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Research Article

Toxic Mechanisms Underlying Motor Activity Changes Induced by a Mixture of Lead, Arsenic and Manganese

Vanda Andrade[1], M Luísa Mateus[1], M Camila Batoréu[1], Michael Aschner[2] and AP Marreilha dos Santos[1]*

[1]Instituto de Investigação do Medicamento, iMed.UL, Faculdade de Farmácia, Universidade de Lisboa, Lisboa, Portugal

[2]Department of Molecular Pharmacology, Albert Einstein College of Medicine, NY, USA

***Corresponding Author**: AP Marreilha dos Santos, Instituto de Investigação do Medicamento, iMed.UL, Faculdade de Farmácia, Universidade de Lisboa, 1649-003 Lisboa, Portugal.

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Abstract

Pb, As and Mn are neurotoxic metals, present as mixtures at various settings. All metals are known to interfere with cholinergic/dopaminergic neurotransmission and motor function. The main objective of this work was to assess metal mixture effects of lead (Pb), arsenic (As) and manganese (Mn) on motor activity, and to evaluate the role of each mixture component as well as their additive/synergic interactions on dopaminergic and cholinergic neurotransmission. Wistar rats were treated with 8 doses of each single metal, Pb, As and Mn, or a triple metal mixture. Motor activity was evaluated along with cholinergic/dopaminergic neurotransmission, using brain acetylcholinesterase (AChE-Br) activity and serum prolactin (PRL-S) levels, respectively. Brain concentrations of Pb, As, Mn were also quantified. The metal mixture induced decreased motor activity relative to all other groups with factor analysis revealing close proximity between AChE-Br and motor activity. Pb brain levels increased significantly as compared to all the other groups, while β coefficients of multiple regression showed that this metal was the most effective in changing AChE-Br. Significant effects of interactions among the three metals on the activity of this enzyme were also noted for the metal mixture. In conclusion, co-exposure to Pb, As and Mn mixture alters the cholinergic system and motor activity to a greater extent than the dopaminergic system. Additive/synergic interactions between Pb, As and Mn may have a relevant role in mediating these events.

Keywords: *Metals; Pb/As/Mn-Mixture; Cholinergic/Dopaminergic Neurotransmission; Additive/Synergic Interactions*

Abbreviations

Pb: Lead; As: Arsenic; Mn: Manganese; Ache- Br: Brain Acetylcholinesterase; PRL-S: Serum Prolactin; CNS: Central Nervous System; PD: Parkinson's Disease; DA: Dopamine; QL: Quantification Limit; ALA: Delta-Aminolevulinic Acid; HPLC: High Performance Liquid Chromatography; SD: standard deviations.

Introduction

Humans are daily exposed to a cocktail of heavy metal toxicants. These ubiquitous elements are present in different combinations in the environment, derived mostly from polluted air and contaminated food [1,2]. Though adverse effects emanating from their single exposures are widely known, there is a paucity of information on the toxicity of low dose mixtures [2]. Pb and As are among the six most toxic pollutants [3]. Despite of its essentiality [4], exposure to excessive Mn levels is also of great public health concern due to its extensive use in the metal industry [5]. The three metals/metalloids are known to be present in mixtures [6].

It is established that Pb, As and Mn are neurotoxic [5-8]. Their neurotoxic effects include a spectrum of biochemical, morphological, behavioural and physiological abnormalities, whose duration may be transient or persistent. Depending upon their severity, some of these abnormalities can have life-threatening consequences and more commonly, result in diminished quality of life [9]. Pb causes damage to various organs, including the CNS. Even low doses may cause motor and neurobehavioral changes [10]. Brain dysfunction due to

prolonged exposure to low levels exposure to As has also been recognized [11], and is characterized by altered locomotor activity and sensory-motor function [12]. Exposure to high levels of Mn may lead to a neurodegenerative disease referred to as manganism, which shares multiple analogies with PD [13].

Pb, As and Mn accumulate in the CNS. The nucleus accumbens, which is a major part of the ventral striatum, is preferentially susceptible to Pb-induced neurotoxicity [14,15]. As accumulates in several brain regions, including the striatum and predominantly in the pituitary [16,17]; Mn preferentially accumulates in globus pallidus and striatum [18,19]. Cholinergic and dopaminergic outputs to these brain areas are critical for several CNS functions, including motor function [18,20-24].

