Use of norway rats as sentinel species in a contaminated urban basin: exploring the relationship between environmental lead and cerebellar cytoarchitecture

Uso de la rata noruega como especie centinela en una cuenca urbana altamente contaminada: explorando la relación entre el plomo ambiental y la citoarquitectura cerebelosa

Scaltritti, Martin*[1]; Tripodi, Mariel A.[1]; Pandolfi, Matias[2†] ; Muschetto, Emiliano[1]; Hancke, Diego[1]; Suárez, Olga V.[1]

[1]Laboratorio de Ecología de Roedores Urbanos, DEGE - IEGEBA (UBA-CONICET). [2]Laboratorio de Neuroendocrinología y Comportamiento en Peces y Anfibios, DBB- IBBEA (UBA - CONICET).
*** martinscaltritti@gmail.com**

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Abstract. Norway rats (*Rattus norvegicus*) have been used extensively as sentinels in environmental studies of lead exposure. This metal can affect every organ in the body, mainly the nervous system. The Matanza-Riachuelo Basin (MRB) is one of the most polluted basins in the world and presents a gradient of toxic metal contamination along its length. The overall goal of this study was to determine the relationship between environmental lead concentration and changes in the cerebellar cytoarchitecture of *R. norvegicus*. Six capture sites located in the MRB were characterized with soil and water presence and nine morphological variables were measured in the cerebellum of thirty-two rats to evaluate cytological damage. The number of cells per area of the molecular layer was negatively associated with the concentration of lead in the kidney and with age. Also, a negative association was found between the nucleus-to-cytoplasmic ratio of Purkinje cells and the concentration of lead in the soil of the study sites. The results obtained in this study show that lead contamination in the MRB could produce changes in the cytoarchitecture of the cerebellum of the rats living there. Despite the inherent limitations of studying wild rats, this work sheds light on how concentrations of lead lower than those used in experimental works can still generate effects on cerebellar cytoarchitecture.

Key words: Norway rat; Heavy metals; Pollution; Cytoarchitecture; Ecotoxicology.

Resumen. La rata noruega (*Rattus norvegicus*) ha sido utilizada extensivamente como especie centinela en los estudios medioambientales sobre exposición al plomo. Este metal puede afectar a todos los órganos del cuerpo, principalmente al sistema nervioso. La Cuenca Matanza-Riachuelo (CMR) es una de las cuencas más contaminadas del mundo y presenta un gradiente de contaminación por metales tóxicos a lo largo de su extensión. El objetivo general de este estudio fue determinar la relación entre la concentración ambiental de plomo y los cambios en la citoarquitectura cerebelosa de *R. norvegicus*. Se caracterizaron seis puntos de captura situados en la CMR con presencia de plomo en suelo y agua y se midieron nueve variables morfológicas en el cerebelo de treinta y dos ratas para evaluar el daño citológico. El número de células por área de la capa molecular se asoció negativamente con la concentración de plomo en el riñón y con la edad. Asimismo, se encontró una asociación negativa entre la relación núcleo-citoplasma de las células de Purkinje y la concentración de plomo en el suelo de los lugares de estudio. Los resultados obtenidos en este estudio muestran que la contaminación por plomo en la CMR podría producir cambios en la citoarquitectura del cerebelo de las ratas que allí viven. Teniendo en cuenta las limitaciones inherentes al estudio de ratas salvajes, este trabajo arroja luz sobre cómo concentraciones de plomo inferiores a las utilizadas en trabajos experimentales pueden aún generar efectos sobre la citoarquitectura cerebelosa.

Palabras clave: Rata noruega; Metales pesados; Contaminación; Citoarquitectura; Ecotoxicología.

INTRODUCTION

Lead is one of the most abundant and toxic metals on earth, affecting practically all organs and systems of the human body being unable to be transformed to harmless forms (Sanín *et al.* 1998). We have been using Pb for centuries in multiple applications such as pigments, makeup, batteries, fuel additives, glass, electronic components, paints, etc. (White *et al.* 2007). Lead is a key metal pollutant introduced by human activities into soil. Although lead use has been drastically reduced worldwide in the last decades, we still have significant morbidity (Rezaee *et al.* 2022) and mortality, causing more than one million deaths in 2017 (Meyer *et al.* 2008; WHO 2018; IHME 2019). Lead poisoning is still a serious problem in the USA, where blood Pb levels ≥5 µg/dL and ≥10 µg/dL, in children, increases in a stepwise fashion for poor neighborhoods with housing built pre-1950 (Hauptman *et al.* 2023).
Lead seriously affect the nervous system (Clasen *et al.* 1974; Holtzman *et al.* 1987; Florea and Büsselberg 2006; White *et al.* 2007; Naqi 2015; Nesta *et al.* 2016; Balza *et al.* 2022; Hauptman *et al.* 2023) causing ataxia, impaired neurological development, and fetal growth, learning difficulties, attention disorders, behavioral issues, coma, seizures, and hyperirritability (ATSDR 2007; Balza *et al.* 2022). In the nervous system, one of the regions with great susceptibility to the toxic effects of lead is the cerebellum (Fonnum and Lock 2000), where the reduction of cell density and alterations in cell shape and size in its layers were observed in experimental studies with high doses of lead (Mcconnell *et al.* 1979; Lorton and Anderson 1986; Patrick and Anderson 2000; Sidhu and Nehru 2004; Naqi 2015). Sidhu and Nehru (2004) emphasized various factors related to lead toxicity which can affect the normal cytoarchitecture of the cerebellum, causing disturbances in its normal functions. Among these factors are mentioned the integrity of the blood-brain barrier, the role of lead-binding proteins, the presence of many cellular scavengers, the disturbance of redox enzyme systems such as glutathione and the induction of stress and oxidative damage (Adonaylo and Oteiza 1999), all detrimental to cell survival (Zelikoff *et al.* 1988).
The Matanza-Riachuelo Basin (MRB), Argentina, is one of the most polluted basins in the world (Bernhardt and Gysi 2013) and metals, like lead, represent the most common heavy metal pollutants (Ronco *et al.* 2008; Mendoza *et al.* 2015). This basin is one of the most urbanized and industrialized areas in the country with more than 16,000 settled industries, and four million residents, living in precarious conditions, with unhealthy sanitary practices, exposed to severe environmental pollution (INDEC 2010; Johnson 2015). Studies conducted by Matanza-Riachuelo Basin Authority (Autoridad de Cuenca Matanza-Riachuelo, ACUMAR) reported that residents of MRB neighborhoods are exposed to environmental lead with blood lead values above the CDC blood lead reference of 3.5 micrograms per deciliter (µg/dL) (CDC lead blood references in children, 2021).
To assess the human risk of exposure to metals, the use of animal species as sentinels is useful (Timbrell 1998; Ceruti *et al.* 2002; Sures 2003; Vidal-Martínez and Wunderlich 2017). Sentinel species serve as early warning indicators, providing advance notice of emerging environmental issues that may impact both the ecosystem and human wellbeing (O'Brien *et al.* 1993). We previously reported that rats (*Rattus norvegicus*) are a good sentinel species of lead contamination in urban ecosystems (Tripodi *et al.* 2020 a, b). We found that rats captured in the MRB were chronically exposed to lead, and they had bioaccumulation of this metal in their soft tissues (liver and kidney) and genotoxic damage, both strongly associated with environmental lead (Tripodi *et al.* 2020 a, b). Based on these results, the aim of this study was to evaluate lead-related damage in the cerebellum of *R. norvegicus* captured in the MRB.

