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Brain oxidative stress and selective behaviour of aluminium in specific areas of rat brain: potential effects in a 6-OHDA-induced model of Parkinson's disease

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Abstract

The ability of aluminium to affect the oxidant status of specific areas of the brain (cerebellum, ventral midbrain, cortex, hippocampus, striatum) was investigated in rats intraperitoneally treated with aluminium chloride (10 mg Al^{3+}/kg/day) for 10 days. The potential of aluminium to act as an etiological factor in Parkinson's disease (PD) was assessed by studying its ability to increase oxidative stress in ventral midbrain and striatum and the striatal dopaminergic neurodegeneration induced by 6-hydroxydopamine in an experimental model of PD. The results showed that aluminium caused an increase in oxidative stress (TBARS, protein carbonyl content, and protein thiol content) for most of the brain regions studied, which was accompanied by a decrease in the activity of some antioxidant enzymes (superoxide dismutase, catalase, glutathione peroxidase). However, studies *in vitro* confirmed the inability of aluminium to affect the activity of those enzymes. The reported effects exhibited a regional-selective behaviour for all the cerebral structures studied. Aluminium also enhanced the ability of 6-hydroxydopamine to cause oxidative stress and neurodegeneration in the dopaminergic system, which confirms its potential as a risk factor in the development of PD.

Keywords

6-hydroxydopamine, aluminium, antioxidant enzymes, lipid peroxidation, Parkinson's disease, protein oxidation.

Abbreviations used: 6-OHDA, 6-hydroxydopamine; BBB, blood– brain barrier; CAT, catalase; DA, dopamine; GPx, glutathione peroxidase; MAO, monoamine oxidase; Mit, mitochondria; PCC, protein carbonyl content; PD, Parkinson's disease; PTC, protein thiol content; ROS, reactive oxygen species; SOD, superoxide dismutase; TBARS, thiobarbituric acid reactive substances; TH, tyrosine hydroxylase; TH-ir, TH immunoreactivity.

The human organism is constantly and inevitably exposed to aluminium, a ubiquitous metal which is the third most abundant element in the Earth's crust, representing 8% of total components (Martin 1997). Although, no biological function has yet been attributed to it (Yokel 2002), aluminium is a toxicant implicated in dialysis encephalopathy (Alfrey *et al.* 1976), osteomalacia (Parkinson *et al.* 1979), non-iron responsible anemia (Elliott *et al.* 1978), and also linked to many other diseases including Alzheimer's disease (Exley 1999; Gupta *et al.* 2005), Parkinson's disease (PD; Yasui *et al.* 1992), and amyotrophic lateral sclerosis (Kurland 1988).

Its ubiquity and extensive use in products and processes coupled with its ability to cause neurodegeneration have made aluminium a cause of health concern (Exley 1999; Yokel 2000; Zatta *et al.* 2003). The daily reported mean dietary intake of 3.5 mg may be increased by the frequent use of aluminium-containing antiperspirants and non-prescription drugs (Weburg and Berstad 1986; Flarend *et al.* 2001), as well as by occupational exposure (Meyer-Baron *et al.* 2007). Aluminium entry into the brain occurs mainly through the blood–brain barrier (BBB). Although the mechanism(s) responsible for aluminium transport at the BBB remains unclear, it has been reported that aluminium can penetrate into the brain as a complex with transferrin by a receptor-mediated endocytosis (Roskams and Connor 1990) and bound to citrate via a specific transporter, the system $Xc^{)}$ (L-glutamate/L-cysteine exchanger) is the most recently accepted candidate (Nagasawa *et al.* 2005). The apparently long half-life of aluminium in brain tissue has been used to explain its easy accumulation in the brain (Yokel *et al.* 2001; Sánchez-Iglesias *et al.* 2007b), which together with the long life of neurons could be related to the elevated levels of aluminium found in the brain of some patients suffering PD (Yasui *et al.* 1992) and Alzheimer's disease (Perl and Brody 1980). However, this fact should not be interpreted as the primary cause of those disorders.

Nevertheless, and despite all hitherto reported data concerning aluminium neurotoxicity, the precise molecular mechanisms responsible for its neurotoxicity remain largely unknown. Even though it is not a transition metal, and consequently does not undergo redox reactions, numerous publications have detailed an increase in the formation of reactive oxygen species after aluminium exposure (Nehru and Anand 2005).

Most of the studies performed both *in vitro* and *in vivo* have attributed its neurotoxicity to the lipid peroxidation caused by the interaction between reactive oxygen species and cell membranes (Gutteridge *et al.* 1985; Zatta *et al.* 2002), thus considering the latter as the main targets of the oxidant-mediated damage. Furthermore, aluminium appears to affect the brain activities of several antioxidant enzymes (Julka and Gill 1996). In addition, its ability to affect the expression of dopamine (DA) receptors D_1 and D2 (Kim *et al.* 2007) as well as the functionality of mitochondria (Niu *et al.* 2005) has also been documented. However, the controversy surrounding these findings is such that both pro-oxidant (Zatta *et al.* 2002; Exley 2004) and antioxidant (Oteiza *et al.* 1993a; Abubakar *et al.* 2004a) properties have been attributed to this metal. The usefulness of the studies to clarify the accepted involvement of aluminium in the pathogenesis of some neurodegenerative process has also been brought into question (Savory and Ghribi 2007).