Pb can interfere with the cholinergic system by altering AChE-Br activity, given its ability to mimic Ca2+ thus enhancing this enzyme's activity [25,26]. Pb can also affect dopaminergic neurons, affecting turnover and DA utilization [10]. Neurochemical alterations of the cholinergic system have been also reported following As exposure, including altered AChE-Br activity [27]. Furthermore, increased striatal DA levels have been noted after exposure to As, suggesting that the nigrostriatal dopaminergic system is a target for this metalloid [12]. Exposure to Mn has been reported to alter AChE-Br activity, causing changes of neuronal excitability in the basal ganglia. These effects may explain, at least in part, the similarity between manganism and PD [28]. Mn has also been shown to induce dopaminergic neurodegeneration in the substantia nigra of rats [13,29].

Pb, As and Mn are also known to interfere with enzymes of the heme synthetic pathway, increasing heme precursor concentrations [30-32]. The steps in the heme pathway most vulnerable to heavy metal inhibition are those associated with the enzymes delta-aminolevulinic acid dehydratase, uroporphyrin decarboxylase and coproporphyrinogen oxidase [33,34]. Heme precursors can accumulate in the brain and may lead to neurotoxicity [35]. In fact, the need for future studies to examine the relationship between heavy metals and urinary porphyrin levels as biomarkers of neurological dysfunction has been previously recognized [34]. Notably, increased excretion of urinary porphyrins concomitant with decreased motor activity has been described [36].

Considering the information described above, we posited that Pb, As and Mn present in mixtures may interfere with optimal cholinergic and dopaminergic neurotransmission, and that additive/synergic effects might occur upon exposure metal mixtures, altering cholinergic/dopaminergic control of motor activity in a pattern distinct from single metal exposures.

Accordingly, the aim of this work was: i) to assess the influence of cholinergic and dopaminergic modifications induced by the mixture of Pb, As and Mn on motor activity; ii) to evaluate the role of Pb, As, Mn and heme precursor accumulation in brain on cholinergic and dopaminergic neurotransmission, and iii) to ascertain additive/synergic interactions between Pb, As and/or Mn, and evaluate the effects of these interactions on cholinergic and dopaminergic neurotransmission.

Materials and Methods

Animals: Male, Wistar rats were purchased from Charles River Laboratories®, Barcelona, weighing 165–206 g. The rats were housed in an independent room with controlled temperature, humidity and a 12-h light/dark cycle. All animals had free access to water and food, supplied as pellets. All experiments were carried out in accordance to criteria outlined in the guiding principles of the European Community Council Directive (89/609/EEC) for the care and use of laboratory animals.

Chemicals: Chemicals were obtained from the following sources: manganese standard for Atomic Absorption Spectrometry (AAS) from Fluka; arsenic standard solution for AAS (H_3AsO_4), nitric acid 65% suprapure (HNO_3), delta-aminolevulinic acid standard from Merck; lead acetate trihydrate puriss. [Pb (CH_3CO_2)2•3H_2O], lead for AAS standard solution, manganese chloride tetrahydrate ($MnCl_2$•4H_2O; 99.99%), sodium (meta)arsenite purum (AsO_2Na; ≥ 99%), 2-Propanol for HPLC (C_3H_8O; 99.5%), 5,5 –dithiobis (2-nitrobenzoic acid) (DTNB), acetylthiocholine (ATCh), diethyl ether ($C_4H_10_0$), ethopropazine, ethyl acetate ($C_4H_8O_2$), ethyl acetoacetate p.a. ($C_6H_{10}O_3$), methanol HPLC grade (CH_4O; ≥99.9%), sodium phosphate dibasic (Na_2HPO_4), sodium phosphate monobasic (NaH_2PO_4) from Sigma–Aldrich. Pure standards of porphyrins (10 nM) were obtained from Porphyrin Products, Frontier Scientific.

Experimental design: A repeated exposure assay was performed: after a 15-day acclimation period, the animals were randomly assigned to 5 groups. The rats were treated with eight daily doses of each single metal, Pb (5 mg/Kg bw), As (60 mg/L), and Mn (10 mg/Kg bw), or the same doses in a triple metal mixture. A control group was also used. 24 h after the last dose the rats were anesthetized with pentobarbital (20 mg/kg, i.p.), blood was collected by cardiac puncture and serum was obtained by centrifugation. Upon sacrifice the brains were dissected out and stored at -80oC for further analyses [33].

Determination of motor activity parameters: Motor activity was evaluated in an open-field apparatus. Two behavioural parameters were determined: the number of squares crossed with all paws (ambulation's) and the number of times that both forelegs were raised from the floor (rearing's) were assessed over a five-minute period.