MATERIALS AND METHODS

Study area

The MRB is located in the Province of Buenos Aires and is bordered to the south by the City of Buenos Aires, Argentina. It has an area of 2,047 km², 22% of which corresponds to urban areas and 55% to rural areas (Faggi and Breuste 2015). The most urbanized and industrialized area of the MRB is located near the mouth of the basin in the La Plata River (Mendoza *et al.* 2015). The main industries located in this area are tanneries, meat-processing plants, chemical, and metallurgical industries (Malpartida 2003). Moving away from these industrialized areas, there is a transition to a mixed urban-rural landscape (ACUMAR 2018), becoming a landscape with predominant agro-industrial activities. This urban-rural pattern is consistent with the gradient from the highest values of environmental lead in the lower basin and lower values towards the middle and high basins (Tripodi *et al.* 2021).

Rodent trapping

Thirty-two rats (*Rattus norvegicus*) (*Table 1*) were captured between July and September 2014 at 6 sites located along the MRB (sites named S1 to S6) (*Figure 1*). Rats were captured using live cage traps as described in Tripodi *et al.* (2020, b). Traps were placed

Table 1. Morphometric and lead concentration in organs and environment data from the 32 *Rattus norvegicus* specimens used in this study.

ID	Site	Sex	Age (Days)	Weight (g)	Body length (cm)	Pb Kidney (µg/g)	Pb Liver (µg/g)	Pb Water (mg/L)	Pb Soil (µg/g)
1	S1	Female	196	236	19	13.268	0.789	0.0125	404.4
2	S1	Male	104	256	19	6.041	0.01	0.003	116.0
3	S1	Female	95	288	19.5	7.831	1.449	0.0125	404.4
4	S1	Female	66	71	15	4.103	0.161	0.0465	96.9
5	S1	Female	112	271	22	6.904	0.01	0.0465	96.9
6	S1	Male	169	287	22	0.17	1.831	0.013	404.43
7	S1	Female	99	66	14.1	1.310	0.025	0.005	5.80
8	S1	Male	176	497	26	3.758	0.19	0.0035	197.5
9	S1	Female	121	148	19	1.774	0.01	0.003	116.0
10	S1	Female	188	134	15	6.686	0.436	0.0125	404.4
11	S1	Female	45	55	13.8	0.298	0.01	0.013	404.43
12	S2	Female	226	266	21	4.136	0.259	0.0125	404.4
13	S3	Male	178	233	20	37.745	0.204	0.003	116.0
14	S2	Female	43	238	22.7	7.99	0.438	0.0125	404.4
15	S3	Male	235	71	14.1	1.315	0.01	0.005	5.80
16	S2	Female	44	300	20	10.094	0.663	0.0125	404.4
17	S2	Male	28	34	11	3.121	0.041	0.008	31.3
18	S2	Male	352	111	17.1	13.748	0.161	0.0465	96.90
19	S3	Female	139	405	24	3.095	0.077	0.0035	197.5
20	S3	Female	95	92	15	1.401	0.025	0.005	5.80
21	S4	Male	178	298	23	1.655	0.01	0.003	116.0
22	S4	Male	269	282	23.4	2.798	0.738	0.0125	404.4
23	S4	Male	203	112	18.5	13.639	0.255	0.0465	96.9
24	S4	Male	202	249	21	25.123	0.465	0.0035	197.5
25	S4	Female	400	531	24.5	1967	0.19	0.005	5.80
26	S5	Male	483	270	20.5	1.600	0.107	0.0125	404.4
27	S5	Male	21	287	22	0.549	1.831	0.0125	404.4
28	S5	Male	20	35	11	0.438	0.079	0.008	31.3
29	S6	Male	15	35	11	0.157	0.079	0.005	31.25
30	S6	Male	22	301	22.4	2.687	0.098	0.0035	197.5
31	S5	Female	27	55	13.8	1.028	0.010	0.0125	404.4
32	S5	Male	29	67	13.5	1.226	0.010	0.005	5.80

in transects close to the shores of the watercourses and had been monitored every morning for four consecutive days. The animals were anesthetized with ketamine (40 mg/kg) and acepromazine (2.5 mg/kg), weighed, measured, and sexed. Age was estimated using the paired eye-lens weight of the crystalline according to the equation proposed by Hardy *et al.* (1983). Kidney and liver samples were taken from each specimen to quantify lead concentrations (*Table 1*).