Consequently, the aim of this present study was to investigate the *in vivo* effects induced by aluminium on indices of oxidative stress in the cerebellum, ventral midbrain, cortex, hippocampus, and striatum of rat brain. We quantified the levels of lipid peroxidation (thiobarbituric acid reactive substances, TBARS) and the oxidant status of proteins (protein carbonyl content, PCC; protein thiol content, PTC), as well as an *in vivo* assessment of the effects of aluminium on the activity of certain antioxidant defence enzymes, which included superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase (CAT). To shed some light on the molecular mechanisms involved, the effects of aluminium on the *in vitro* activity of antioxidant enzymes have also been investigated. Finally, taking into account the high levels of aluminium found in the substantia nigra of some patients suffering PD (Yasui *et al.* 1992), we investigated its ability to modify the capacity of 6-hydroxydopamine (6-OHDA) administered intraventricularly in an experimental model of PD (Soto-Otero *et al.* 2002; SánchezIglesias *et al.* 2007a) to cause oxidative stress in ventral midbrain and striatum and to induce neurodegeneration in striatal dopaminergic terminals.

Materials and methods

Chemicals

Aluminium chloride hexahydrate, bovine serum albumin, CAT, cytochrome *c*, desipramine hydrochoride, 3,3'-diaminobenzidine, 2,4-dinitrophenylhydrazine hydrochloride, 5,5'-dithiobis-(2-nitrobenzoic acid), EDTA, GPx, glutathione reductase, hydrogen peroxide, ketamine/xylazine, mouse monoclonal antibody to tyrosine hydroxylase (TH), thiobarbituric acid, xanthine, and xanthine oxidase were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Avidin–biotin-peroxidase complex and biotinylated secondary antibody were purchased from Vector (Burlingame, CA, USA). The water used for the preparations of solutions was Milli-RiOs/QA10 grade (Millipore Corp., Bedford, MA, USA). All remaining chemicals used were of analytical grade and were purchased from Fluka Chemie AG (Buchs, Switzerland).

Animal treatment

Adult male Sprague–Dawley rats (200–250 g) were used to perform the *in vivo* studies. All animals were housed individually in polypropylene cages to reduce extraneous trace element contamination in a room equipped with 12 h light/dark automatic light cycles, maintained at 22 ± 1°C with a relative humidity of 65%. All experiments were carried out in accordance with the 'Principles of laboratory animal care' (NIH publication No. 86-23, revised 1996) and approved by the corresponding committee at the University of Santiago de Compostela. Aluminium dosage was adjusted according to animal's body weight just before each experiment. All rats were allowed a standard maintenance diet (A04, Panlab S.L., Barcelona, Spain) and water *ad libitum*. Animals were randomly assigned to two experimental groups. The first group was subdivided into two subgroups, each consisting of ten animals: rats in subgroup A were daily i.p. injected with aluminium chloride in saline (NaCl 0.9%) at a dose of 10 mg Al^{3+}/kg for 10 days; rats in subgroup B were injected with the same volume of saline over the same period. The second group was subdivided into four subgroups of 11 animals each: rats in subgroup C were used as normal (i.e. non-lesioned) controls, and received the corresponding injections of vehicle

for 10 days; rats in subgroup D were i.p. injected with aluminium chloride at a dose of 10 mg Al^{3+}/kg/day for 10 consecutive days; rats in subgroup E received i.p. injections of saline for 10 days and were lesioned on the 8th day (2 h after i.p. injection) with 200 µg 6-OHDA in 3 µL sterile saline containing 0.2% ascorbic acid injected in the third ventricle; rats in subgroup F were i.p. injected with aluminium chloride (10 mg Al^{3+}/kg/day) for 10 days and were lesioned with 6-OHDA in the same way as subgroup E. Injection solutions containing aluminium were prepared by dissolving aluminium chloride in saline, adjusting to pH 4.6 with sodium hydroxide, and waiting the equilibration time necessary to obtain a clear solution. In all cases, the volume injected was 0.5 mL.

Stereotaxic coordinates for intraventricular injection of 6-OHDA were 0.8 mm posterior to bregma, midline, 6.5 mm ventral to the dura, and tooth bar at 0. The solution was injected with a 10 µL Hamilton syringe coupled to a motorized injector (Stoelting, Wood Dale, IL, USA), at 0.5 µL/min, and the cannula was left *in situ* for 5 min after injection. All surgery was performed under ketamine/ xylazine anaesthesia, and 30 min prior to injection of 6-OHDA, rats received desipramine (25 mg/kg i.p.) to prevent uptake of 6-OHDA by noradrenergic terminals. The accuracy of the lesions and cannula placement were confirmed by post-mortem analysis with cresyl violet staining.

Brain samples

At the end of the experimental period animals of subgroups A and B were stunned with carbon dioxide and killed by decapitation. Brains were quickly removed and rinsed with ice-cold saline. Regional brain segments (cerebellum, ventral midbrain, cortex, hippocampus, striatum) according to Paxinos and Watson (2007) were immediately dissected out on an ice plate. These samples were used for the determination of the enzyme activities of GPx, SOD, and CAT and also for the estimation of TBARS, PCC, and PTC. The detailed procedures used are included in Appendix S1.

