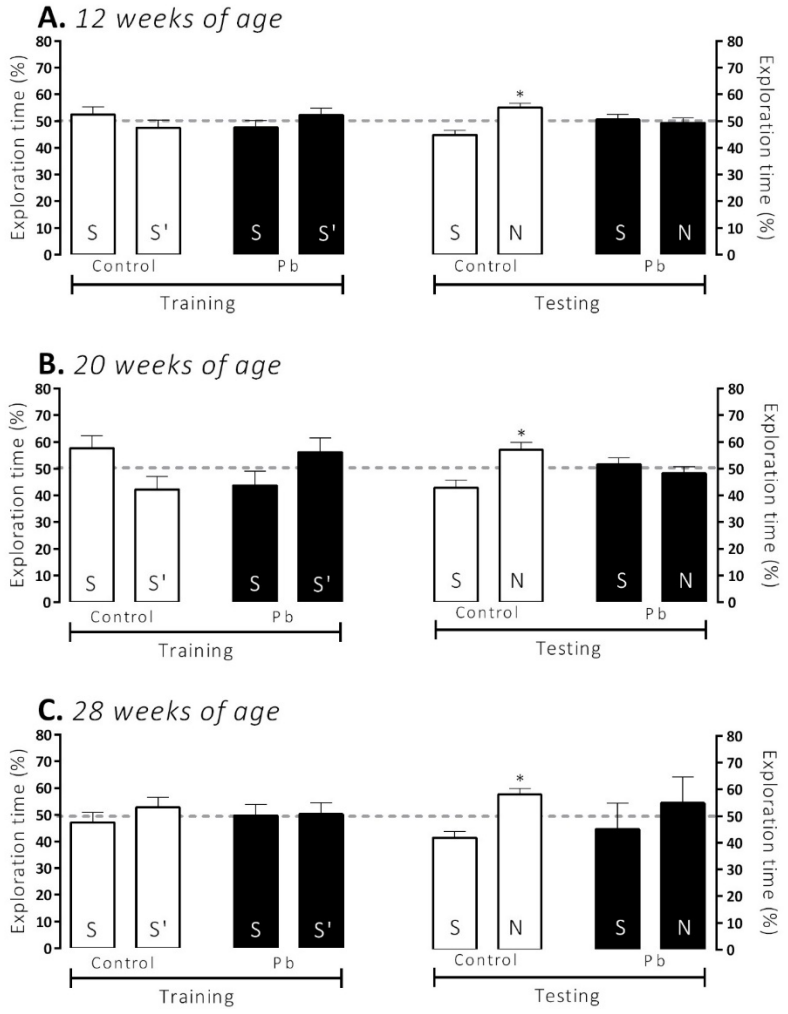

Figure 39 – *Episodic long-term memory evaluation by NOR test of PbI and CTL protocols at 12, 20 and 28 weeks*
Training and testing exploration time percentage data from 12 weeks of age (A), 20 weeks of age (B) and 28 weeks of age (C) are presented. Values are mean ± SEM; *p < 0.05 (paired).

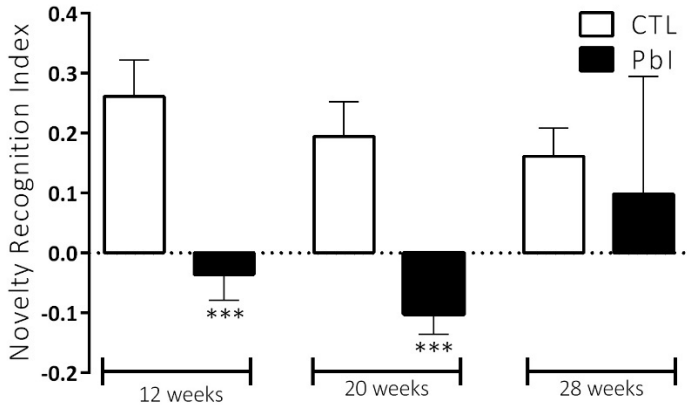

Figure 40 – *Novelty recognition index data of PbI and CTL protocols at 12, 20 and 28 weeks*
Values are mean ± SEM; ***p < 0.001 (unpaired).

Table 30 – *Values of episodic long-term memory evaluation by NOR test of PbI and CTL groups at 12, 20 and 28 weeks*
Values are presented as mean ± SEM. n=10/group; $p < 0.05 (paired); ***p < 0.01 (unpaired).

Group	Age	Exploratory time %				Novelty Recognition Index
		Training		Testing		
		S	S'	S	N	
CTL	12 weeks	53 ± 2.8	47 ± 2.8	45 ± 1.7	55 ± 1.7$	0.26 ± 0.06
Pb		48 ± 2.5	52 ± 2.5	51 ± 1.9	49 ± 1.9	-0.04 ± 0.04***
CTL	20 weeks	58 ± 4.8	42 ± 4.8	43 ± 2.9	57 ±2.9$	0.19 ± 0.06
PbI		44 ± 5.3	56 ± 5.3	52 ± 2.5	48 ± 2.5	-0.10 ± 0.03***
CTL	28 weeks	47 ± 3.7	53 ± 3.7	42 ± 2.3	58 ± 2.3$	0.16 ± 0.05
PbI		50 ± 4	50 ± 4	45 ± 10	55 ± 10	0.09 ± 0.19

Effect on morphofunctional processes in dentate gyrus subregion of the hippocampus

Neurons

Data from ***NeuN*** staining (see ***Figure 41*** and ***Table 31*** for values) showed that an intermittent exposure (early and adult) to lead seem not be affecting neurodegenerative processes in dentate gyrus hippocampal region, even after a period of 8 weeks without lead, and after the second exposure.

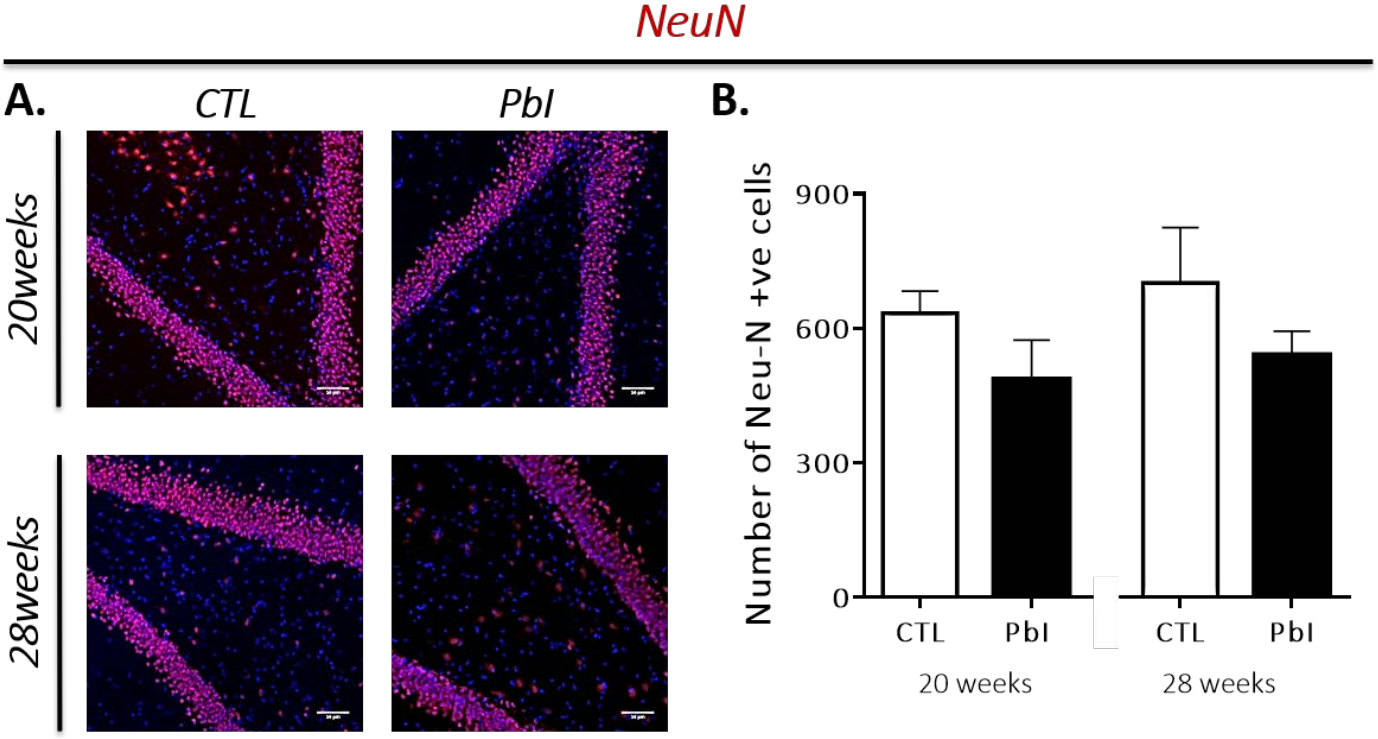

Figure 41 – *Detection and quantification of NeuN in DG hippocampal area of PbI and CTL protocols at 12, 20 and 28 weeks*
Confocal images (A) and quantitative analysis data (B) are represented.
Scale bar is 50µm for staining images. Values are mean ± SEM, $p > 0.05$.