Tissue preparation

Immediately after sacrifice in the capture site, the brain was removed and fixed by immersion in 4% formaldehyde for 48 hours and then preserved in 70% ethanol for further analysis. The cerebellum has a simple structure composed of three layers: molecular layer, Purkinje cell layer, and the granular layer (Fonnum and Lock 2000) (*Figure 2B*, *Figure 3*). The cerebellum

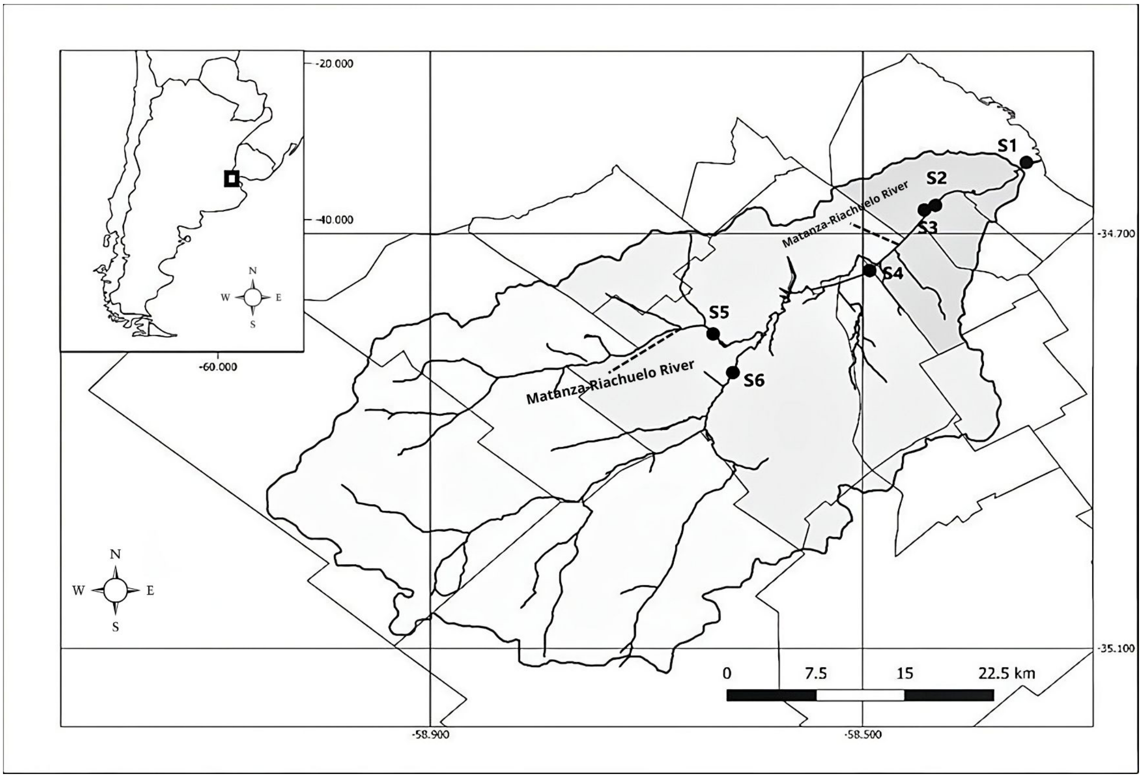

Figure 1. Location of rat's sampling sites (S1 to S6, according to Table 1) in the Matanza-Riachuelo Basin (MRB), indicating main course of Matanza-Riachuelo River (MRR).

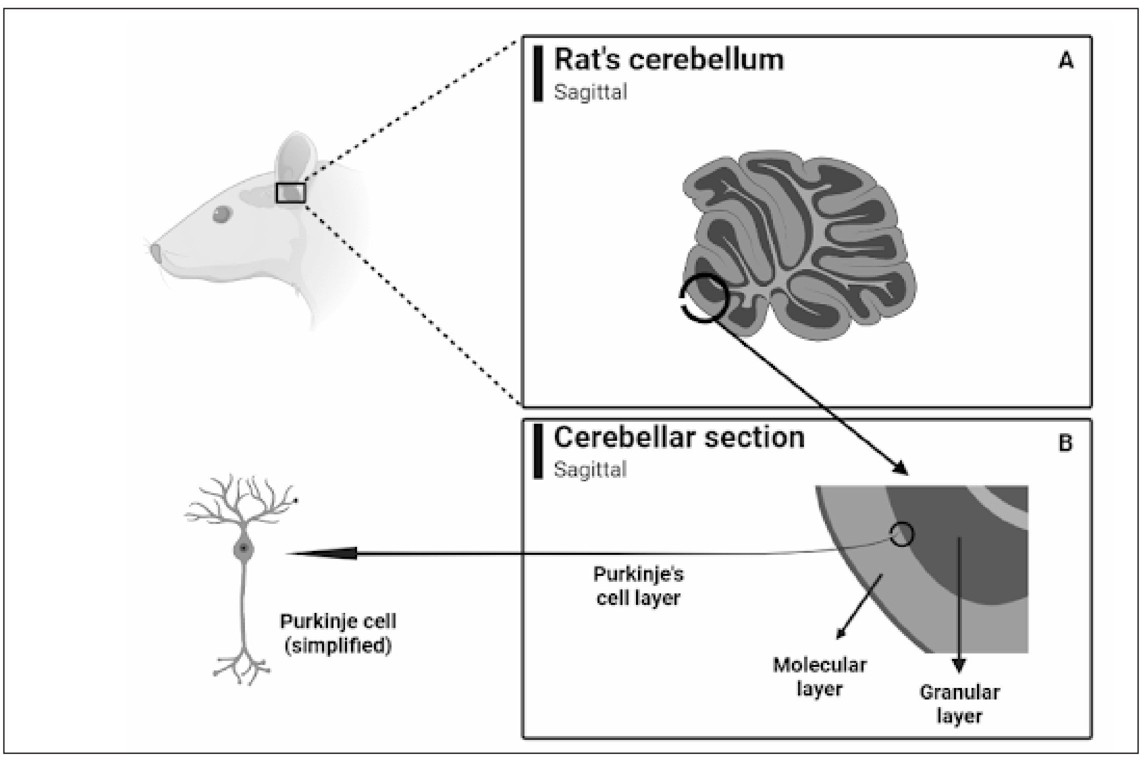

Figure 2. *A)* Schematic sagittal view of rat cerebellum. *B)* Schematic cerebellar section identifying the three cerebellar layers: molecular layer (mainly cellular prolongations with a small number of small neurons and glial cells), granular layer (which contains numerous neurons, together with a smaller number of other types of neurons, the Golgi cells), and Purkinje mono cell layer. The structure of a Purkinje cell is shown simplified.