Forty-eight hours after lesion with 6-OHDA, five rats of subgroups C, D, E, and F were killed according to the above cited procedure to perform biochemical studies because it has been previously reported that the peak for oxidative stress is reached at this time

(Sánchez-Iglesias *et al.* 2007a). In these rats, the levels of the oxidative stress caused by 6-OHDA were estimated by quantification of both lipid peroxidation and protein oxidation in ventral midbrain and striatum.

Preparation of brain mitochondria

Brain mitochondria from Sprague–Dawley rats weighing 200–250 g were obtained by differential centrifugation according to a previously published method (Méndez-Álvarez *et al.* 1997 and protein concentration determined according to Markwell *et al.* (1978), using bovine serum albumin as the standard.

Determination of TBARS, PCC, and PTC

The TBARS, PCC, and PTC determinations for *in vitro* and *in vivo* experiments were performed spectrophotometrically as previously described in Hermida-Ameijeiras *et al.* (2004) and Sánchez-Iglesias *et al.* (2007a), respectively. 2,4-Dinitrophenylhydrazine hydrochloride and 5,5'-dithiobis-(2-nitrobenzoic acid) were used as chemical dosimeters for PCC and PTC determinations, respectively. For *in vitro* experiments, $AlCl_3$ was pre-incubated with brain mitochondria at a final Al^{3+} concentration of 5 µM.

Measurement of the enzyme activities of SOD, GPx, and CAT SOD and GPx activities were measured spectrophotometrically by slight modifications introduced to the methods of McCord and Fridovich (1969) and Flohe and Gunzler (1984), respectively. CAT activity was polarographically measured by a modification to a previous published method (Méndez-Álvarez *et al.* 1998). *In vitro* effect of aluminium on each enzyme activity was estimated using different Al^{3+} concentrations (10, 50, 100 µM) and the following enzyme activities: 1 U for SOD, 0.2 U for GPx, and 20 U for CAT. The detailed methods are included in Appendix S1.

Determination of MAO activity

Monoamine oxidase (MAO) activity was spectrophotometrically measured in mitochondria preparations as previously described (Soto-Otero *et al.* 2001), using

kynuramine as a non-selective substrate and (-)-deprenyl and chlorgiline as irreversible inhibitors for MAO-A and MAO-B estimation, respectively. Different concentrations of Al^{3+} (10, 50, 100 µM) were incorporated to the incubation to assess the effect of aluminium on both MAO-A and MAO-B activity.

Immunohistochemistry

The remaining six rats of subgroups C, D, E, and F were processed for immunohistochemistry. Animals were killed by a chloral hydrate overdose 1 week post-lesion and then processed for TH immunohistochemistry according to a previously published methodology (Rey *et al.* 2007).

Statistical analysis

Data were expressed as the mean ± SD. Statistical differences were tested using one-way ANOVA followed by Bonferroni's test for multiple comparisons and by *post hoc* Student–Newman–Keuls test for immunohistochemistry studies. The statistical significance was set at $p < 0.05$. Normality of populations and homogeneity of variances were verified before each ANOVA.

Results

In vivo effects of aluminium administration on brain lipid peroxidation and protein oxidation

Lipid peroxidation was assessed by the determination of TBARS concentration, and protein oxidation was estimated by both PCC and PTC. As shown in Fig. 1a, animals exposed to aluminium (10 mg Al^{3+}/kg/day for 10 days) exhibited a significant increase in lipid peroxidation in the regions of cerebellum (+159%), ventral midbrain (+54%), cortex (+20%) and striatum (+33%), while there were no significant changes in hippocampus. TBARS levels in the striatum were particularly high when compared with those found in other cerebral regions and in both aluminium- treated and control rats.

Following aluminium treatment, both PCC (Fig. 1b) and PTC (Fig. 1c) were significantly elevated when compared with controls in cerebellum (+26%, +19%, respectively), ventral midbrain (+135%, +15%, respectively) and striatum (+26%, +16%, respectively), while there was a significant decrease in the cortex region (-12%, -16%, respectively) and in the hippocampus (-20%, -26%, respectively). When compared with other areas of non-treated animals, PCC and PTC control levels were significantly higher in cerebral cortex and lower in the ventral midbrain (Figs. 1b and c).

In vivo effects of aluminium administration on the brain activity of different antioxidant enzymes

The effects of aluminium administration (10 mg Al^{3+}/kg/day for 10 days) on the activity of different antioxidant enzymes (SOD, GPx, CAT) were also studied in several brain regions, and the results showed that the i.p. administration of aluminium influenced SOD activity (Fig. 2a). Thus, there was a significant decrease in cerebellum (-26%) and cortex (-21%) of the aluminium-treated group, while a significant increase was noted in hippocampus (+22%). No significant changes were detected in ventral midbrain and striatum when compared with the controls. As depicted in Fig. 2b, exposure to aluminium caused depletion in the activity of GPx in cerebellum (-26%), ventral midbrain (-28%), cortex (-28%), and striatum (-11%). By contrast, GPx activity increased in the hippocampus (+40%). Exposure of rats to aluminium decreased the levels of CAT activity (Fig. 2c) in the cerebellum (-41%), ventral midbrain (-29%), and striatum (-25%) compared with respective controls. CAT activity was not significantly modified in the cortex, but showed a significant increase in the hippocampus (+35%).