Synapses

Data from ***Syn*** staining (see ***Figure 42*** and ***Table 31*** for values) showed that, at 20 weeks of age, animals that underwent an exposure to lead until 12 weeks and were withdrawn from that drinking solution for 8 weeks have a reduction in the synaptic transmission. A second lead exposure for 8 weeks potentiates this change even more, with a synaptic loss being even stronger at 28 weeks of age.

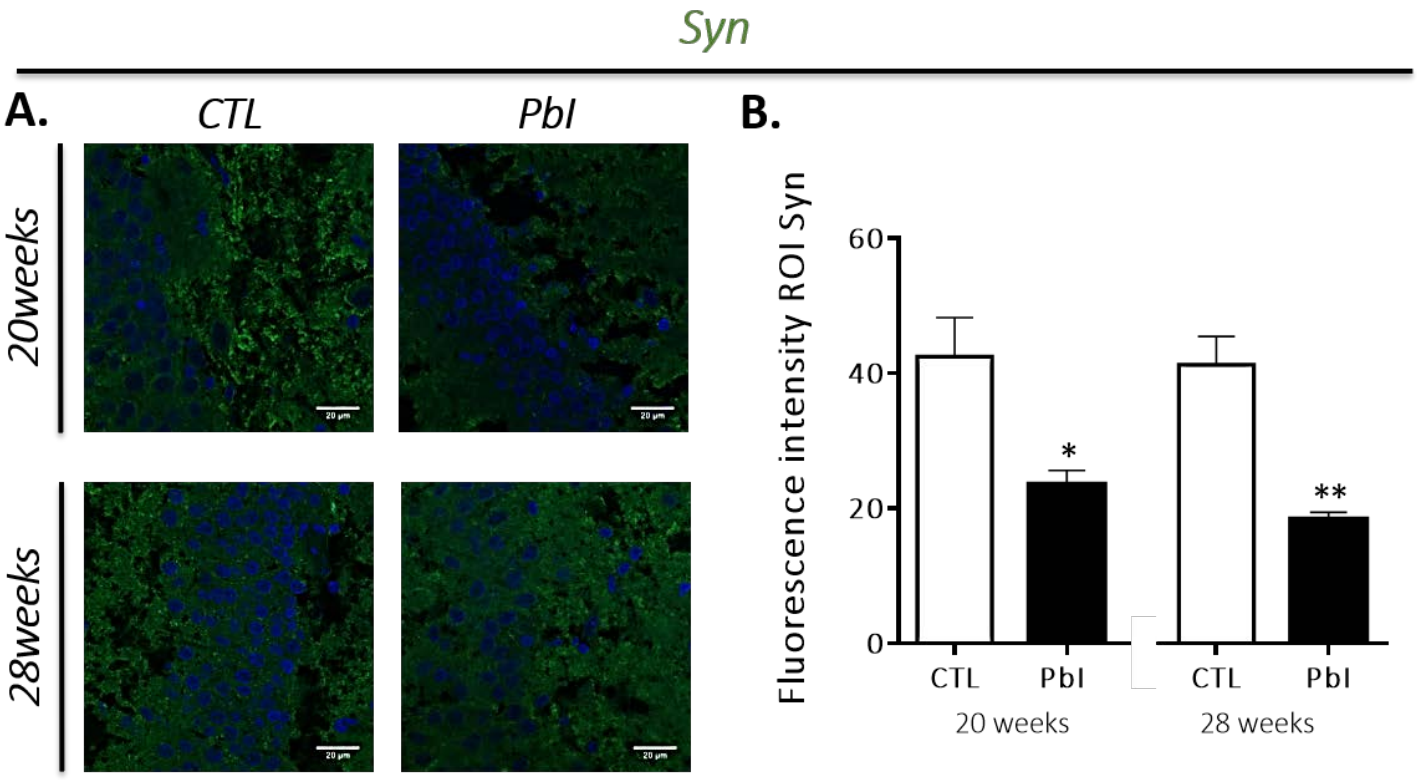

Figure 42 – *Detection and quantification of Syn in DG hippocampal area of PbI and CTL protocols at 12, 20 and 28 weeks*
Confocal images (A) and quantitative analysis data (B) are represented.
Scale bar is 20µm for staining images. Values are mean ± SEM; *p < 0.05; **p < 0.01.

Astrogliosis

Data from ***GFAP*** staining (see ***Figure 31*** and ***Table 22*** for values) showed that the first, early-life lead exposure primarily affects the astrocytic cells, even if a period of lead abstinence was present. A second exposure to lead does not potentiate the changes already present in these animals.

The astrocytic cells are in the activated state within the hippocampus (astrocytes marked with GFAP staining are denser, showing hypertrophic branches and an upregulation for GFAP staining), that is reminiscent of chronic neuroinflammatory mechanisms and alterations in the tripartite synaptic processes for neuronal communication, changes that persist after lead-free period and after a second exposure (from 20 to 28 weeks of age).

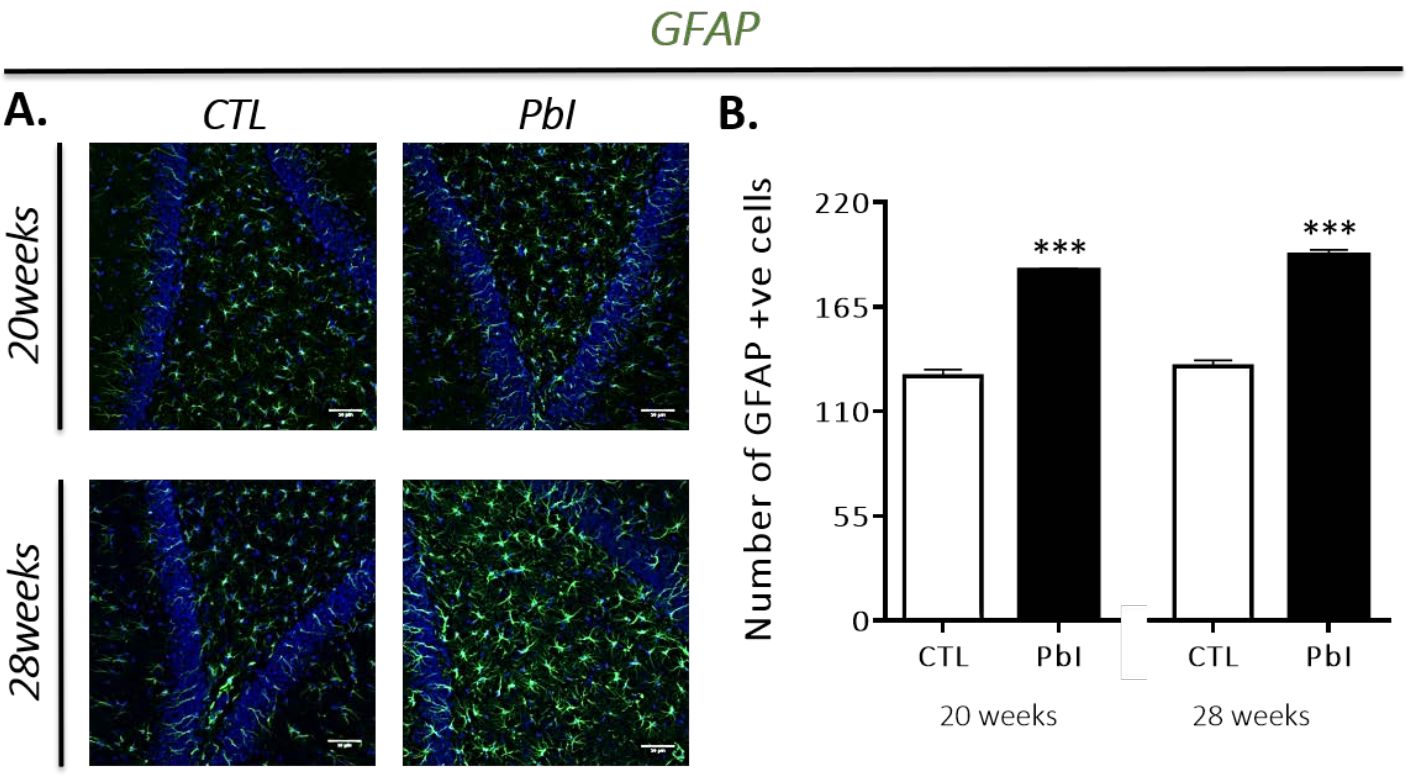

Figure 43 – *Detection and quantification of GFAP in DG hippocampal area of PbI and CTL protocols at 12, 20 and 28 weeks*
Confocal images (A) and quantitative analysis data (B) are represented.
Scale bar is 50µm for staining images. Values are mean ± SEM; ***p < 0.001.

Microgliosis

Data from *Iba-1* staining (see *Figure 44* and *Table 31* for values) showed no visible alterations at the morphological level of the microglial cells, with microglia being in ramified state, small cell bodies and numerous long branching processes at 20 weeks of age. However, after the second exposure, microglial cells became reactive, with loss of branches and upregulation for Iba-1.