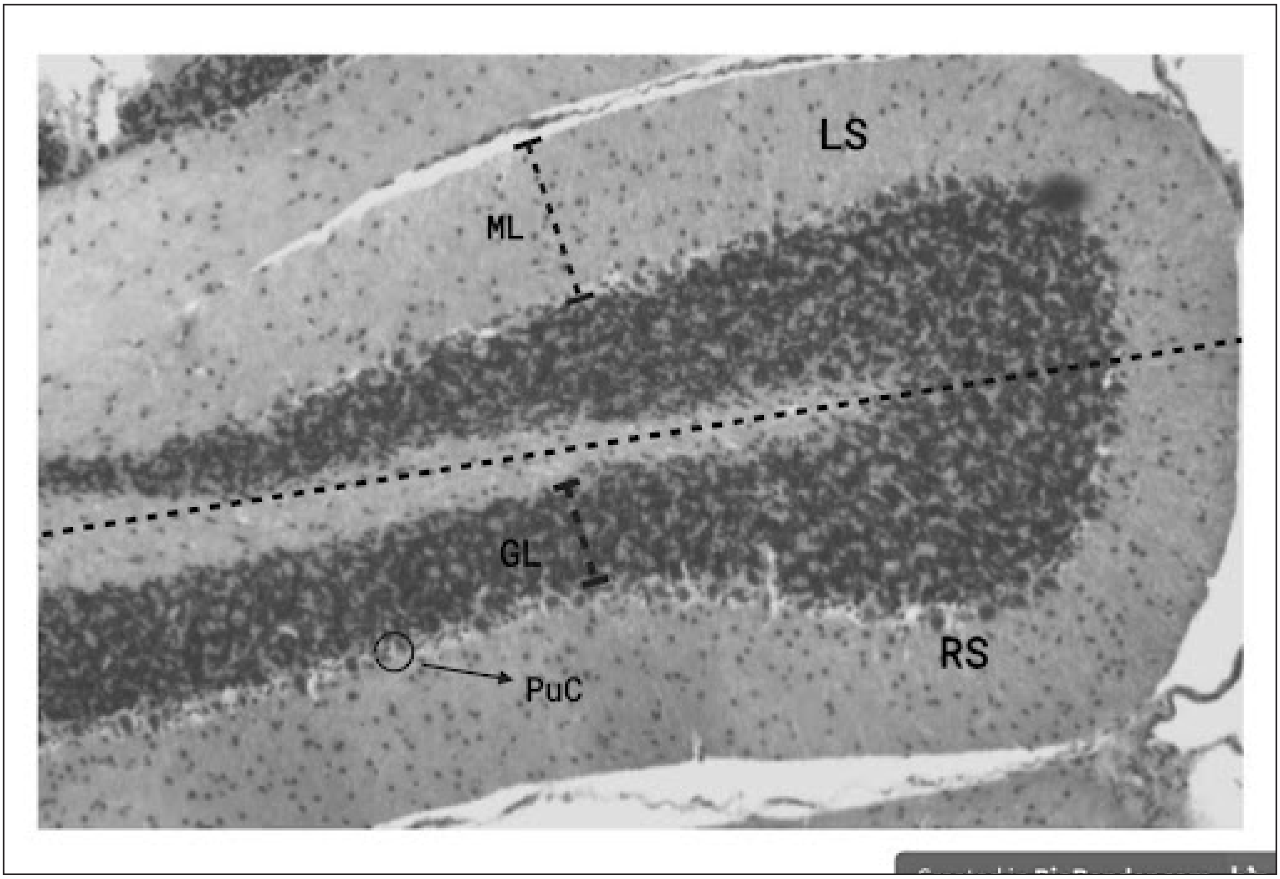

Figure 3. Light microscopy photograph of a cerebellar ridge, with Hematoxylin staining. ML= Molecular Layer. GL= Granular layer. PuC= Purkinje cell monolayer. RS= Right side. LS= Left side. (4X magnification) (Edited with Biorender App).

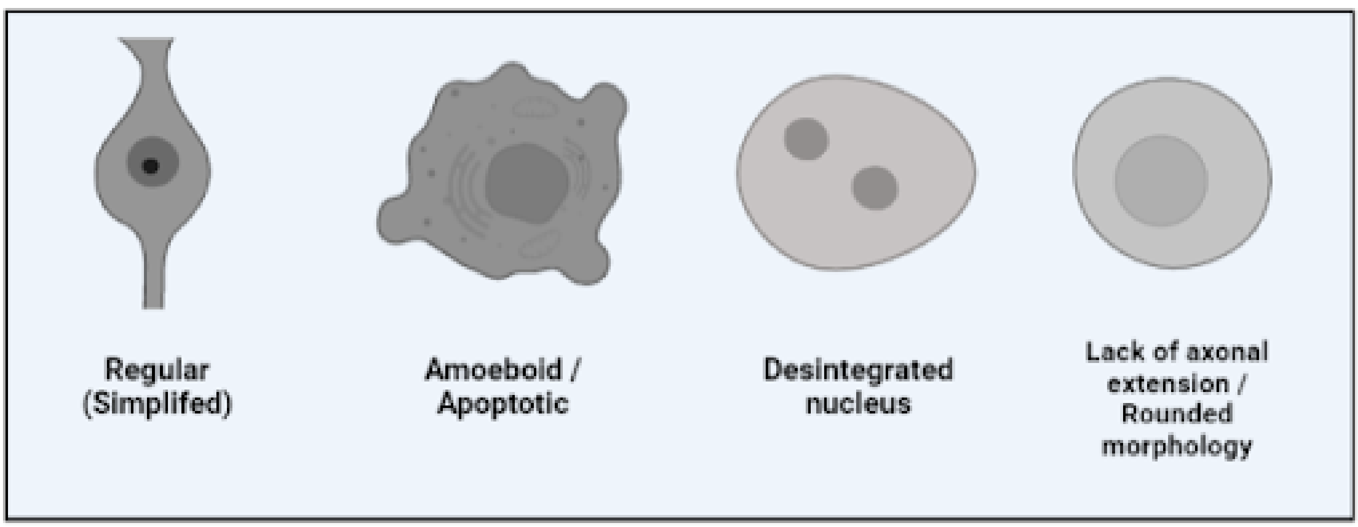

Figure 4. Schematic of Purkinje cells showing regular cell morphology and the anomalies considered in this study: amoeboid or apoptotic cells, disintegrated nucleus, lack of detectable axonal extension, and rounded shape (Made with Biorender App).

from each rat was embedded in paraffin and sectioned into 10-µm thick slices (*Figure 2A*). Four consecutive sections, each separated by 40-µm, were stained with hematoxylin (Fischer *et al.* 2008). In each section, 468 photographs were taken using an Olympus CX31 optical microscope, equipped with a Q-Imaging GO-3 camera and Q-Capture Pro 6.0 licensed software. For image analysis of molecular and granular layer widths, 4X magnification was used, while for the rest of the variables, photographs were taken at 40X magnification. Photographs at 40X mag were taken from right to left, with a movement of 250 µm between photographs. The measurement and determination of the variables were performed using ImageJ 1.8.0 software.