Effects of aluminium administration on the degeneration of DA terminals in the striatum

In control rats, i.e. rats not lesioned with 6-OHDA (subgroup C) and rats treated with aluminium alone (subgroup D), the dopaminergic neurons in the pars compacta of the substantia nigra were intensely immunoreactive to TH, and a dense and evenly distributed TH immunoreactivity (TH-ir) was observed throughout the striatum, which indicated the presence of a dense network of nigrostriatal dopaminergic terminals (Fig. 3a and b). No

significant changes in the density of DA striatal terminals were observed in control rats (i.e. not injected with 6-OHDA; subgroups C and D; Fig. 4). TH-ir terminal density was higher in the control groups (subgroups C and D; Fig. 3a and b) than in rats that were intraventricularly injected with 6-OHDA (subgroups E and F; Fig. 3c and d). We observed a significant decrease in TH-ir terminal density (-27%) in rats intraventricularly injected with 6OHDA when compared with control rats (subgroup-C rats). In rats i.p. treated with aluminium and subjected to intraventricular injection of 6-OHDA (subgroup F), the reduction in the density of DA striatal terminals with respect to the control rats (subgroup D) and rats injected with 6OHDA (subgroup E) was statistically significant (-48% and -28%, respectively; Fig. 4).

Effects of aluminium administration on brain lipid peroxidation and protein oxidation in 6-OHDA-lesioned rats and controls

Once again, the concentration of TBARS was used as an index of lipid peroxidation in striatum and ventral midbrain (Fig. S1a). Both regions of animals i.p. injected with aluminium alone (subgroup D) showed a statistical difference in TBARS production compared with non-lesioned (subgroup C) control rats (+40% in ventral midbrain and +16% in striatum). Intraventricular injection of 6-OHDA resulted in a significant increase in TBARS concentration in both striatum and ventral midbrain when compared with control rats (+29% and +76%, respectively) and to animals treated with aluminium (+12% and +26%, respectively). Ventral midbrain and striatum TBARS levels in rats i.p. treated with aluminium and intraventricularly injected with 6-OHDA were +107% and +40% (respectively) increased in respect to control animals. When compared with rats only treated with aluminium (i.e. without 6-OHDA; subgroup D), the TBARS levels of subgroup-F rats were markedly increased in both regions (+47% in ventral midbrain and +21% in striatum) and there were also significant differences when compared with subgroup E, rats only lesioned with 6-OHDA (+17% in ventral midbrain and +8% in striatum). Protein oxidation was assessed by the determination of PCC and PTC in the samples of striatum and ventral midbrain. No significant changes were noticed in the PTC (Fig. S1c) except for rats lesioned with 6-OHDA when compared with animals

treated only with aluminium (-11%). By contrast, as shown in Fig. S1b, intraventricular injection of 6-OHDA induced a significant increase in the PCC in striatum and ventral midbrain (+33% and +22%, respectively) when compared with control rats. Treatment with aluminium (subgroups D) revealed a significant difference in ventral midbrain and striatum when compared with control rats (+11% and +18%, respectively). On the other hand, in subgroup F (animals treated with aluminium and lesioned with 6-OHDA) the PCC was reduced in both regions (-16% in ventral midbrain and striatum) when compared with the rats lesioned with 6OHDA.

In vitro effects of aluminium on the lipid peroxidation and protein oxidation induced by 6-OHDA autoxidation in brain mitochondrial preparations

As depicted in Fig. S2a, the incubation of 6-OHDA (10 µM) with brain mitochondria at 37°C for 20 min induced a significant production of TBARS when compared with the mitochondria (Mit) and Mit + Al controls (+91% and +116%, respectively). Significant changes in TBARS production (+100%, +127%) were observed when the autoxidation of 6-OHDA occurred in the presence of Al^{3+} (5 µM) when compared with the Mit and Mit + Al controls, respectively. However, the presence of aluminium did not significantly affect the level of TBARS found after the incubation of 6-OHDA in mitochondrial preparations when compared with the Mit + 6-OHDA control. The incubation of 6-OHDA (10 µM) with brain mitochondria at 37°C for 20 min induced a significant increase in the PCC (+7%; Fig. S2b) when compared with the Mit control. However, the results obtained in the presence of Al^{3+} (5 µM) were not significantly different from control values. As can be seen in Fig. S2c, the PTC exhibited a significant reduction (-9%) in the incubation of 6-OHDA (10 µM) with brain mitochondria at 37°C for 20 min when compared with that obtained in the control with mitochondria alone and Mit + Al^{3+}. The presence of aluminium had no significant effect on the PTC found with mitochondrial incubations when compared with controls.

In vitro effects of aluminium on the enzyme activities of GPx, SOD, CAT, and MAO

The results regarding the effect of aluminium on the *in vitro* activities of GPx, CAT,

SOD, MAO-A and MAO-B enzymes are displayed in Table S1. In all cases, no significant differences were detected between assays performed in the presence and in the absence of Al^{3+} (10, 50, 100 µM).

Discussion

Aluminium is a non-redox metal whose accumulation in the brain has been linked to various neurodegenerative diseases (Yokel 2000; Zatta *et al.* 2003). Several hypotheses have been given to explain its reported ability to promote biological oxidations (Exley 2004). Thus, it has been shown to facilitate iron-induced lipid peroxidation (Gutteridge *et al.* 1985); non-iron-induced lipid peroxidation (Verstraeten and Oteiza 2000); non-iron-mediated oxidation of NADH (Kong *et al.* 1992); and non-iron-mediated formation of the hydroxyl radical (Méndez-Álvarez *et al.* 2002). Additionally, it also appears to inhibit several antioxidant enzymes in different parts of the brain (Nehru and Anand 2005).