Also, an increased number of these cells at both 20 and 28 weeks was observed, which is evocative of the microglia activation, becoming reactive, promoting long-lasting pro-neuroinflammatory processes that contribute to central nervous system protection. The period of lead abstinence of 8 weeks was not sufficient for microglial number to recover to normality and the second period potentiated the increase of these cells (evaluation at 28 weeks), which is evocative of microglial activation.

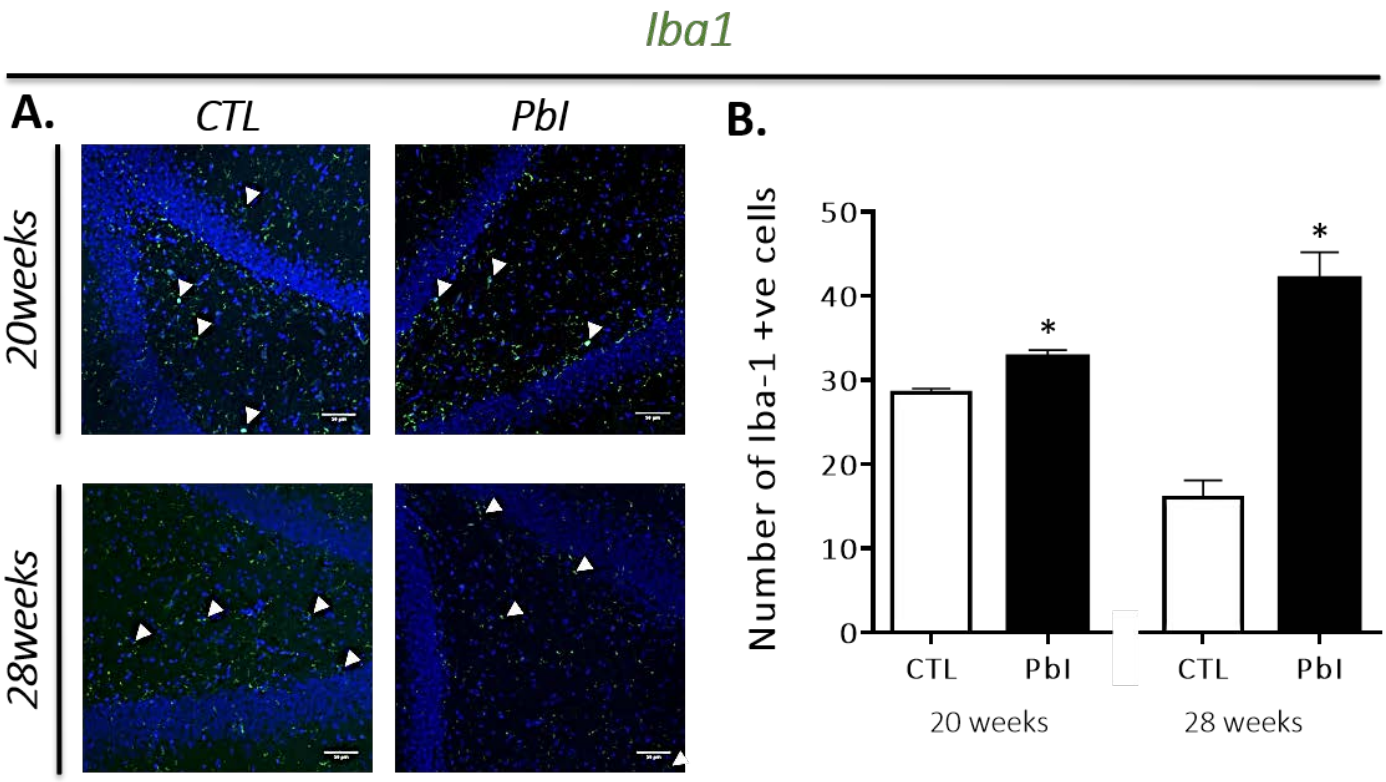

Figure 44 – *Detection and quantification of Iba-1 in DG hippocampal area of PbI and CTL protocols at 12, 20 and 28 weeks*
Confocal images (A) and quantitative analysis data (B) are represented.
Scale bar is 50µm for staining images. Values are mean ± SEM; *p < 0.05.

Table 31 – *Values of NeuN, Syn, GFAP and Iba-1 stainings quantification in DG hippocampal area of PbI and CTL groups at 12, 20 and 28 weeks*
Values are presented as mean ± SEM. n=3/group; *p < 0.05; **p < 0.01; ***p < 0.001.

Group	Age	Number of *NeuN* positive cells	*Syn* staining fluorescence intensity	Number of *GFAP* positive cells	Number of *Iba-1* positive cells
CTL	20 weeks	634 ± 49.7	42 ± 5.8	129 ± 3.5	29 ± 0.5
PbI		486 ± 87.8	23 ± 2.1*	184 ± 1.2***	33 ± 0.9*
CTL	28 weeks	702 ± 122.8	41 ± 4.2	134 ± 3.2	16 ± 2.1
PbI		540 ± 53	18 ± 1.1**	192 ± 3.1***	42 ± 3.2**

4.4 COMPARISON BETWEEN LONG-TERM (PBP), SHORT-TERM (PBS) AND INTERMITTENT (PBI) LEAD EXPOSURE PROFILES AT 28 WEEKS OF AGE

After the physiological, behavioural and immunohistochemistry characterization of all lead exposure groups in three different timepoints (12, 20 and 28 weeks of age) and its comparison with a matching control group, a comparison between all these three developed lead exposure profiles was performed. In summary, the long-term lead exposure (PbP) was exposed from foetal period to 28 weeks of age through animal life, the short-term lead exposure (PbS) was exposed to a single lead exposure from foetal period until 12 weeks of age with no adult exposure until 28 weeks and, finally the intermittent lead exposure (PbI) suffered a double exposure, from foetal period until 12 weeks of age and an adult exposure from 20 to 28 weeks of age.

Basal physiological differences between lead exposed groups

Long-term lead exposure causes the highest increase in blood pressure, while the intermittent lead exposure causes the mildest blood pressure increase, without significant differences between heart rate and respiratory frequency values

Some significant differences were reported regarding blood pressure values (***Figure 45***) between PbS (n=10) PbI (n=10) and PbP (n=10) groups (*A*). Concerning systolic blood pressure (sBP), only a significant difference was reported between PbI and PbP groups (**p < 0.01). Regarding the diastolic blood pressure (dBP), significant differences were reported between PbS and PbP groups (***p < 0.001) and PbI and PbP groups (****P < 0.0001). Also, significant differences were reported concerning the mean blood pressure (mBP) between PbS and PbP groups (**P <0.01) and PbI and PbP groups (****p < 0.0001). No significant differences between groups were observed between groups regarding both, heart rate (*B*) and respiratory frequency (*C*) (p > 0.05).

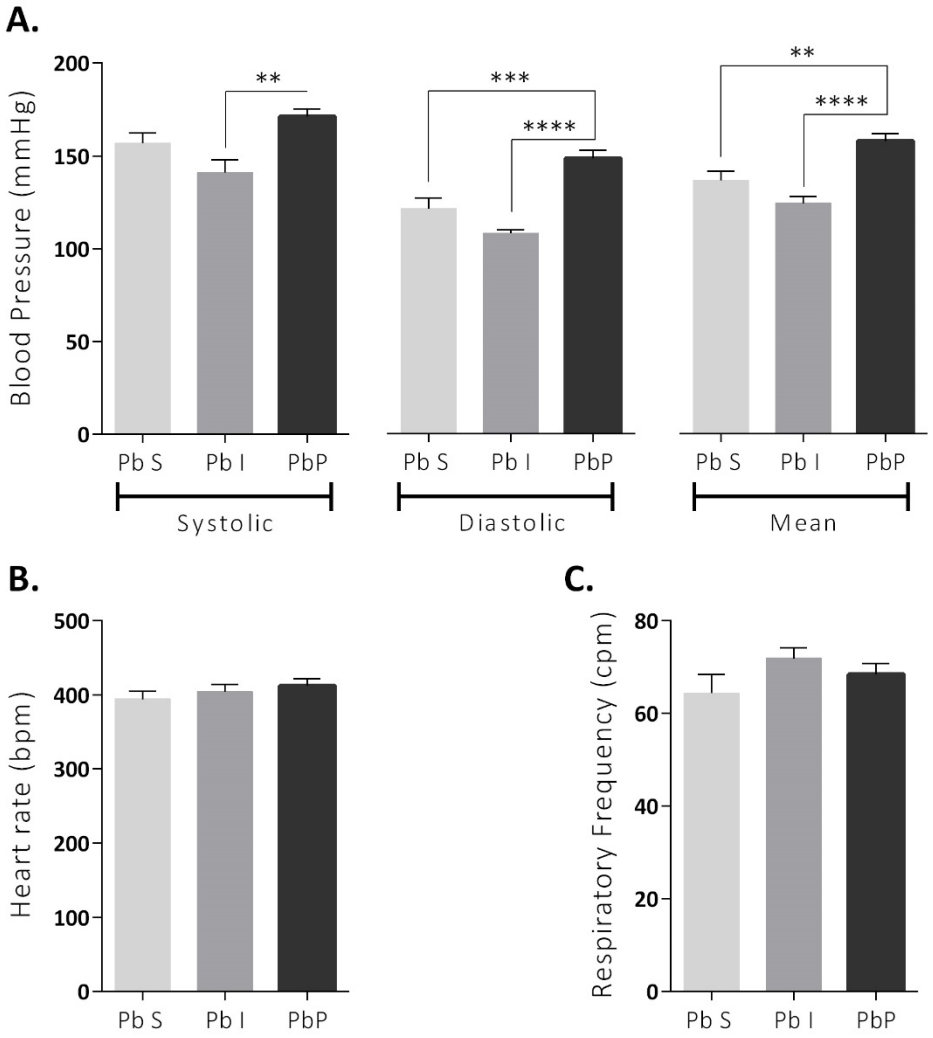

Figure 45 - *Physiological parameters in PbS, PbI and PbP groups*
Significant differences were reported in blood pressure values between groups (A), However, no significant changes were reported in heart rate (B) and respiratory frequency (C). Values are mean ± SEM; **p < 0.01; ***p < 0.001; ****p < 0.0001.