The parameters evaluated in each photograph included: Molecular and granular layer widths; molecular layer cell density; average nuclear and cytoplasmic area of Purkinje cells; nucleus-to-cytoplasmic (N:C) ratio of Purkinje cells; average cell area of molecular and granular layer; and number of abnormal Purkinje cells. The abnormalities include irregular shapes, disintegrated nucleus, lack of axonal extension, and spherical or amoeboid shape (*Figure 4*).

Without making a distinction between the various cell types, all cells with circular or elliptical morphology visible under hematoxylin staining were counted to determine the molecular layer's cell density (*Figure 5*). Within the molecular layer of the cerebellum, a variety of distinctive cell types coexist, including basket cells, stellate cells, an expansive array of parallel fibers originating from the granule cell layer, as well as the flattened dendritic arbors of Purkinje cells. Due to the limitations of hematoxylin staining in distinguishing all these specific cell populations, the present study focused on quantifying the overall cell density of all cells exhibiting a circular or elliptical morphology that were visible with this histological technique (*Figure 6*).

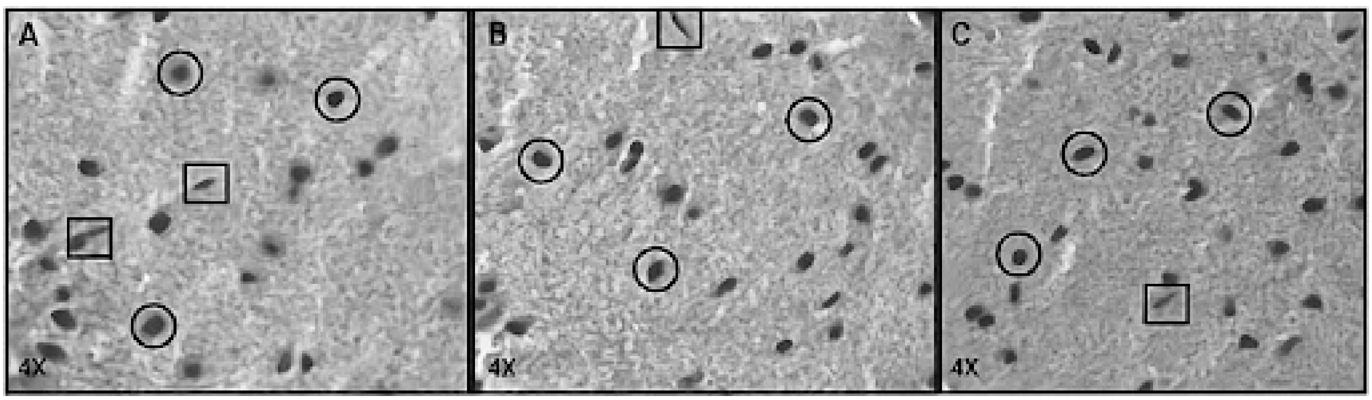

Figure 5. Light microscopy photographs of molecular layer cells at 4x magnification. Circles indicate examples of cells counted and squares indicate cells with morphology not considered for analysis. Images were taken from rats captured in S1, S2 and S3 respectively.

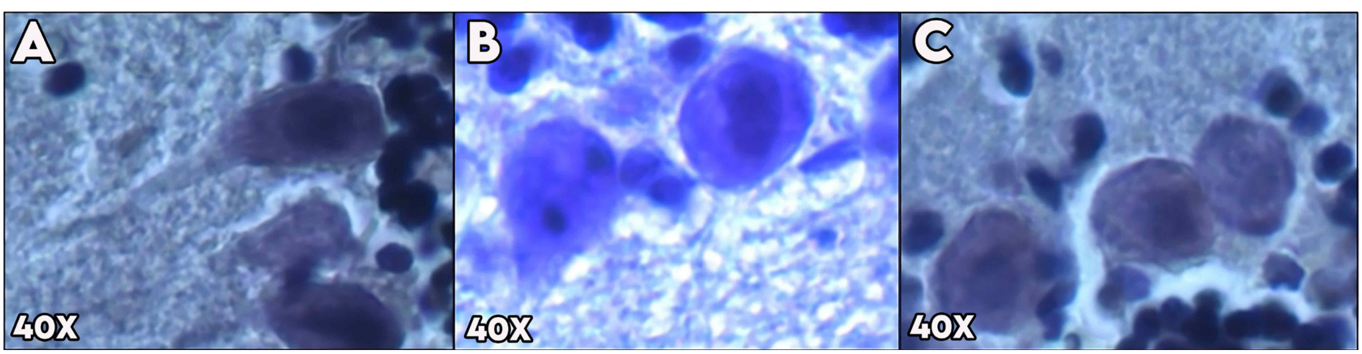

Figure 6. Light microscopy photographs of Purkinje cells with Hematoxylin staining from *R. norvegicus* captured in MRB. *(A)* Purkinje cell of regular morphology (40X magnification). *(B)* Shows a cell with a disintegrated nucleus (left) and a cell without detectable axonal extension (right). *(C)* Cells with "amoeboid" and rounded morphology (40X magnification). Image A was taken from a rat captured in S3. Images B and C were taken from rats captured in S2.

Statistical analyses

The data on environmental lead concentrations in surface water and soil (*Table 2*) and lead concentrations in the organs of the analyzed rats were obtained from a previous study carried out by Tripodi *et al.* (2020 b). The association between the variables measured in the cerebellum of *R. norvegicus* from the six sampling sites and environmental and rat characteristics were analyzed using Generalized Linear Mixed Models. Nine models were performed, one for each dependent variable (Molecular and granular layer widths, molecular layer cell density, average nuclear and cytoplasmic area of Purkinje cells, N:C ratio of Purkinje cells, average cell area of molecular and granular layer, and number of abnormal Purkinje cell). The explanatory variables in each model were environmental lead values of water and soil, characteristics of the captured rats (length, age, weight, sex, reproductive status), and the lead concentration in their liver and kidney. Random factors included, capture site (S1 to S6), rat identification number (1 to 32), and photographic analysis variables (Cerebellar section and ridge, side, photo number). Different types of probability distributions were used to model the dependent variables, including Normal distribution for molecular and granular layer widths, average nuclear and cytoplasmic area of Purkinje cells, and average cell area of molecular and granular layer; Poisson distribution for molecular layer cell density; Beta distribution for N:C ratio of Purkinje cells; and Bernoulli distribution for number of abnormal Purkinje cell. The final model was chosen based on the lowest Akaike's information criterion (AIC) and the fewest explanatory variables.