In this study, aluminium was administered at a dosage regimen to guarantee its accumulation in different areas of rat brain (Sánchez-Iglesias *et al.* 2007b). Aluminium-treated animals remained healthy and showed no signs of toxicity during the treatment. However, certain tiny, white inclusions were observed floating in the abdominal cavity, probably caused by a partial precipitation of the aluminium and previously reported (Abubakar *et al.* 2004a).

Previous studies have shown results ranging from no significant changes in TBARS levels (Oteiza *et al.* 1993b), to an increase in TBARS levels (Sharma and Mishra 2006), and a significant decrease in TBARS levels (Abubakar *et al.* 2004b). Our data clearly demonstrate that aluminium exposure caused a significant increase in the levels of TBARS in most of the brain areas studied, but particularly so in the cerebellum. Although, some authors have reported a decrease (Esparza *et al.* 2003) or no changes (Deloncle *et al.* 1999) in the levels of TBARS, our results are in total (Esparza *et al.* 2005; Nehru and Anand 2005; Dua and Gill 2001; Jyoti *et al.* 2007) or partial agreement (Julka and Gill 1996) with most previously published data. Curiously, we found no significant changes in the levels of TBARS in the hippocampus. In our opinion, the

apparent contradictory results found in the literature are due to the use of different chemical forms for aluminium exposure (Julka and Gill 1996; Esparza *et al.* 2003) and/or to the use of different administration routes (Deloncle *et al.* 1999; Jyoti and Sharma 2006) as we have demonstrated recently (Sánchez-Iglesias *et al.* 2007b).

Another index to assess oxidative stress is the oxidant status of proteins. We found that the effects caused by aluminium on both PCC and PTC were not homogenous for all areas studied. Indeed, our data showed that aluminium provoked an increase in both PCC and PTC in the cerebellum, ventral midbrain, and striatum, whereas a decrease was found in the cortex and hippocampus. The most noteworthy effect of these results is the lack of correlation between the increase observed in the PCC, ostensibly a consequence of a situation of oxidative stress, and the apparently contradictory increase in PTC. The increase in the PTC may be a consequence of increased GSH production (Khanna and Nehru 2007) and its subsequent capacity to reduce disulfide groups in proteins. Our results contrast with previous findings that aluminium exposure affects neither PCC (Oteiza *et al.* 1993b) nor PTC (Oteiza *et al.* 1993b) in whole brain. Others report a significant increase of PCC in both hippocampus (Jyoti and Sharma 2006) and cortex (Jyoti *et al.* 2007) and a significant loss of PTC in whole brain (Dua and Gill 2001). Once again, this variability in relation to previous data appears to corroborate the importance of the chemical speciation of aluminium and the route of administration. Interestingly, the aluminium salt used not only affects the accumulation of this metal in different cells but also modulates its toxic effects (Le´vesque *et al.* 2000). Despite these findings and taking into account the complexity of the aluminium speciation chemistry (Bharathi *et al.* 2008), it is very difficult to gain insight into the relation between the particular chemical speciation of aluminium in the brain and its biological effects. Nevertheless, our results clearly show that aluminium can create a situation of oxidative stress in most brain areas. This is mainly a consequence of the fact that aluminium is a strong Lewis acid which allows it to react with the superoxide anion and form a metal–superoxide complex ($AlO^{\bullet 2+}$), which is a more potent oxidant than the superoxide anion and putatively has a high catalytic potential, as suggested by Kong *et al.* (1992) and later discussed by Exley (2004).

These changes were accompanied by significant variations in the activity of SOD, GPx, and CAT. Our results indicate that all the neural tissues examined, except hippocampus, showed similar behaviour patterns and emphasize a significant decrease in the enzyme activity of most antioxidant systems, and agree with previous studies (Julka and Gill 1996; Dua and Gill 2001; Abubakar *et al.* 2004a; Nehru and Anand 2005; Jyoti *et al.* 2007). Our *in vitro* experiments show no significant modification in the enzyme activity of either of SOD, GPx, and CAT, which we interpret as a declination of the gene expression of antioxidant enzymes (González-Muñoz *et al.* 2008). This reduction in antioxidant enzyme activity helps to explain the brain oxidative stress observed following aluminium administration. Furthermore, the reduction in GPx activity also explains the increase in the ratio GSH/GSSG previously reported by other authors (Esparza *et al.* 2003; Khanna and Nehru 2007), which helps explain the increase in the PTC found after aluminium exposure. The inability of aluminium to affect both MAO-A and MAO-B activity discards their involvement in the aluminium-induced brain oxidative stress. Data showing a partial disagreement with our findings have also been reported (Esparza *et al.* 2005), but is possibly because of the regimen of aluminium administration. A putative explanation for the increase in the activity of SOD, GPx, and CAT in the hippocampus (Sánchez-Iglesias *et al.* 2007b), may be the existence of a compensatory mechanism because of the ability of cells to increase the expression of antioxidant enzymes when exposed to high levels of toxicants (Gechev *et al.* 2002), an effect which could overcome the ability of moderate levels of aluminium to inhibit gene expression of antioxidant enzymes. Another interpretation could be the confinement of aluminium in granulovacuoles of neurofibrillar tangles because of its high capacity to interact with tau proteins in hippocampal cells (Walton 2006), thus reducing the presence of free aluminium and consequently its disposition to affect the activity of antioxidant enzymes and generate oxidative stress. Although, our findings contrast with those of Julka and Gill (1996), similar results were also found by Esparza *et al.* (2003).