Baroreceptor and chemoreceptor reflexes differences between lead exposed groups

Significant impairment of baroreflex was caused by permanent and intermittent lead exposures without changes in the chemoreflex sensitivity

The baroreflex gain (A) was significant different between the PbS group and PbI group (PbS vs PbI - *p < 0.05) and the PbS and PbP groups (PbS vs PbP - *P<0.05), without significant differences between PbI and PbP groups (p > 0.05) (n=10/group, **p < 0.01).

As for the chemoreflex sensitivity (*B*), no significant differences were reported between the three groups (n=10/group, p > 0.05). Data shown in ***Figure 46.***

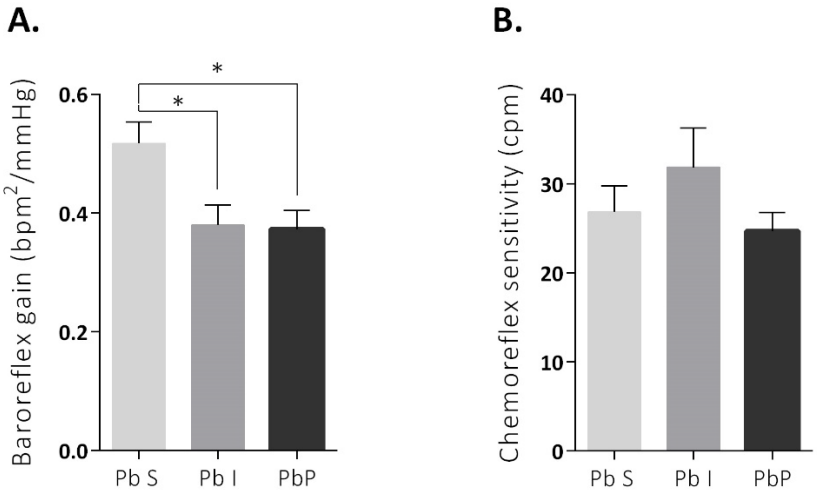

Figure 46 – *Baroreceptor gain and chemoreflex sensitivity evaluation in PbS, PbI and PbP groups at 28 weeks*
Significant differences were reported between groups in the baroreflex gain (A). No significant differences were observed in the chemoreflex sensitivity (B). Values are mean ± SEM; *p < 0.05.

Autonomic output differences between lead exposed groups

Permanent lead exposure causes the strongest sympathoexcitation without any changes in the parasympathetic tone

A significant difference was observed in LF parameter (*A*) by the PbP group, when compared to the PbS animals (n=10/group; *p < 0.05). No significant differences were reported in the HF (*B*) and LF/HF (*C*) parameters (p > 0.05), data shown in *Figure 47*.

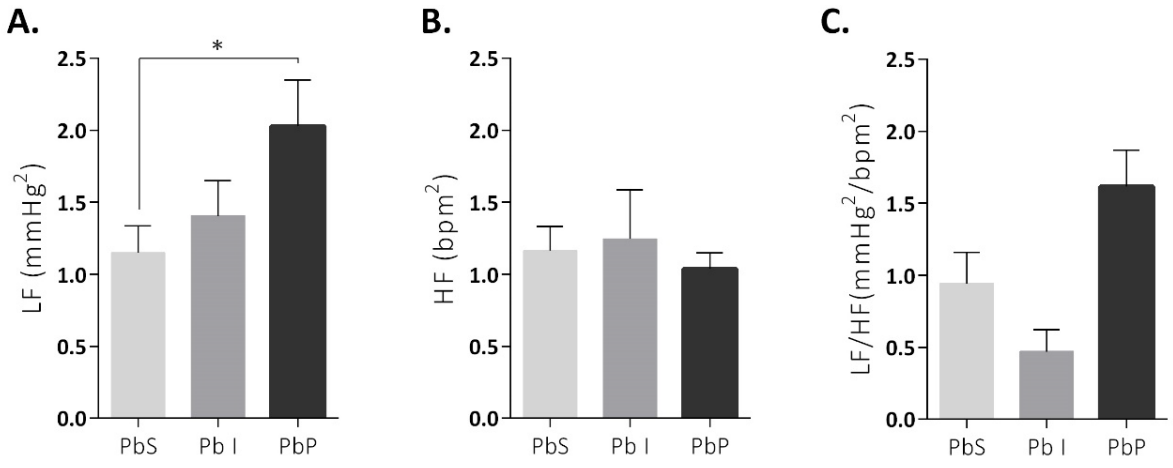

Figure 47 *- Autonomic function evaluation of PbS, PbI and PbP groups at 28 weeks*
Low frequency band– LF (A), high frequency band - HF (B) and LF/HF index (C) are presented. A significant difference was observed in LF band in the PbP group when compared to the PbS group. No other significant modifications between groups were observed in the LF and HF bands and LF/HF index; *p < 0.05.

Behavioural differences between lead exposed groups

Permanent lead exposure leads to a strong anxiety levels without changing the locomotor and exploratory activity

In EPM test (***Figure 48***) and concerning the percentage of presence time (*A*), only a significant difference between PbS and PbP groups was observed regarding the percentage of time in the open arms (n=10/group; *p < 0.05). No significant differences were depicted in the percentage of time spent in closed arms and, also in the number of entries (*B*), either in the open and closed arms, between the three groups (P < 0.05).

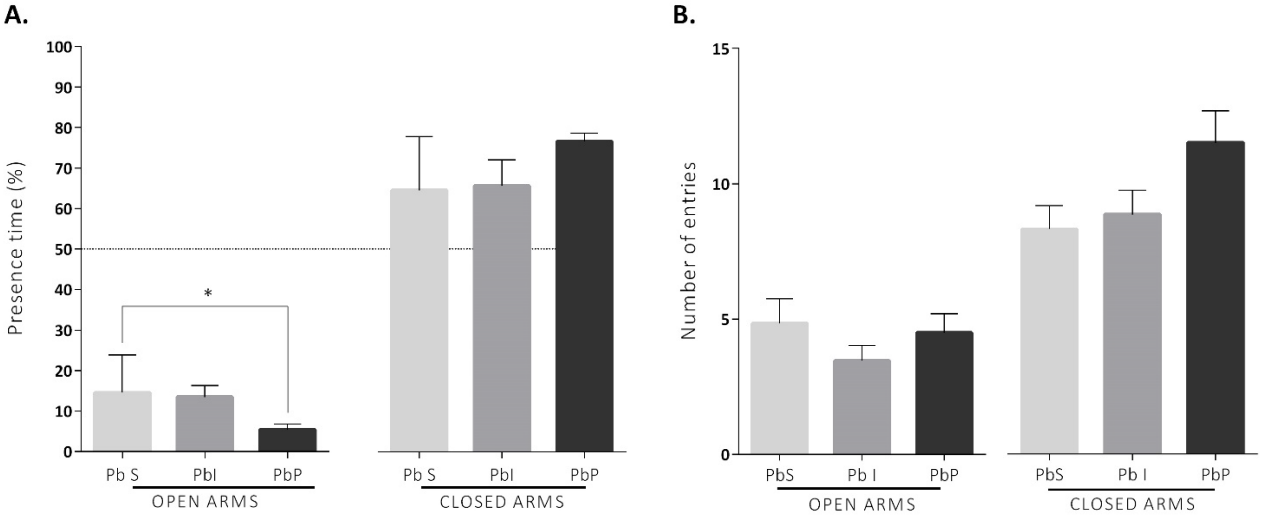

Figure 48 – *Anxiety behaviour assessment by EPM test in PbS, PbI and PbP groups at 28 weeks*
A significant decrease of percentage of time was reported between PbS and PbP group (A) without any differences in the percentage of time in closed arms and number of entries in both, open and closed arms (B).
Values are mean ± SEM; *p < 0.05.