Evaluation of cholinergic function: Cholinergic function was assessed by analyses of AChE -Br activity, slightly modified from the Ellman's method (1961) by Worek., *et al* (1999) [37]. Fifty mg of brain homogenates were used in the presence of ethopropazine (20 µM), as pseudocholinesterase inhibitor. Readings were performed in a Hitachi spectrophotometer. Repeatability expressed as coefficient of variation (CV %) was 12.2%. The results are expressed as nmol AChE/min/g brain protein.

Evaluation of dopaminergic function: PRL -S levels were used as a biomarker of the dopaminergic function [38]. The levels of this neurohormone were quantified by an enzyme immunoassay (Citomed®) and the plates were read with an Anthos Zenyth 3100 microplate detector. The QL was 0.6 ng/mL.

Determination of heme precursor's levels in the brain: Heme precursors are presented as the sum of ALA, uroporphyrins and coproporphyrins. To determine brain ALA levels, 1 mL of supernatant (2,500 rpm, 10 min) from brain homogenates or ALA standards was added to 1 ml of acetate buffer (pH 4.6) and 0.2 mL of acetoacetate. After mixing (5 sec) and incubation (100°C, 10 min), 3 mL of ethyl acetate were added. The samples were agitated (15 sec), centrifuged (2000 rpm, 3 sec) and Ehrlich reagent was added to the organic phase. Spectrophotometric readings were performed at 553 nm. The QL was 0.012 mg ALA/L. The extraction of uro- and coproporphyrins from the brain was performed according to the method described by [39]. For separation and quantification, a HPLC in a Hewlett Packard Agilent 1100 HPLC system, with a LiChrospher 100 Merck RP18 column (125mm x 4mm, 5µm) was used. The fluorescence detector was set at an excitation wavelength of 395nm and an emission wavelength of 620nm. The mobile phase consisted of methanol 100% (solvent A) and sodium phosphate monobasic (50 mM), pH 3.5 (solvent B). A gradient was used starting at the mobile phase A:B (30:70%) for the first 3 min, changed linearly to A:B (80:20%) for 10 min, maintained equal for 3 more min. Next, the column was equilibrated with 30% of solvent A for 5 min. The flow rate was 1.0 mL/min. Dried porphyrin standards (10 nM) recovered with HCl (3M) were used to obtain a calibration plot with R2=0.9998. The determined QLs were: 7.7 nmol/L for uroporphyrins and 17.2 nmol/L for coproporhpyrins. The results are expressed as mg of heme precursors per g of brain protein.

Determination of Pb, As and Mn levels in the brain: 80 mg of each brain sample was digested by Microwave-Assisted acid digestion (900 W, 30'') using Parr Microwave Acid Digestion Bombs® with 2.9 mL of oxidizing acid mixture containing 4:1 (v/v) HNO_3 65% suprapure: H_2O_2 30%. The digested solutions were transferred to volumetric flasks and the volume was complemented with deionized water; samples were kept at 4°C until analysis. The metal concentrations were determined by graphite furnace atomic absorption spectrophotometry (GFAAS) in a PerkinElmer AAnalyst™ 700 equipped with a WinLab 32 for AA software. Daily calibration curves for each element were obtained with standard solutions. The QLs were 3.8 µg Pb/L, 22.5 µg As/L and 5.2 µg Mn/L. The results are expressed as mg of Pb, As or Mn per g brain protein.

Determination of brain protein contents: Determined according to Bradford protein assay [40] using bovine serum albumin as the standard.

Statistical analysis: Statistical analysis was performed with the SPSS 16.0 statistical package for Windows (SPSS, Inc., Chicago, IL, USA). For each treated group data are presented as means ± SD of the variation as % relative to the control group mean values. All the parameters were compared by Mann-Whitney tests to assess differences between groups. Factor Analysis is a data reduction statistical method

that groups similar variables into dimensions. It can be used to determine the pattern of inter correlations among variables, since variables that are correlated with one another are grouped. In this type of analysis higher proximity between variables Component Plots in Rotated Space indicate higher association among them, with Varimax rotation method serving to more precisely clarify the output [41].