We used an animal model of PD induced by 6-OHDA administration in the third ventricle to assess the ability of aluminium to affect both the index of oxidative stress in the nigrostriatal system and the extent of lesions of the DA system in the striatum. Our

results showed that aluminium causes an enhancement in 6-OHDA-induced lipid peroxidation and protein oxidation (except for PTC in ventral midbrain). The administration of aluminium alone (i.e. without 6-OHDA) also caused a significant difference in the levels of both lipid peroxidation and protein oxidation for PCC. Although, these results are in partial agreement with the results obtained with non-lesioned rats, they do not agree with the *in vitro* results obtained using brain mitochondria preparations, in which we found no significant effect because of the presence of aluminium. Evidently, these findings corroborate the importance of the *in vivo* studies in this kind of investigation, which appears to be a consequence of the tight relationship existing among the different molecular mechanisms coexisting within the brain. Our immunochemical study showed that aluminium administration alone caused no significant change in TH-ir striatal terminals. In contrast, lesion with 6-OHDA caused a loss of TH-ir striatal terminals, which was significantly increased with the administration of aluminium. Obviously, these data show the ability of aluminium to increase the capacity of 6-OHDA to cause nigrostriatal neurodegeneration. Evidently, the importance of these data is increased by the ability of neuromelanin to bind aluminium which may be the cause of the high concentration of aluminium found in the brain of certain patients suffering PD (Yasui *et al.* 1992). Furthermore, our results also help us understand the reported ability of aluminium to potentiate etiological agents and accelerate the progression of a disease (Exley and Esiri 2006). In conclusion, aluminium acts mainly as a pro-oxidant probably by reducing the activity of defence antioxidant enzymes. In addition, the increase in oxidative stress, together with enhanced 6-OHDA-induced neurodegeneration, suggest the participation of free radical-induced oxidative cell injury in mediating aluminium neurotoxicity. Interestingly, we demonstrated that aluminium neither enhanced lipid peroxidation nor decreased antioxidant enzyme activities in the hippocampus. Given that aluminium content was markedly increased in the hippocampus (Sánchez-Iglesias *et al.* 2007b), this dual effect could be related to the biphasic behaviour of aluminium previously reported by Abubakar *et al.* (2004a). In our opinion, the high concentration of aluminium in the hippocampus may possibly induce an increase in defence enzyme levels, which could cause the steady state of TBARS levels after

aluminium exposure. However, in the remaining cerebral areas, antioxidant enzymes have a lower activity, which is interpreted as a consequence of the postulated oxidant potential of $AlO_2^{\bullet 2+}$ (Kong *et al.* 1992; Exley 2004) and consequently the factor responsible for the oxidative impairment found in those brain areas. Finally, we assume that the distinct cerebral areas exhibit different sensitivities to aluminium, which can be partly explained by the differences in the brain barrier mechanisms. In fact, our data lean towards the concept of the blood–brain regional barrier introduced by Zheng *et al.* (2003). Evidently, the hypothesis that it would be more accurate to talk about a blood–hippocampal barrier and a blood–striatum barrier, for example, needs to be corroborated and characterized by future research.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Figure S1 Levels of TBARS (a), protein carbonyl content (b), and protein thiol content (c) in both ventral midbrain and striatum of different groups of rats: control, control + Al-treated, 6-OHDA-lesioned, 6-OHDA-lesioned + Al-treated.

Figure S2 *In vitro* effects of aluminium on TBARS formation (a), protein carbonyl content (b), and protein thiol content (c) induced by the autoxidation of 6-OHDA in mitochondrial preparations from rat brain.

Table S1 *In vitro* effects of aluminium on the enzyme activities of SOD, GPx, CAT, and MAO.

Appendix S1 Supplementary methods.