Table 2. Average lead concentration in water, soil, and organs of *R. norvegicus* captured at each sampling site. Data extracted from Tripodi *et al.* (2020 b). The EPA maximum contaminant level for lead in drinking water is 0.015 mg/L (Advisory Committee on Childhood Lead Poisoning Prevention (ACCLPP), 2012). The Canadian Council of Ministers of the Environment stipulates the limit levels of lead in soil for agricultural, residential, commercial, and industrial use at 70, 140, 260 and 600 mg/kg respectively (Canadian Council of Ministers of the Environment, 1999).

		Lead concentration			
Site	n	Water (mg/L, Mean ± SD)	Soil (mg/kg, Mean ± SD)	Kidney (µg/g, Mean ± SD)	Liver (µg/g, Mean ± SD)
S1	11	0.017 ± 0.016	241.0 ± 162.3	4.74 ± 3.94	0.45 ± 0.64
S2	5	0.018 ± 0.016	268.3 ± 187.8	7.82 ± 4.36	0.31 ± 0.24
S3	4	0.004 ± 0.001	81.3 ± 93.3	2.40 ± 1.23	0.08 ± 0.09
S4	5	0.014 ± 0.019	164.1 ± 150.6	9.04 ± 10.29	0.33 ± 0.28
S5	5	0.010 ± 0.003	250.1 ± 211.5	0.97 ± 0.48	0.41 ± 0.80
S6	2	0.006 ± 0.003	114.4 ± 117.6	1.42 ± 1.79	0.09 ± 0.01

All statistical analyses were performed using the R program version 4.0.3 with the nlme version 3.1-157 package used for models with Normal distribution, lme4 version 1.1-29 for models with binomial and Poisson distributions, and glmmTMB version 1.1.3 for a model with Beta distribution. These packages were chosen for their ability to handle mixed-effects models with different probability distributions for the dependent variables.

RESULTS

The sites closest to the mouth of the Matanza-Riachuelo Basin (S1 and S2) presented the highest average values in molecular and granular layer width (*Table 3*), the smallest average granular and molecular layer cell size in the cerebellum and the highest percentage of Purkinje cells with anomalies (28.4%) (*Figure 6*). On the other hand, the highest average cell density of the molecular layer and the highest average N:C ratio of Purkinje cells were found in the rats at the site farthest away from the mouth of the MRB (S6).

The cell density of the molecular layer was negatively associated with the concentration of lead in the kidney and with the age of the rats captured in the MRB (P value < 0.05) (*Figure 7*). In addition, the average N:C ratio of Purkinje cells of the rats captured in the CMR was negatively associated with soil lead concentration of the sampling sites (P value < 0.05) (*Figure 8*).

Table 3. Comparative cerebellar evaluation in the specimens of *R. norvegicus* captured in the MRB for each sampling site (S1 to S6). Values represent mean ± SD (S1 to S6).

Variable	Site					
	S1	S2	S3	S4	S5	S6
Molecular layer width (µm, Mean ± SD)	168.4 ± 35.9	186.9 ± 35.9	167.5 ± 36.4	174.7 ± 35.8	158.6 ± 33.4	151.0 ± 37.4
Granular layer width (µm, Mean ± SD)	109.4 ± 33.3	128.5 ± 32.2	124.6 ± 33.5	122.8 ± 32.6	112.7 ± 32.1	117.3 ± 35.0
Molecular layer cell density (Number of cells. µm-², Mean ± SD)	0.0021 ± 0.0005	0.0019 ± 0.0005	0.0017 ± 0.0005	0.0018 ± 0.0005	0.0023 ± 0.0005	0.0027 ± 0.0006
Nucleus-to-cytoplasmic (N:C) ratio of Purkinje cells (Mean ± SD)	0.19 ± 0.07	0.22 ± 0.08	0.22 ± 0.07	0.22 ± 0.07	0.22 ± 0.07	0.24 ± 0.07
Purkinje cell nucleus area (µm², Mean ± SD)	25.95 ± 12.5	32.06 ± 13.07	34.84 ± 12.37	32.25 ± 12.8	34.55 ± 12.54	38.77 ± 12.1
Purkinje cytoplasmic area (µm², Mean ± SD)	138.24 ± 37.4	148.1 ± 35.5	159.7 ± 37.6	151.8 ± 36.6	157.7 ± 37.2	161.3 ± 37.0
Abnormal Purkinje cells proportion (Abnormal cells.total cells ¹, Mean ± SD)	0.114 ± 0.317	0.284 ± 0.451	0.127 ± 0.333	0.232 ± 0.422	0.100 ± 0.301	0.037 ± 0.189
Molecular layer cell area (µm², Mean ± SD)	16.98 ± 4.78	17.85 ± 4.73	20.01 ± 5.24	21.5 ± 5.81	18.71 ± 4.57	20.4 ± 5.55
Granular layer cell area (µm², Mean ± SD)	10.27 ± 3.33	10.12 ± 2.46	12.84 ± 3.37	12.90 ± 3.55	10.55 ± 2.76	14.4 ± 4.47