Thus factor analysis was also performed to describe the relationship among motor activity parameters, ambulation and rearing counts, and neurotransmission functions, cholinergic and dopaminergic functions. The method was applied in order to group common variables into 2 dimensions permitting the evaluation of the relationship between AChE-Br activity and PRL-S levels to motor activity. Additionally, standardized β regression coefficients were obtained by multiple regressions and used to analyse the relative load [42] of Pb, As, Mn and heme precursor accumulation in brain to AChE-Br and PRL-S modifications. Factorial ANOVA was also performed to test if interactions between Pb, As and Mn could have a significant effect on the AChE-Br activity or PRL-S concentrations. For all analysis the significance of the results was considered when p values were less than 0.05.

Results

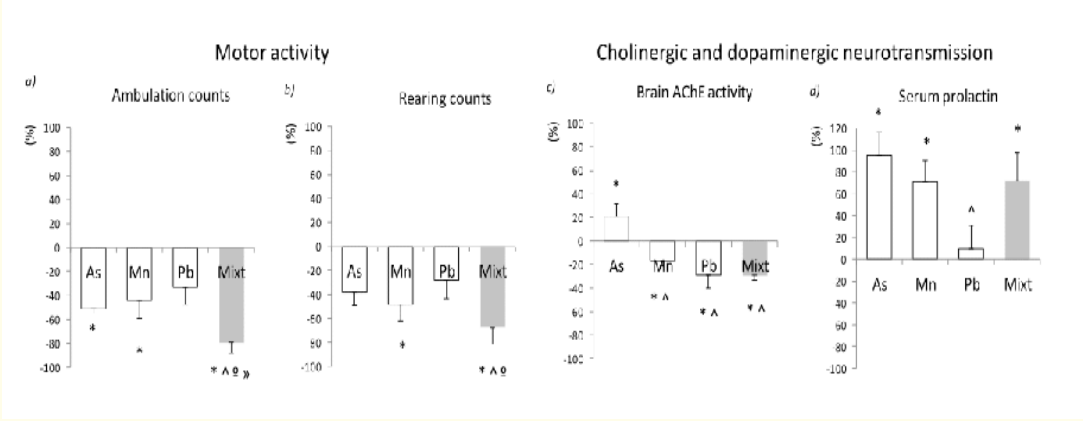

Figure 1: *Motor activity evaluated by ambulation (a) and rearing (b) counts and, cholinergic and dopaminergic functions, which were evaluated by AChE activity in brain (c) and serum PRL levels (d), respectively. Determinations were performed in As, Mn, Pb, metal mixture (Mixt) and control groups. Data are expressed as mean± SD of the variation in % relative to the control group. N=6 in each group. All groups were compared by Mann-Whitney tests: +, *, ^, º and » are p < 0.05 versus PD, C, As, Mn and Pb.*

The As treated group exhibited a significant ($p < 0.05$) decrease in ambulation counts, while the Mn treated rats showed a significant decrease in both motor activity parameters compared with the controls ($p < 0.05$) (Figure 1 a and b). Rats treated with the metal-mixture exhibited decreased ambulation and rearing counts, which were significantly different from all the other groups ($p < 0.05$) (Figure 1 a and b), except for rearing activity when compared with the Pb-treated group ($p < 0.05$) (Figure 1 b).

AChE-Br activity in the As treated rats was significantly ($p< 0.05$) higher than in controls (Figure1 c), while inversely, in the metal mixture, Pb and Mn exposed groups the activity of the enzyme was significantly ($p < 0.05$) lower than controls and As treated rats (Figure 1 c). Additionally, rats treated with the metal mixture, As or Mn exhibited increased PRL-S concentrations, which were significantly ($p < 0.05$) different from the controls (Figure 1 d).

Relationships among neurotransmission functions and motor activity parameters.