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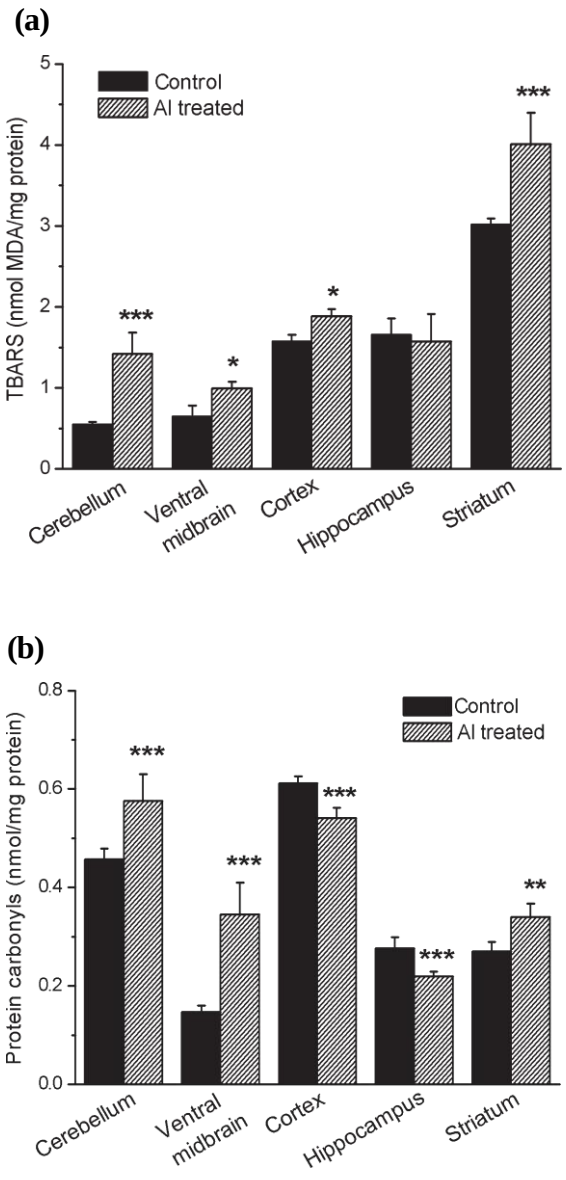
Fig. 1 Levels of TBARS (a), protein carbonyls (b), and protein thiols (c) in different brain areas of rats i.p. treated with saline (control) or aluminium chloride (10 mg Al^{3+}/kg/day) for 10 days. Abbreviation: Al, aluminium. Data are expressed as mean ± SD from five rats (n = 5). Significance of differences among groups was assessed by a one-way ANOVA followed by a Bonferroni's test. Asterisks denote values significantly different from the corresponding control; *p < 0.05, **p < 0.01, and ***p < 0.001.



(c)

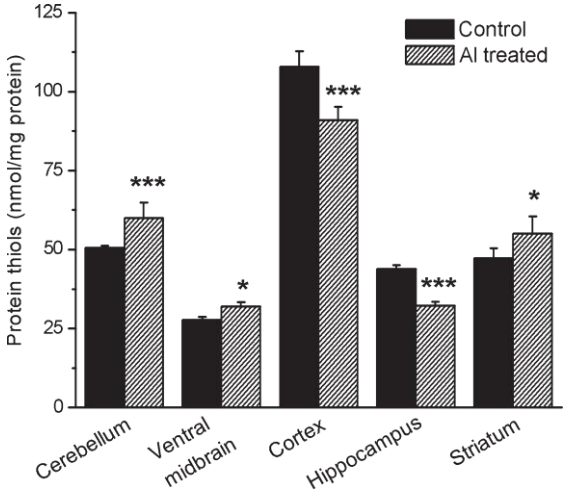
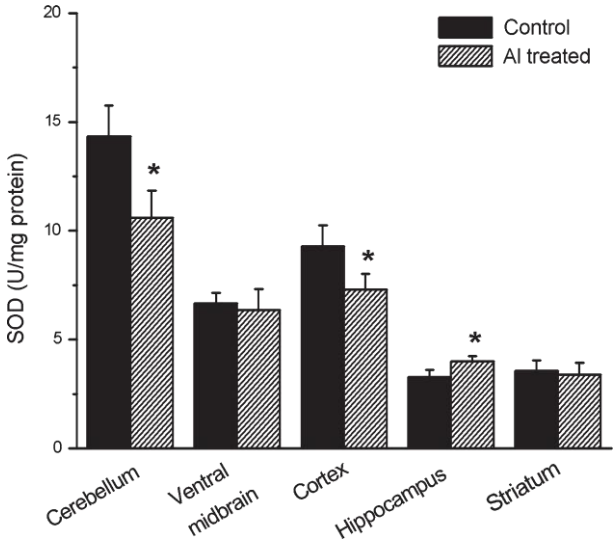

Protein thiols (nmol/mg protein)
125
100
75
50
25
0
Control
Al treated
***
*
***
***
*
Cerebellum
Ventral midbrain
Cortex
Hippocampus
Striatum

Fig. 2 Enzyme activity of: SOD (a), GPx (b), and CAT (c) in different brain areas of rats i.p. treated with saline (control) or aluminium chloride (10 mg Al^{3+}/kg/day) for 10 days. Abbreviation: Al, aluminium. Data are expressed as mean ± SD from five rats (n = 5). Significance of differences among groups was assessed by a one-way ANOVA followed by a Bonferroni's test. Asterisks denote values significantly different from the corresponding control; *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$.

(a)



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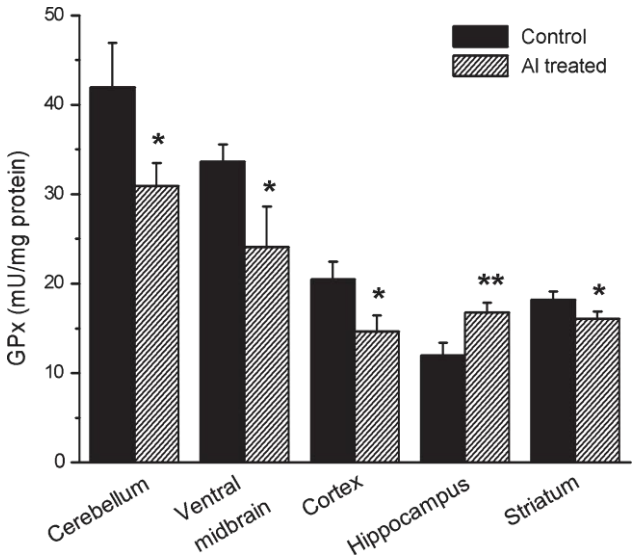