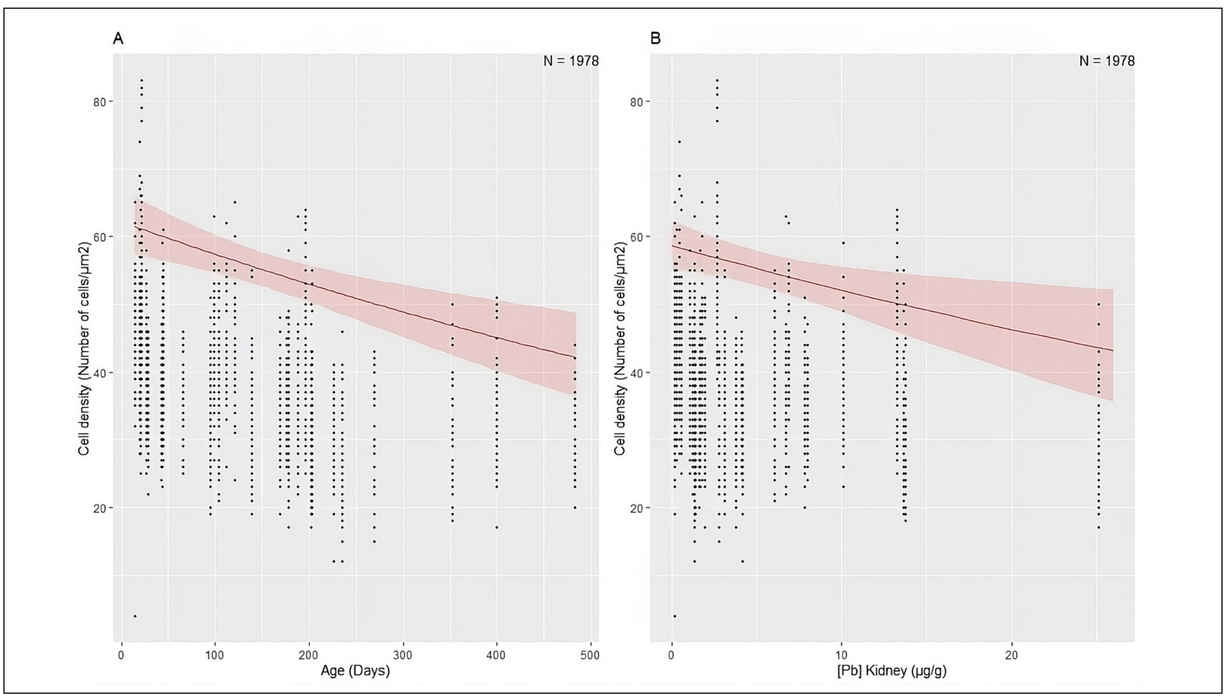

Figure 7. Relation between molecular layer cell density (Number of cells/µm) and age (P value < 0.05) (A) and kidney lead concentration (µg/g) (P value < 0.05) in *R. norvegicus* individuals captured in the MRB (B) Red areas indicate the confidence interval. Upright the number of observations is indicated.

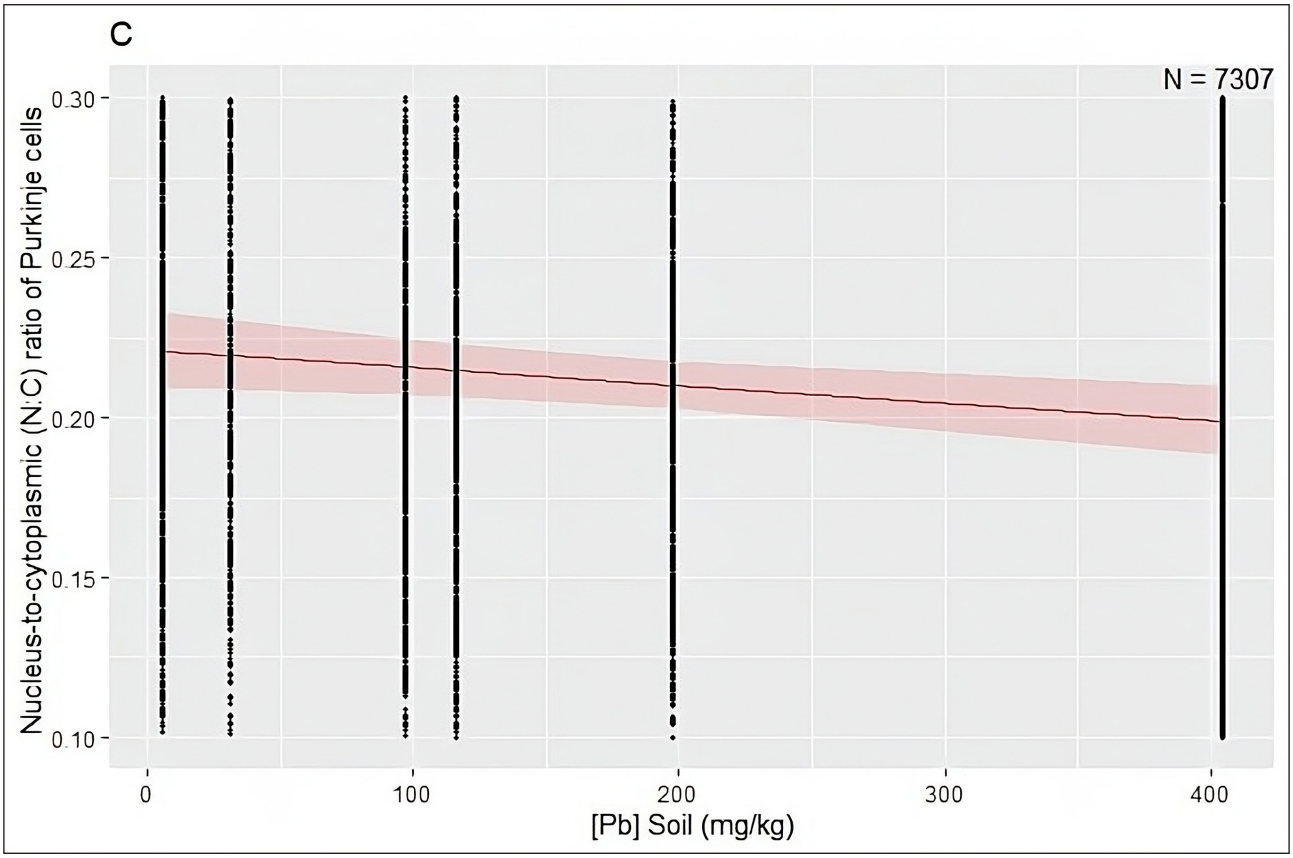

Figure 8. Relation between nucleus-to-cytoplasmic (N:C) ratio of Purkinje cells and soil lead concentration at sampling sites (P value < 0.05). Red areas indicate the confidence interval. Upright the number of observations is indicated.

DISCUSSION

In this work, we found that rats of the MRB showed cytoarchitecture changes in their cerebellum associated with lead exposure. First, the concentration of lead in the kidneys was negatively associated with the cell density in the molecular layer of the cerebellum of rats living in the MRB.