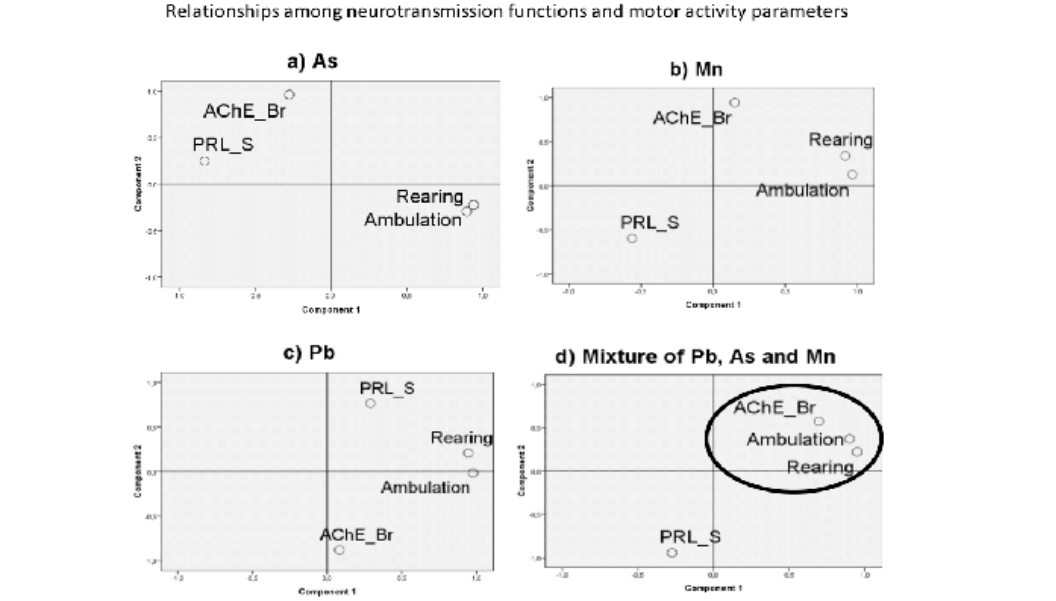

Figure 2: *Relationship between cholinergic and dopaminergic neurotransmission and motor activity. Component Plots in Rotated Space illustrate the association of AChE activity in brain (AChE-Br) and serum PRL levels (PRL-S), with changes in ambulation and rearing counts in As (a), Mn (b), Pb (c) and metal mixture (Mixt) (d) treated animals. Plots were obtained by Factor Analysis extracting two components and using the Varimax rotation method. N=6 in each group.*

The analysis of the relationships among cholinergic and dopaminergic neurotransmission and motor activity revealed that in all single metal treated groups both neurotransmission parameters had analogous distances for ambulation or rearing activities (Figure 2 a, b and c). Nevertheless, a slight higher proximity among motor activity parameters and PRL-S levels in the Pb group and AChE-Br activity in the Mn group was detected. In contrast, in the metal mixture treated group, the neurotransmission parameter AChE-Br activity had the closest and marked proximity to both motor activity parameters (Figure2 d).

Metals and heme precursors in brain

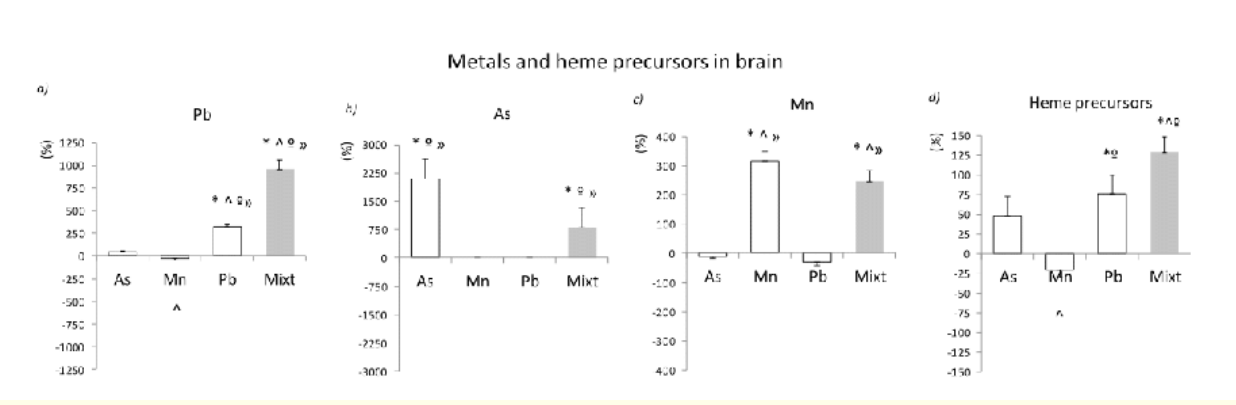

Figure 3: *Brain concentrations of Pb (a), As (b), Mn (c) and heme precursor levels (ALA, uroporphyrins plus coproporphyrins) in As, Mn, Pb and metal mixture (Mixt) treated groups. N=6 each group. Data are expressed as mean± SD of the variation in % relative to the control group. In C, Mn and Pb groups, As levels were not quantified, since they were lower than the QL. All groups were compared by Mann-Whitney tests: *, ^, º, and » are p < 0.05 versus C, As, Mn and Pb.*

Brain Pb levels were significantly higher in the metal mixture-treated group compared to all the other groups ($p < 0.05$) (Figure3 a), while in the Pb-treated group the levels of this metal was significantly higher than in controls and the other single metal treated groups ($p < 0.05$) (Figure3 a). As levels in the As- and metal mixture-treated rats were significantly higher relative to all the other groups ($p < 0.05$) (Figure3 b). Brain Mn levels were significantly higher ($p < 0.05$) in the Mn- and metal mixture-treated groups compared to control, As- and Pb-treated groups ($p < 0.05$) (Figure3 c).