The open field test performance of PbS, PbI and PbP groups is presented in ***Figure 49***. Regarding the total number of entries (*B)*, the significant differences between PbP and PbS group (n=10/group; ***p < 0.0001) and PbP and PbI group (****p < 0.0001). No significant differences were observed in the presence time in the centre parameter (*A*) between groups. Also, no significant differences were reported in the total travelled distance (*C*) and average velocity (*D*) between the three groups. (NS p > 0.05).

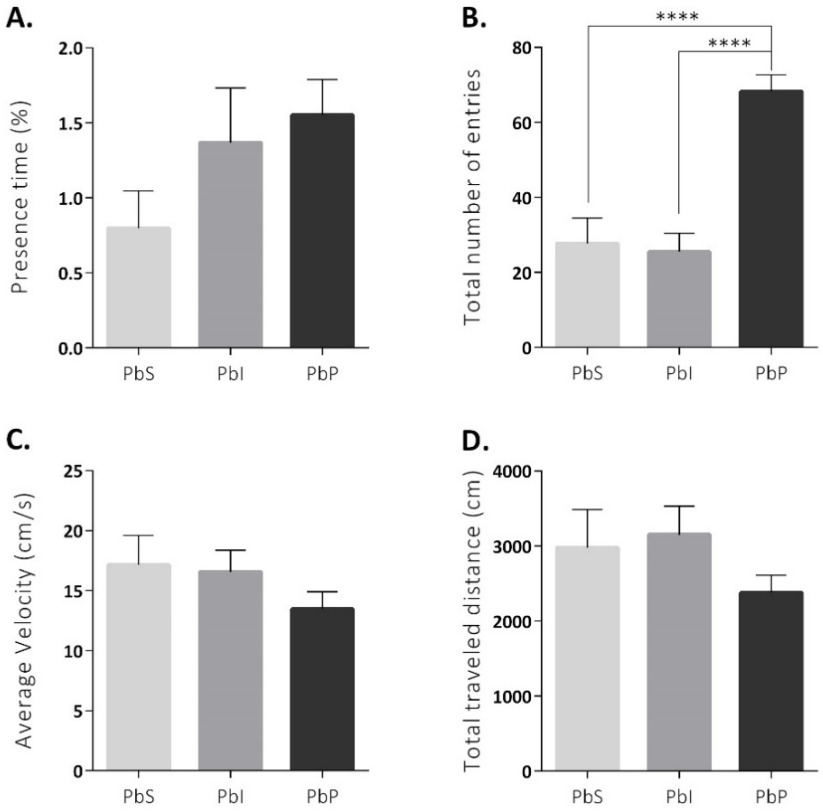

Figure 49 – *Locomotor and exploratory activity assessment by OFT of PbS, PbI and PbP groups at 28 weeks*
Significant differences were found between PbP group and PbS and PbI groups (A). No changes were observed in the total travelled distance (B) and average velocity (C) between groups. Values are mean ± SEM; ****p < 0.0001.

Memory differences between lead exposed groups

Lead exposure profiles caused no changes in the working and episodic long-term memory

Spatial working learning and memory assessment performance is presented in ***Figure 50***. Regarding the percentage of spontaneous alternation *(A)* and total number of entries (*B*) calculated from Y-Maze test, no significant differences between PbS, PbI and PbP groups were reported (n=10/group; NS p < 0.05).

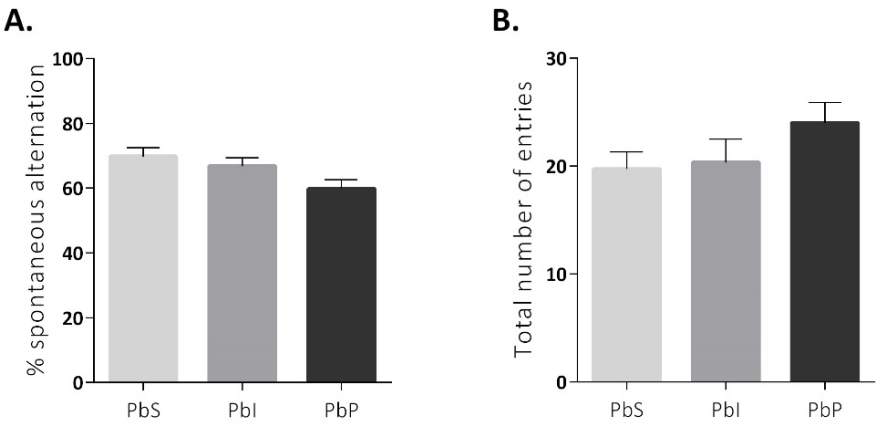

Figure 50 - *Performance in Y-Maze test by PbS, PbI and PbP groups at 28 weeks*
There are no statistical differences between the lead exposed groups in % of spontaneous alternations (A) and total number of entries (B). Values are mean ± SEM.

Episodic long-term memory assessment by NOR test performance of PbS, PbI and PbP groups at 28 weeks are shown in *Figure 51*. No significant differences were found between groups in the novelty recognition index calculated from the exploration time percentage data in the testing day (n=10/group; NS $p > 0.05$) as all groups show a similar pattern of exploration of the objects.

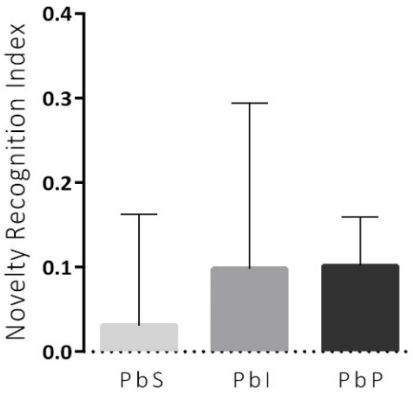

Figure 51 - *NOR test performance results in different phases of the test of the PbS, PbI and PbP groups at 28 weeks of age*
No significant differences between the three groups were found in the novelty recognition index (C) calculated from data in the testing day. Values are mean ± SEM.

Neuronal, synaptic and glial differences between lead exposed groups

Intermittent lead exposure causes loss of synapses, the most prominent reactive microgliosis without differentiating in the neuronal number and astrogliosis

Figure 52 depicts differences in the immunohistochemistry data between PbP, PbS and PbI exposure protocols. NeuN stained tissues (*A*) show no significant differences between the three lead exposed groups, PbS, PbI and PbP (n=3/group; NS $p > 0.05$)). A significant decrease in the Syn staining (*B*) fluorescence intensity quantification was shown by the PbI group when compared to PbS (**$p < 0.01$) and PbP group (**$p < 0.01$). The comparison of quantification of GFAP (*C*) and Iba-1 (*D*) showed that no significant differences were reported between groups in the number of astrocytes (NS $p > 0.05$) and a significant increase by the PbI group was reported when compared to the PbS group (*$p < 0.05$).

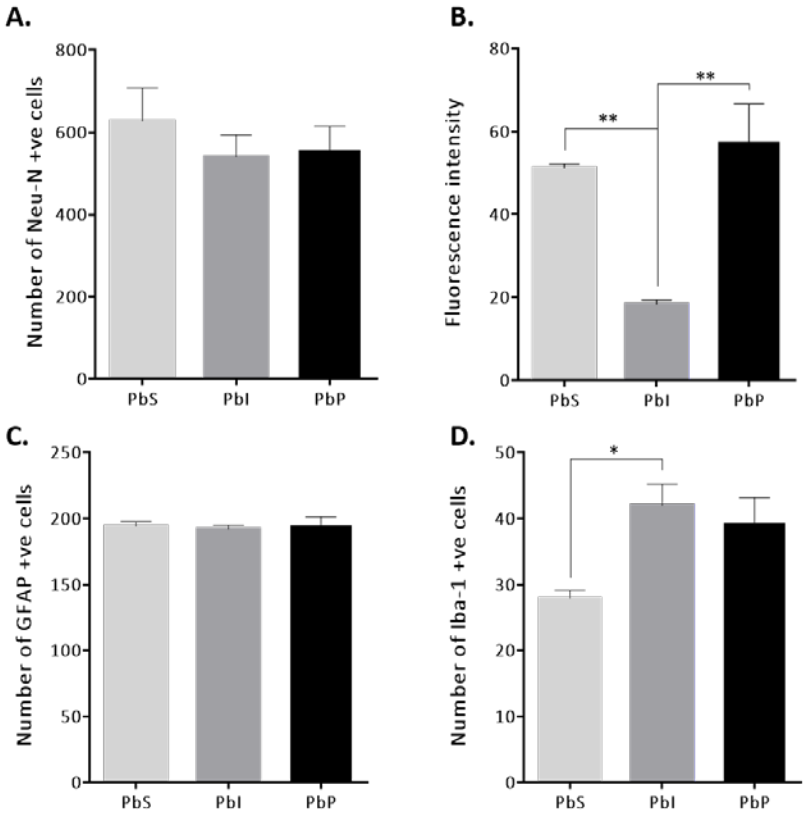

Figure 52 - *Quantification of NeuN, Syn, GFAP and Iba-1 in DG hippocampal area of PbS, PbI and PbP groups at 28 weeks.* No differences were reported in NeuN positive cells between PbI and CTL groups (A). Significant decrease between PbI group and PbS and PbP groups were observed in the Syn staining quantification (B). The number of GFAP positive cells (C) showed to be similar between the three groups, however, an increase in the PbI group, when compared to the PbS group was reported in the number of Iba-1 positive (D). Values are mean ± SEM; *$p < 0.05$; **$p < 0.01$.