Cell loss is one of the most documented effects of lead poisoning in the CNS (Sidhu and Nehru 2004; Mousa *et al.* 2015; Naqi 2015), and according to Adonaylo and Oteiza (1999), a reduction in cell density in the molecular layer may be caused by cell death due to oxidative stress caused by chronic lead exposure. Although Ma (1996) suggests a critical value of 25 µg/g of lead in the kidneys in wild mammals for diagnosing chronic lead exposure in natural environments, the lead values in the kidneys of the specimens used in this study did not exceed 9 µg/g. Despite this, it was still sufficient to cause changes in the cellular density of the cerebellum. Naqi (2015) and Mousa *et al.* (2015) found similar results in experimental studies where the administration of high doses of lead in rats resulted in a reduction in the cell density of the molecular layer of the cerebellum. This result could imply that high concentrations of bioavailable lead in natural environments can generate changes in the cell density of the cerebellum even at concentrations much lower than those administered experimentally.

Additionally, a negative association was found between the cell density of the molecular layer of rat's cerebellum and their age. There is a well-documented reduction in cerebellar volume and loss of cells in the different layers of the cerebellum, particularly Purkinje cells, as individuals age (Andersen *et al.* 2003; Andersen 2004). This correlation could be because, as individuals age, there is a reduction in the efficiency of cerebellar functioning in general, explained by a reduction in the integration of information in the senescing cerebellum. On the other hand, as Purkinje cells are the final destination of the afferent pathways of the cerebellar cortex (Purves *et al.* 2001), their loss could affect the cell density of the other layers. Our finding is consistent with the results reported by Zhang *et al.* (2006), who found a negative correlation between age and cell density in all three layers of the cerebellum in mammals.

Under normal conditions, the N:C ratio is relatively constant for each tissue, cell type and species (Stevenson 1935), making it an important indicator in the determination and analysis of atypia and malignancy in cells (Vaickus and Tambouret 2015; Moore *et al.* 2019). We found that the N:C ratio of Purkinje cells was negatively associated with soil lead concentration at the sampling sites. This could be a cellular response due to lead poisoning, causing either atrophy of the nucleus, an increase in total volume (McConnell *et al.* 1979) due to increased cytoplasmic activity, or both simultaneously, which could explain the negative association found in our study. However, no relationship was found between lead in soil and changes in neither nuclear nor total area of Purkinje cells separately. Previous studies have found contradictory results in the relationship between lead concentration and changes in cytoplasmic and nucleus size in Purkinje cells. For example, Naqi (2015) showed that the administration of high doses of lead in rats caused degenerating and decreasing in total size of Purkinje cells, while Mcconnell *et al.* (1979) found a swelling/increasing in the size of Purkinje cells with higher concentrations of lead. These conflicting results could be due to differences in the doses of lead administered, in timing and duration of exposure, or there could be threshold concentrations of lead above which one or the other effect occurs. In addition, it could also be due to the methodology used for tissue fixation, so these results must be taken with caution. Future studies should consider perfusion of the animals instead of formaldehyde fixation, for example, to prevent possible volumetric changes associated with cell death. Future investigations employing more specialized staining methods will be necessary to provide a deeper assessment of how lead exposure impacts the distinct cellular types within the molecular layer of the cerebellum.

More studies are needed to clarify the effects of lead in the nuclear and cytoplasmic area of Purkinje cells (and therefore the N:C ratio). Previous research by our group had shown that the lead concentrations in soft tissues (kidney and liver) of *R. norvegicus* were a good indicator of lead contamination in water and soil in the MRB (Tripodi *et al.* 2020 b). Lead in MRB is therefore bioavailable, and this study's results indicate that there is enough lead in soft tissues to affect the cytoarchitecture of the cerebellum (e.g., by reducing the molecular layer's cell density).

The Canadian Council of Ministers of the Environment (1999) suggests soil lead limits in 70, 140, 260 and 600 mg/kg for agricultural, residential, commercial, and industrial use, respectively. Four of the six sites analyzed in our study have soil lead concentrations that exceeded the recommended limit for residential use, which is especially concerning given that among these sites there is a shantytown (S2) and the association found in this study between lead contamination and signs of cerebellar damage in rats. This highlights the importance of using sentinel species that live near humans in the analysis of exposure to contaminants, considering that even concentrations of lead much lower than those used experimentally have an impact on cerebellar cytoarchitecture.

In this regard, certain response variables analyzed in this study (proportion of abnormal Purkinje cells, molecular layer width, and total and nucleus area of the Purkinje cell) show trends that correspond to expected outcomes but lack significant associations with explanatory variables. This could also be related to each specimen's lead bioaccumulation and exposure extent, as well as its age, sex, or nutritional status. Obtaining more data would help in determining whether some of the trends observed in the non-significant response variables could be attributed to a lack of power in the analyses performed due to the number of samples used. Although the effects of lead on the central nervous system (CNS) have been well studied, most of the research has been conducted in experimental studies using lead concentrations that exceed those found in natural environments. However, by studying wild rats in areas near human populations, we can learn how wild populations respond to contaminants like lead in natural environments.

Finally, because of the heavy pollution in the MRB, other neurotoxic metals like cadmium and arsenic may have a synergistic effect with lead, even at low concentrations. Previous research has found that certain metals, such as lead and arsenic, have a significant synergic neurotoxic effect (Mejía *et al.* 1997; Singh *et al.* 2017). This observation is especially pertinent given the presence of both metals in the MRB (Tripodi *et al.* 2021). However, further investigation is necessary to fully understand their synergistic effect, especially at the natural concentrations found in the basin.

This work contributed to the understanding of the damage in the CNS of wild rats caused by exposure to environmental contaminants such as lead in the MRB. We highlight the importance of knowing how species that live in close relationship with humans, such as *R. norvegicus*, respond to exposure to neurotoxic pollutants, considering the variations in exposure between species.

Given that rats do not explore the environment in the same way as humans, live in burrows and are closely associated with water sources, there could be a greater intake of lead contained in the soil and water and consequently a higher neurotoxic damage. Considering that *R. norvegicus* is a commensal species of humans, and that the MRB is a highly populated area with inhabitants in a disfavored socioeconomic situation living on the floodplain of rivers, residing in precarious

housings and with a lack of access to health centers, this work highlights the potential and neglected health risk to which people are exposed to.
Finally, studies in this type of environment involve several problems such as high crime rates, theft of traps, and periodic flooding, all of which make it difficult to obtain an optimal sample size. This work sheds light on how lower concentrations of lead than those used in experimental work can still generate effects on cerebellar cytoarchitecture of rats. Furthermore, there might be a synergistic effect with other neurotoxic metals, so further studies including the different types of metals in the basin are needed.

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