Brain heme precursor levels in the As treated group showed a trend for an increase relative to the controls ($p > 0.05$). The accumulation of heme precursors in the Mn treated group was significantly lower than in the As group ($p < 0.05$). Treatment with Pb caused a significant increase in brain heme precursor levels when compared with controls and the Mn-treated group. The mixture-treated group exhibited the highest level of heme precursors with statistical significance ($p < 0.05$) compared to all other groups, except the Pb-treated rats (Figure 3d).

Table 1: Multiple regression was performed using As, Mn, Pb and heme precursors concentrations in the brain as independent variables, to predict brain AChE activity (dependent variable) in mixture treated rats. Standardized β coefficients reflect the relative contribution of each parameter to AChE activity modification.

Table 1 shows that in mixture treated rats Pb concentration in the brain is the most prominent factor to change AChE-Br activity and evinces an inverse relationship between these parameters (β=-1.571). Both As and Mn brain levels show a positive relationship with the activity of AChE-Br, with Mn levels possessing a higher β coefficient than As concentrations (β=0.611 and β=0.387 respectively). The concentration of heme precursors in the brain was found to be less important in influencing the AChE-Br activity (β=-0.110) (Table 1).

Parameter	β coefficients (standartized)
As_Br	0.387
Mn_Br	0.611
Pb_Br	-1.571
Heme Precursors_BR	-0.110

Table 1: *Multiple regression was performed using As, Mn, Pb and heme precursors concentrations in the brain as independent variables, to predict brain AChE activity (dependent variable) in mixture treated rats. Standardized β coefficients reflect the relative contribution of each parameter to AChE activity modification.*

$R^2 = 0{,}695;\ p < 0.05$

To better understand the influence of heme precursor accumulation on AChE-Br activity and PRL-S concentrations, one-way ANOVA was performed. No significant effects ($p>0.05$) of brain heme precursor accumulation on cholinergic/dopaminergic parameters were noted.

Effect of metals interactions in brain AChE activity.

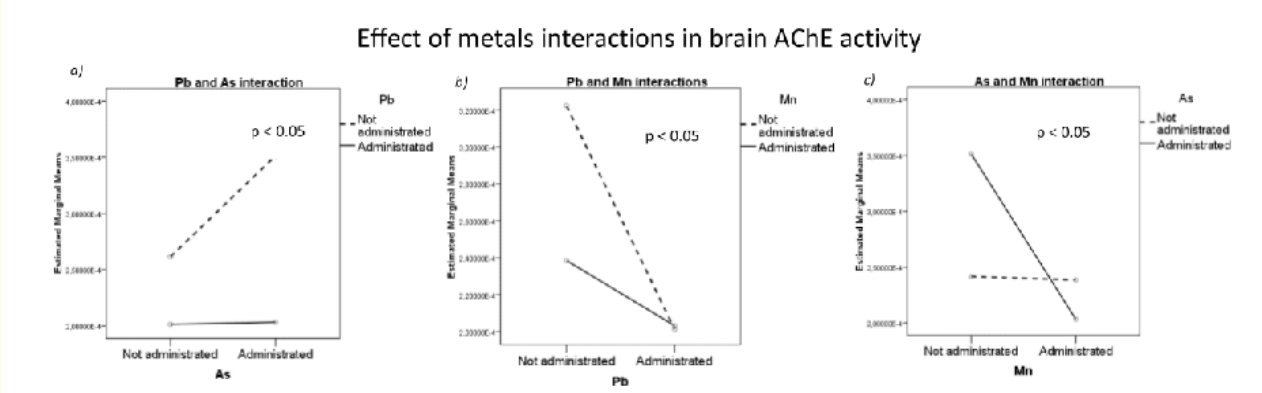

Figure 4: *Effects of metal interactions on brain AChE activity were assessed by factorial ANOVA. Plots of estimated marginal means illustrate the interactions between Pb and As (a), Pb and Mn (b) and As and Mn (c). Non-parallel lines indicate the existence of interaction, with higher difference between the two line's slopes being indicative of a higher degree of interaction. The effect of the interactions in brain AChE activity was significant when p< 0.05.*

Interactions between the three metals were detected (Figure 4 a, b and c), and their combined effects on AChE-Br activity were statistically significant ($p < 0.05$) (Figure 4 a, b and c).