5 DISCUSSION

The current comparative study provides additional insight into the association between the physiological dynamics and lead intoxication evoked by different profiles of lead exposure.

First, independently of the type of lead exposure profile, the current study reveals a clear association between lead exposure, hypertension and cardio-respiratory reflex impairment. And, even after a lead free-period, only the sympathetic nervous system activity and the baroreflex function were re-established to physiological conditions. This pattern of association is similar with other clinical situations, such as hypertension, acute heart ischemia or heart failure[46,119–121].

Second, we have showed an involvement of sympathetic nervous system in modulation of the baroreceptor reflexes responses or hypertension development on lead intoxication. In fact, in our study, the overactivity of the sympathetic nervous system is concomitant with baroreceptor reflex impairment and/or hypertension. This exaggerated sympathetic tone associated with mild hypertension, observed in three different lead exposure profiles, can result from an increased intrinsic activity of the brain stem vasomotor neurons or can be secondary to a reduction of the inhibitory potency of baroreceptors [122,123].

Third, all groups exposed to lead, evaluated in different time points, had behavioural changes, namely anxiety, hyperactivity and/or long-term impairment memory. This can be, in part, explained by the immunohistochemistry studies from the present study, that demonstrated that independently of lead exposure profile, lead-induced reactive astrogliosis and microgliosis in dentate gyrus of hippocampus of rats.

Finally, in our model of intermittent exposure we demonstrated, for the first time, that an intermittent lead exposure causes adverse health effects, i.e, hypertension, sympathetic overactivity, increased chemoreflex sensitivity and baroreflex impairment, similar to a long-term exposure, however less pronounced.

Therefore, exposures to lead during the developmental phase can alter the normal course of development, with lifelong health consequences.

I. Effects of different lead exposures on physiological parameters

Increased blood pressure is synonymous of hypertension, which is one of the main causes of mortality and morbidity. It has been broadly defined that low-level lead exposure is one of the causes of hypertension in humans, and it has already been reproduced in various animal models of chronic lead exposure[39,40,42,72].

In the present study, all lead exposed rats showed an increased *arterial blood pressure* (≈ 15 mmHg). In PbP group evaluated at 12, 20 and 28 weeks the average increase in systolic blood pressure was 37, 32, 51 mmHg and in diastolic blood pressure was 38, 51and 57 mmHg, respectively.

In PbS and PbI groups evaluated at 20 weeks and in PbS group evaluated at 28 weeks it was an average increase in systolic blood pressure (66 and 36 mmHg, respectively) and in diastolic blood pressure (45 and 30 mmHg, respectively). Thus, during the 16 weeks period without lead exposure, a clearance of lead occurred with repercussions on blood pressure values. However, the effect on blood pressure values was similar to the PbS and PbI groups that only had 8 weeks without lead exposure. It remains to be determined if a clearance for a longer time can render these animal models' normotensive.

However, we can notice that, at 28 weeks, PbI group, the new animal model of lead exposure developed, has a less pronounced hypertension when compared to PbP group, which might suggest that the duration of Pb exposure is more relevant than the time of exposure. In PbI group, the average increase in systolic blood pressure was 21 mmHg and in diastolic blood pressure was 18 mmHg.

It is interesting that the effect on diastolic blood pressure produced by lead exposure was more evident than that of systolic blood pressure. The same pronounced effect on diastolic blood pressure was observed in men exposed to lead[124]. This can be explained by the fact that lead has a greater effect on the vascular smooth muscle, which contributes for peripheral resistance. Actuality, lead exposure affects the vascular response to vasoactive agents, induces endothelial damage, reducing the NO bioavailability and increasing endothelin levels[125,126] .

The increase in blood pressure reported here has been well documented in previous studies using rodent models of chronic low levels of lead exposure[40,42]. An increase in blood pressure of rats occurs at low lead exposure levels of approximately 0.1 to 100 ppm, which is similar to the exposure levels seen in the environment. Similar observations were obtained in a population-based study, showing that blood lead levels as low as 20–30 μg/L increase blood pressure, even after adjustment for potential confounders such as age, sex, smoking habits, alcohol intake, waist circumference, and educational level[127]. Moreover, while low to moderate lead level exposures (BLLs <30 μg/dL) show only

a low degree of association with hypertension, higher exposures (primarily seen in occupational settings) increase the risk for hypertensive heart disease and cerebrovascular disease as latent effects[128].

Although the pathogenesis of hypertension promoted by lead is multifactorial, the mechanisms for lead-induced hypertension have been extensively examined. The involvement of renin-angiotensin and sympathetic nervous systems[44,129], oxidative stress[130], circulating catecholamine levels[131], beta-adrenergic receptors[42], Na^+/K^+ ATPase[39], and endothelial factors[126] as well as renal dysfunction[132] have been implicated in lead-induced hypertension.

Despite the recorded hypertension evoked by different profiles of lead exposure, these animals did not have significant changes in ***heart rate*** when compared to controls. This leads us to conclude that neither early nor lifelong postnatal Pb exposure, at these doses, affect heart rate in adulthood. In contrast, others have shown that developmental exposure to other toxicants, such as manganese or mercury, can increase heart rate[133,134]. Studies in humans that evaluated heart rate showed inconsistent findings[40].

Regarding ***respiratory frequency***, only the intermittent exposure (PbI group), evaluated at 28 weeks) and the long-term exposure group (PbP) whose evaluation was performed at 12 and 28 weeks had an increase in respiratory frequency. This increase was concomitant with a higher chemoreceptor reflex sensitivity, indicating that lead triggered an overall alert-like reaction which could contribute not only to a higher respiratory rate, but also to blood pressure increase.

In occupational lead exposure, it was established that workers have higher frequencies of respiratory symptoms, higher serum and urine lead concentration, but lower pulmonary function tests values[135].

II. Effects of different lead exposures on cardio-respiratory reflexes

All lead exposed groups had a higher ***chemoreceptor reflex sensitivity***. However, it seems that PbP groups had a tendency of lower chemoreflex sensitivity that may be related to an already installed remodelling process due to a persistent stimulation which is not the case in the intermittent exposure (PbI). The PbS at 20 and 28 weeks and the PbI at 20 weeks, despite a lead-free period, didn't show significant improvements in chemoreflex function.

The permanent increased sensitivity to chemoreceptor reflex suggests the involvement of a protective sympathoexcitatory reflex in the maintenance of oxygen homeostasis but mostly appears to be an important piece of the internal defence mechanisms to manage the progression of lead toxicity[136–138].

Moreover, the observed chemoreflex facilitation was accompanied by the impairment of ***baroreflex function***, a specific component of the defence reaction[120,139,140], suggesting that lead toxicity may impair central autonomic areas leading to a higher sympathetic tone. The exception was PbS group that was exposed from foetal period until 12 weeks of age and then had 16 weeks period without lead exposure. Even the PbS and PbI groups that had 8 weeks period without lead exposure showed chemoreflex facilitation and baroreflex impairment. Thus, only a higher period without lead exposure is capable to improve baroreflex function, without significant changes in chemoreflex function. However, it is uncertain if a longer period (>16 weeks) without exposure could reverse the chemoreflex hypersensitivity.

Therefore, our autonomic reflexes data indicate that lead exposure from foetal period, independently of the duration, induces a permanent chemoreceptor dysfunction and a temporary baroreceptor impairment that can be partially responsible for high blood pressure values. This dysfunction could have been evoked by the foetal exposure to lead, resulting in impairment of nervous system development.

In fact, lead primarily affects the central nervous system, particularly the developing brain. During pregnancy, lead stored in bone is released into blood and, since lead has the ability to pass through the blood-brain barrier, it becomes a source of exposure to the developing foetus[24,32]. Additionally, children are at a greater risk than adults of suffering from the neurotoxic effects of lead, because they absorb 4 to 5 times as much ingested lead as adults from a given source[1,24,141]. This can result in lasting cognitive impairment , that has been already described previously in several prospective epidemiological studies[32,142].

III. Effects of different lead exposures on autonomic nervous system

To clarify the mechanisms underlying this autonomic reflex dysfunction, data from autonomic tone evaluation was essential. In fact, our data showed that the PbP exposed group have a lasting sympathoexcitation, which is in accordance with previous studies that indicated central and peripheral sympathetic hyperactivity after chronic lead exposure[44,45]. A study in 9-11-year-old children with low blood Pb levels (≤ 3.76 μg/dL) also found increased sympathetic nervous system activity during rest and, paradoxically, a depressed sympathetic response during the stressful computer task[143].

In fact, the PbP exposure, by stimulating the carotid chemoreflexes, induces sensory long-term facilitation, and drives an elevated sympathetic tone from the hindbrain leading to hypertension, and despite the 8-week period without lead exposure, the sympathetic activity in the PbS and PbI groups evaluated at 20 weeks does not diminish significantly. On the other hand, as shown by the results of the PbS group, a lead-free period of 16 weeks is sufficient for the reestablishment of the sympathetic nervous system activity, similar to the physiological condition.