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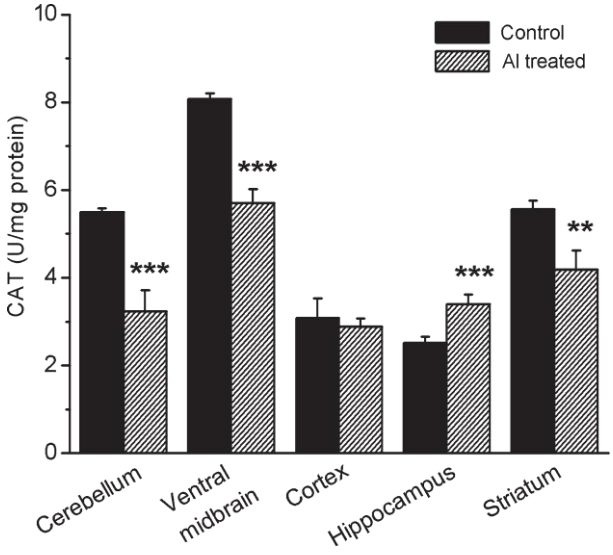
Control
Al treated
CAT (U/mg protein)
10
8
6
4
2
0
***
***
***
**
Cerebellum
Ventral midbrain
Cortex
Hippocampus
Striatum

Fig. 3 Microphotographs showing changes in the striatal TH-ir 1 week post-lesion in rats injected intraventricularly with vehicle (i.e. controls; a), i.p. treated with aluminium (10 mg Al^{3+}/kg/day) for 10 days (b), lesioned intraventricularly on the 8th day with 200 µg 6-OHDA (c), or i.p. treated with aluminium for 10 days and lesioned on the 8th day with 6-OHDA (d). Scale bar, 1 mm.

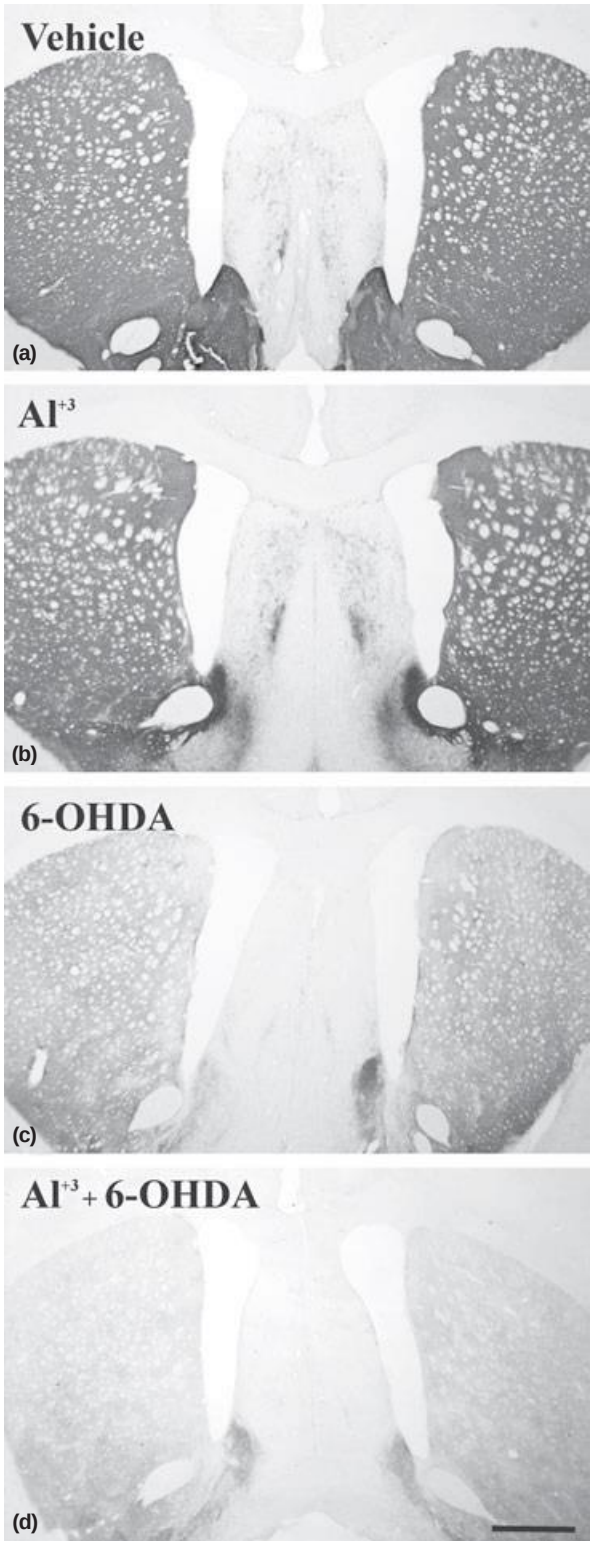

Fig. 4 Density of striatal dopaminergic terminals estimated as optical density, 1 week post-surgery in the different experimental groups. Abbreviation: TH-ir, tyrosine hydroxylase immunoreactivity. Optical densities are expressed as percentages of the values obtained in the control groups. Data are expressed as mean ± SD obtained from six animals (n = 6). Mean that differ significantly are indicated by a different letter (p < 0.05, one-way ANOVA and *post hoc* Student– Newman–Keuls test).