Similar tests were performed to detect interactions between these metals and levels of PRL-S, establishing an interaction between As and Mn ($p < 0.05$) and this dopaminergic marker.

Discussion

Metals are ubiquitous elements in the environment, and the effects induced by exposure to their mixtures at low concentrations [2] remain a public health concern. Pb, As and Mn are neurotoxic metals/metalloids included in a list of toxicants of primary interest for federal agencies [6,43]. However, there is paucity of information on the interactions between these metals and their effects on brain function. Here, we posited that co-exposure to these metals exerts additive/synergic effects on the cholinergic/dopaminergic control of motor activity, with greater motor effects than those induced by exposures to a single metal.

Motor activity reflects the integrated output of the sensory, motor and associative processes of the nervous system in case of the absence of systemic toxicity [12]. Open field tests were performed to obtain data pertaining to ambulation and rearing activity. In general the metal mixture induced a decrease in both motor activity relative to controls and all single treated groups (Figure 1 a and b), attesting to increased toxicity in the co-exposure mixture group.

To evaluate cholinergic neurotransmission, we determined AChE-Br activity. Exposure to the mixture decreased AChE-Br activity, and similar cholinergic changes were noted in the Pb and Mn exposed groups (Figure 1 c). However, the administration of As increased the enzyme's activity (Figure 1 c). Pb-, As- and Mn-induced changes in AChE-Br activity have been previously described [44-46]. The reported effects of Mn are contradictory, with some studies reporting decreased AChE-Br activity while others reporting an increase. Variation in several factors, such as age, dose, route of administration, genetic background and frequency of exposure may reflect this inconsistency in response [45]. These factors might also explain discrepancies in the enzyme's activity upon Pb or As exposures [46-48].

PRL is a neurohormone secreted by the anterior pituitary gland. PRL levels in serum are commonly used to assess dopaminergic function. In fact DA itself can regulate the release of PRL, with DA input from the hypothalamic tuberoinfundibular dopaminergic system being responsible for the secretion of PRL in the pars distalis. Therefore, a decrease in DA turnover in the hypothalamus may lead to a corresponding increase in PRL levels [38]. Levels of the hormone significantly ($p < 0.05$) increased in all treated groups compared with

controls with the exception of the Pb exposed animals (Figure 1 d), consistent with decreased dopaminergic activity (Figure 1 d). Corroborating observations were previously reported in response to Pb, As and Mn exposures [10,12,13].

Factor analysis was performed to assess relationships between neurotransmission parameters and motor activity. An accentuated proximity of AChE-Br activity to both motor activity parameters was apparent in the metal mixture treated group (Figure 2d), suggesting that cholinergic modifications induced by the metal mixture are likely more relevant than dopaminergic changes for the noted decrease in motor activity. Nevertheless, we posit that the decreased dopaminergic activity, inferred by an increase PRL-S levels (Figure 1 d), might have played a secondary role in decreasing ambulation and rearing activities. In fact, the co-localization of Pb, As and Mn in the striatum is established [14-19]. All three metals interfere with both the cholinergic and dopaminergic systems [12,13,25,27-29], and there is a reciprocal circuity between both neurotransmitter systems in this brain area. Cholinergic interneurons express D1-like and D2-like DA receptors through which complex modulation of the cholinergic tonus is achieved (activation or inhibition). Furthermore, cholinergic receptors are expressed on striatal dopaminergic terminals and their activation controls DA release [49]. Thus, acetylcholine plays a major role in the modulation of the activity of dopaminergic neurons. Accordingly, modifying one of these neurotransmitter systems is likely perturb the other [50]. The balance between the two neurotransmitter systems controls cognitive functions, reward action and motor functions, with modifications in the dopaminergic/cholinergic pathway causing symptoms inherent to PD [49,51].