However, we can notice that, at 28 weeks, PbI group has a less pronounced sympathoexcitation when compared to PbP group, which might suggest that the duration of Pb exposure is more relevant than the time of exposure.

The autonomic data from the present study indicates that the impaired autonomic regulation likely contributed to the baro and chemoreflex dysfunction observed in these Pb groups. Other studies already described that chronic lead exposure induces sympathoexcitation by peripheral and central stimulation, increasing the activity of sympathetic receptors (α2 and β1) and promoting increased plasma concentration of adrenaline and noradrenaline[44].

Sympathetic nervous activity is also affected by baroreceptor reflexes, as they provide a tonic inhibitory influence, controlling peripheral vasoconstriction and cardiac output[144]. Therefore, our data show that lead exposure, independently of the duration, induces a baroreceptor dysfunction that could be due to chronic resetting to hypertensive state. This dysfunction could have been evoked by the foetal exposure to lead experienced by all groups, resulting in impairment of nervous system development.

Our hypothesis is that the autonomic dysfunction, seen in lead exposures, may have been mediated through central autonomic controls and the major mechanism inducing changes in cardiorespiratory reflexes would be similar to that already suggested for other pathologies, which also runs with sympathetic overexcitation, chemoreflex facilitation and baroreflex impairment[46,119,121,131,145–147]. This mechanism may involve neuronal pathways linking the hypothalamus to lower brainstem nuclei, in particular, the PVN-NTS axis[120,148,149].

IV. Different profiles of lead exposure and blood lead levels

Blood lead levels, mainly lead levels in the erythrocytes, is representative of soft tissue lead and is the primary biomarker used for the assessment of lead exposure, both for screening and diagnostic purposes and for biomonitoring body burden and absorbed doses of the metal[32]. Other currently available biomarkers, such as bone or teeth (for past exposures), faeces (for current gastrointestinal exposure), or urine (for organic lead) are sometimes more useful than blood for the assessment of lead exposure[32]. In the study presented here, blood lead concentrations in PbS at 20 and 28 weeks and PbI rats at 20 weeks were much higher than those in control rats but lower than PbP rats (5.8±0.7 and 3.7±0.4 versus 27.9±2.3 and 20.9±10.7 μg/dL, respectively). Despite the lower blood lead levels in PbI at 20 weeks and PbS at 20 and 28 weeks, caused by a lead-free period of 8 and 16 weeks, an elevation of blood pressure produced by these profiles of exposure was confirmed by the present investigation.

Regarding the intermittent model of lead exposure (PbI), the blood lead levels, evaluated at 28 weeks, were similar to those obtained in the group permanently exposed to lead, which might suggest that, in this type of lead exposure assessment, the time is more relevant than the duration of Pb exposure.

Studies in humans, also demonstrated that immigrant women and children had elevated blood lead levels[150,151]. In refugee children, the levels were almost 14 times higher than that of US children aged 1–5 years based on the most recent National Health and Nutrition Examination Survey (NHANES) data. For that reason, the American Academy of Pediatrics recommends that children who have been adopted or emigrated from countries where lead poisoning is prevalent should be screened for elevated blood lead levels[152]. Considering the amount of lead acetate ingested, we would expect to get a higher increase in blood lead levels. The apparent reason for that is the standard chow used. This chow is rich in iron (0.18 g/Kg) that is known to decrease the susceptibility to lead toxicity, possibly through a lower lead absorption. The importance of an adequate iron diet was also demonstrated in a recent prospective study that showed that iron-deficient children in Boston aged 1–4 years were at significantly increased odds of developing lead poisoning[153].

V. Effects of different lead exposures on neurobehavioral function

Few studies have attempted to model neurobehavioral changes in young animals following low level exposure, and there is a needed to identify tests that are sensitive to the neurobehavioral changes that may occur. Mechanisms of action are not yet known; however, results have suggested that hippocampus/dentate gyrus may be uniquely vulnerable to early low-level lead exposure.

Studies in children have suggested that many neurobehavioral functions are modified by early chronic low-level lead exposure. These include memory and learning, visual attention, abstract problem-solving, cognitive set-shifting, and motor dexterity[68,154–161] .

The results of the present study clearly demonstrate that lead exposure since foetal period, independently of the age or the lead exposure profile, produced a significant long-term ***anxiety-like behaviour*** in adult rats, as indicated by elevated plus-maze (EPM) test results, together with an increase in ***hippocampal astrocytes and microglial cells***. It has already been shown that chronic lead exposure can produce behavioural disturbances, including anxiety, in human and in animal models[85,162]. Also, there is a strong evidence suggesting an association between hippocampal dysfunction and the behavioural deficiencies observed in experimental animals following neonatal Pb exposure[57,81].

In our results, we can notice that the young animals (12 weeks) were more anxious than the older ones (20 and 28 weeks). In fact, a study on the ontogeny of anxiety-like behaviour in laboratory healthy rodents has showed that adolescent rats demonstrated a higher anxiety-like response than adults[163]. The hypothesis that adolescent rats have a more noticeable response to stressors than adults is being confirmed by these results.

Moreover, in the open field test (OFT), it was observed (although it was not shown in the results) that rats exposed to lead, independently of the exposure profile, spent more time exploring the periphery of the arena, usually in contact with the walls. This tendency to remain close the walls, called *thigmotaxis*, can be also used as an index of anxiety in rats. Even though, it is a normal rodent behaviour, animals showing a more prominent *thigmotaxic* behaviour are considered to be more anxious[102].

Also, a long-term lead exposure from foetal period until 20 weeks of age induces ***hyperactivity***, showed by the increased average velocity, without changing the general locomotor activity and exploration behaviour. Studies within the past decade have indicated that certain environmental factors, including exposure to environmental pollutants can induce hyperactivity. Recent studies showed that Pb exposure may be associated with higher risk of clinically diagnosed ADHD in children, even at low levels[164–168].

Although the PbS group also presented anxiety, it was less pronounced when compared to the permanently exposed group (PbP) or to the PbS and PbI groups evaluated at 20 weeks. This leads us to conclude that a 16 weeks lead-free period can cause beneficial behavioural effects by reducing the anxiety level. It remains to be determined if a longer lead-free period can render these animal models not anxious.

As for the ***spatial memory and learning***, no impairment was depicted, regardless the time or duration of exposure, as no alterations in the Y-Maze test results were observed. Regarding ***long-term memory***, only the PbP evaluated at 12 weeks and the PbS and PbI evaluated at 20 weeks showed an impairment in NOR test performance, namely in *Novelty index,* as compared to controls. And this impairment of memory observed in the present study cannot be attributed to the locomotor activity impairment or anxiety behaviour presented by these animals, since it was similar to the matching control group. Therefore, anxiety was not a factor due to the long process of habituation to the arena of the Novel object recognition test. Moreover, anxiety and locomotor activity were also tested during the test, showing no differences between groups (data not shown in this thesis).

However, although there were no significant differences in *Novelty index* results in the other exposed groups, we can notice that these animals had a similar exploration time in the two objects in the testing day of the NOR test (the novel and the familiar object), allowing us to conclude that they don´t recognize the new object as novelty. This, because, when animals are exposed to a familiar and a novel object, they approach frequently and spend more time exploring the novel than the familiar one due to animal's normal explorative curiosity when in the presence of novelty, which was not the case in the Pb-exposed animals.

This difference in NOR test performance can be due to lead-induced impairments of the hippocampus, in part explained by the ***synaptophysin, astrocytes and microglia expression*** in the dentate gyrus region. The PbS and PbI at 20 weeks and PbI at 28 weeks of age groups had a decrease in hippocampus/ dentate gyrus region synapses. The first, had a preeminent long-term memory impairment and the second had a similar exploration time in the two objects (indicative of some level of long-term memory impairment). Moreover, a permanent exposure until 20 weeks (PbP) promotes an increase in synapses in the dentate gyrus. Thus, lead exposure disturbs synaptogenesis of dentate gyrus, which could lead to synaptic plasticity impairment in the hippocampus.

In fact, decreases and increases in the synaptic activity are an answer for synaptic strengthening or weakening over time, which has been given a name of synaptic plasticity. Synaptic plasticity in one of the main molecular processes behind learning and memory, and memories are being described by interconnected networks of synapses in the brain[30,169].

Lead exposure has been already implicated in the impairment of synaptic plasticity in the developing hippocampus, but the mechanism remains unclear[170]. A reduction in the length of dendritic field and the number of dendritic branches of hippocampal dentate granule cells in the developing hippocampus after lead exposure has been reported[171,172]. Also, other studies have reported that developmental lead exposure causes alteration of NMDAR subunit ontogeny and disruption of its downstream signalling[30,173], which are associated with deficits in hippocampal long-term potentiation (LTP)[174]. A more recent study concluded that developmental lead exposure alters synaptogenesis through inhibiting canonical Wnt pathway[170].

As the DG subregion of the hippocampus is a substrate for both cognition and mood regulation[175], it was important to evaluate whether the different exposure profiles led to morphological[48] alterations in this area. Immunohistochemistry evidence indicate that all exposed groups showed an increase in hippocampal astrocytes and microglial cells. Glial cells that englobe astrocytes and microglia in the CNS execute a variety of important functions, maintaining a complex interdependency between neurons and glia[176]. Neuronal damage[37] has been already described as one of the key elements of Pb toxicity. However, recently, neuroglia has been the focus as a target of low-level lead exposure toxicity[34].

In our study, to better understand and correlate the changes that has been reported in animal behaviour with molecular alterations within the brain, more specifically, the cognitive impairment of the animals that have been exposed to lead, we focused our attention to both, neuronal and glial changes in the dentate gyrus. Astrocytes along with endothelial cells make up the blood brain barrier (BBB). Astrocytes perform homeostatic regulatory functions, exerting a fine control of the CNS extracellular environment, as well as, are one of the key elements of tripartite synapse. Also, astrocytes are involved in the long-term potentiation, that is crucial for synaptic plasticity, learning and memory[116,118]. Astrocytes play a crucial role in the inflammation induction in the brain, interplaying with microglia, upon antigen presence[34,116]. Also, upon pathological conditions, as lead toxicity, maladaptive reactive astrogliosis[118] is triggered causing the depletion of glutamine, therefore reduction of synaptic GABA causing hyperexcitability of hippocampal neuronal circuits, due to a decrease in glutamine [55,116,118]. Glial fibrillary acidic protein (GFAP) is an astrocytic intermediate filament protein that is induced during periods of reactive astrocytic gliosis[36].

On a morphological level, reactive astrogliosis ranges from mild to prominent[116,118], the last often being accompanied by glial scarring. In this study, the morphological evaluation of astrocytes, stained with GFAP in DG showed that exposure to lead enhanced the astrocytic reactivity, more specifically, a persistent maladaptive as GFAP immunoreaction was greatly enhanced within individual

cells, the density of the cells was much higher within the area and the branching processes of the cells were hypertrophic. To corroborate the morphological evaluation, a quantification of number of astrocytes within the area was performed as it has been already described that not only morphological changes, but also a number of astrocytes is increased upon lead exposure[36,48].

Our results showed that the developmental period of exposure to lead, from foetal period until 12 weeks of age, leads to a strong reactive astrogliosis that persists through adulthood in all groups of exposed animals (permanent – PbP, intermittent – PbI and single exposure – PbS). Exposure to lead during adulthood does not cause changes in the astrogliosis, which persists reactive through time, even of the absence of exposure is of 16 weeks (PbS group). These findings are consistent with other that reported[1] that the major lifelong changes in the GFAP gene expression occur during the developmental period, due to immaturity of blood-brain barrier, offering little protection and low resistance to lead toxicity because of the lack of high-affinity lead-binding protein in astrocytes that sequester lead and remove it from mitochondria[177].

Nevertheless, GFAP is the major marker of astrocytes, studies showed that it is not exclusively produced by astrocytes, but also is present, in very small quantities in oligodendrocytes, that are responsible for neuronal myelinisation. The demyelination of neurons has been already described as one of the major toxicological changes due to lead exposure[178]. Thus, the changes in the GFAP expression may reflect various glial populations that respond differently to Pb.

Microglial cells are the neuroinflammatory sensors and are usually present in the resting state in the brain. Studies have already confirmed that, microglia, morphologically, are defined as extremely motile cells with processes and branches for territorial scanning in the brain[117,179]. In the pathogen presence, microglia are activated, becoming reactive microglia, changing morphologically to an ameboid form and increasing in number of cells substance release to combat the pathogenic attack. These substances could also be damaging to the surroundings of the activated microglia, leading to oxidative stress. The migrating nature of these cells permits a rapid response to the injury in the nervous system, thus leading to the fast proliferation and phagocyting of the cells and their parts[179]. Iba1 (ionizing calcium-binding adaptor molecule 1) has been already described by various studies as being a novel protein that it is singly expressed in microglia and its upregulation is a marker of activation of these cells. This polyclonal antibody does not cross-react with neurons or astrocytes[180].

Morphological evaluation of animals at 20 weeks showed that exposure to lead until 12 weeks of age and exposure to lead until 20 weeks of age does not disrupt the microglial cells in the dentate gyrus, presenting microglial cells with a small cell bodies and branching processes for territorial

scanning. However, the results from quantification of the Iba-1 stained tissue showed that an increase in the number of resting state microglial cells was provoked by exposure to lead from foetal period until adulthood (PbP group observations). This is consistent with other results observed before that show that due to the changes in the neurogenesis, and the fact that lead is present in the brain, increase in the number of microglial cells happens[55,181]. This increase persists even after a long abstinence from lead exposure, which was observed in the PbS group of animals.

Remarkably, a different pattern of microgliosis was observed in the animals that were exposed to lead twice (PbI group, from foetal to 12 weeks of age and from 20 to 28 weeks of age). After the second exposure to lead, the microglia was activated, with branching loss and upregulation to Iba-1 staining. Also, an increase in the number of cells from 20 to 28 weeks was observed which is indicative of not only disruption of microglia but also a process of neuroinflammation in the dentate gyrus, causing cognitive and behavioural changes in animals. This activation is consistent with other results that show that neuroinflammation is one of the key features of lead toxicity[34]. Together with activation of astroglia, the intermittent exposure to lead causes the neuroinflammatory activation, delaying their promoting assistance in the neuronal differentiation and maturation in the hippocampus, a specific brain region that maintains active neurogenesis and prevents neurodegeneration in whole life.

It is important to mention that all the neurobehavioral impairments observed in the present study were not the result of nonspecific stimulation. In fact, the different lead exposures did not affect body weight gain of dams or offspring development, neither locomotor activity.

Changes in long-term memory that have been identified in the lead exposed animals are one of the main neurobehavioral disruptions that have the most devastating effects during the lifetime of the brain health. The early alterations in the hippocampus (and more specifically in the dentate gyrus) are the potential mechanisms for neural pathways formation disruptions[182] , memory dysfunction, learning impairment during development and weakened neurogenesis during adulthood and aging[183]. All these alterations have been already linked to an increase in vulnerability to cognitive decline, neurodegenerative diseases and dementia[157,184].

The neurobehavioral results presented here are not surprising, since exposure to lead began in a very critical period, the foetal period, and the primary site of Pb action is the central nervous system, where Pb exposure is associated with several neurobehavioral and psychological alterations[19,58,185,186]. During the prenatal period in mammalian species, the rapid growth of the central nervous system makes the foetus particularly vulnerable to insults[187,188]. This phenomenon is evident from the results of several independent (and prospective) human studies which indicate that maternal stress during pregnancy is associated with adverse neurodevelopmental outcomes in the child later in life, including

attention-deficit/hyperactivity disorder, autism, and anxiety disorders[188]. Also, in low-level exposure to lead during early childhood was shown to be inversely associated with neuropsychological development and neurobehavioral-cognitive performance[189].

Similar to human studies, the current research in animals exposed to different lead profiles has clearly demonstrated that behavioural changes, namely anxiety, hyperactivity and/or long-term impairment memory can be a consequence of developmental lead exposure.

Therefore, the lead-induced alterations in rat development observed here due to ingestion of lead water (the most common route of contamination in the general population) are important in terms of searching for similar developmental alterations in human beings exposed to low levels of lead.

6 CONCLUSIONS AND FUTURE PERSPECTIVES

As the result of this work, we provide the first direct experimental evidence that an intermittent lead exposure has detrimental effects on cardiorespiratory control, on anxiety, cognitive impairment and on synaptic, astrocytic and microglial functions. The current work adds to our understanding of the complex interactions between sex, level of Pb exposure and developmental window of Pb exposure on cognitive processing and mammals´ physiology.

In summary, our experiments have provided several main findings to support our hypothesis that the exposure in utero produces behavioural, functional, and structural deficits that can be irreversible as they were apparent, in this study, at adult age.

As future perspectives, we intend to evaluate the effect of different lead exposure profiles, in target organs already removed and where lead is primarily distributed, such as heart, liver and kidneys. Also, correlations between target organs lead levels, blood lead levels, lead exposure profiles and physiological and neurobehavioral parameters should be performed.

In order to evaluate the physiological changes through the time course of animal lives, telemetric sensors should be implanted for continuous haemodynamic assessment of these subjects to better understand the most relevant timepoints of lead toxicological effects in conscious animals.

Moreover, since the chemoreflex dysfunction and anxiety behaviour were maintained after a 16 week-period without lead exposure, future pharmacological studies in central areas should be tested in order to reverse these dysfunctions towards normality.

Other central brain regions should also be evaluated, specially the amygdala, hypothalamus and centres of autonomic regulation for traces of morphofunctional changes within these areas that could account for the alterations that were observed in the animal models in this study. Similarly, some other studies must be performed to assess neurogenesis and neural morphological alterations, possible myelinic loss, electrophysiological alterations and other behavioural changes that lead could be responsible for within various lead exposure protocols.

Since sex differences in the neurodevelopmental effects of early lead exposure have been reported in humans, future research should focus on health effects differences between males and females exposed to different lead exposure profiles.

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