We also evaluated the relative influence of each metal and heme precursor accumulation in the brain to AChE-Br activity modifications. Standartized β coefficients of multiple regressions, using the activity of AChE-Br as a dependent variable, were compared. The inverse relationship observed between Pb levels and AChE-Br activity (β=-1.571) (Table 1) is in agreement with the Pb-induced decrease in the enzyme's activity (Figure 1 c). Notable is also the observation that Pb levels in brain had the highest absolute β coefficient value (Table 1) of all independent parameters included in the regression equation. This suggests that the levels of brain Pb might be the most important factor in modifying AChE-Br activity. The metal-mixture-treated group also exhibited decreased levels of As and Mn, as compared with the respective single exposed groups (Figure 3 b and c); while the degree of decrease of Mn levels in brain was higher than As (Figure 3 b and c), the value of the β coefficient of Mn was also higher than the one determined for As (Table 1). These outcomes are suggestive of dose-dependent effects of As or Mn on AChE-Br activity.

Pb, As and Mn are known to interfere with enzymes of the heme synthesis biosynthetic pathway, leading to increased heme precursors body burden. The exposure to Pb, As or Mn results in increased ALA levels, As exposure increases uroporphyrins concentrations and coprporphyrins levels are increased by both Pb or As [30-32]. Porphyrins can accumulate in the brain and induce neurotoxic events [35]. In agreement, the highest accumulation of heme precursors in the brain was detected in the metal mixture treated rats ($p < 0.05$) (Figure 3d). ALA´s deleterious effects in the brain include the induction of oxidative stress, conditions known to interfere with motor behaviour [35,52]. Additionally, in vitro experiments have shown that ALA and porphobilinogens, which are reduced forms of porphyrins, inhibit presynaptic release of acetylcholine [53]. Herein, the β coefficient value of heme precursor levels in the brain indicated that the contribution of this parameter in modifying AChE-Br activity was smaller than Pb, As or Mn levels (Table 1). Moreover and unexpectedly, one-way ANOVA showed that heme precursors accumulation in the brain did not have a significant effect ($p >< 0.05$) on neither AChE-Br activity nor PRL-S levels ($p > 0.05$). Previous work from our laboratory indicated that urinary ALA levels and changes in the urinary porphyrin profile could possibly attest to neurotoxic effects on motor activity in rats co-treated with a mixture of Pb, As and Mn [33,54]. It is conceivable only acetylcholine is affected by heme precursors [53] with AChE being spared. Alternatively heme precursor accumulation in the brain may affect motor activity by other distinct mechanisms. Notably, ALA competes for the binding of GABA to GABA receptors, possibly provoking some of the symptoms of acute porphyria [55]. GABA neurotransmission in the nucleus accumbens and other parts of the corpus striatum influences motor activity [56] with stimulation of GABA-B receptors inhibiting CNS function [57]. Additionally is has been proposed that direct toxic effects of porphyrin precursors on the nervous system, as well as intracellular metabolic derangement resulting from heme deficits contribute to neurologic disorders [58].

Along with the effects of Pb, As and Mn on AChE-Br activity, we tested by factorial ANOVA whether additive/synergic interactions between these metals might affect the function of this enzyme. Non-parallel lines in Figure 4 a, b and c suggest interactions between Pb, As and Mn. The similarity of the angle between the two lines in the three figures (Figure 4 a, b and c) suggests also that the degree of the three interactions was analogous. Moreover significant ($p < 0.05$) effects of these interactions were detected on AChE-Br (Figure 4 a, b and c). In contrast, only the interaction between As and Mn had a significant effect ($p < 0.05$) on PRL-S concentrations. This outcome might explain the higher damage to the cholinergic control of motor activity in co-exposures to Pb, As and Mn, when compared with the dopaminergic routes (Figure 1 a and b). Paradoxically, there was a higher proximity among motor activity parameters and PRL-S in the Pb treated group (Figure 2 c), suggesting that Pb's effects on the cholinergic system might be mediated via dopaminergic dysregulation. However in the metal mixture group, despite the presence of higher Pb brain levels compared with rats treated with Pb alone (Figure 3a), factor analysis showed AChE-Br activity is the neurotransmission parameter closest to motor activity (Figure2d). Thus, it is plausible that in the metal-mixture-treated group, the effects of the interactions among brain metals/metalloids on the function of AChE-Br superimposed on the effects of treatment with Pb alone.

Overall the outcomes from this work suggest: that the mixture of Pb, As and Mn induce increased motor activity toxicity, which is exerted through mechanisms different than the ones occurring upon exposures to each one of the elements alone; the co-exposure to these mixture induce higher damage of the cholinergic control of motor activity as compared with dopaminergic function; the effects of the interactions among Pb, As and Mn may have a relevant role to these events.

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