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Exposure of young rats to diphenyl ditelluride during lactation affects the homeostasis of the cytoskeleton in neural cells from striatum and cerebellum

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ABSTRACT

In the present report we examined the effect of maternal exposure to diphenyl ditelluride $(PhTe)_2$ (0.01 mg/kg body weight) during the first 14 days of lactational period on the activity of some protein kinases targeting the cytoskeleton of striatum and cerebellum of their offspring. We analyzed the phosphorylating system associated with glial fibrillary acidic protein (GFAP), and neurofilament of low, medium and high molecular weight (NF-L, NF-M and NF-H, respectively) of pups on PND 15, 21, 30 and 45. We found that $(PhTe)_2$ induced hyperphosphorylation of all the proteins studied on PND 15 and 21, recovering control values on PND 30 and 45. The immunocontent of GFAP, NF-L, NF-M and NF-H in the cerebellum of 15-day-old pups was increased. Western blot assays showed activation/phosphorylation of Erk1/2 on PND 21 and activation/phosphorylation of JNK on PND 15. Otherwise, p38MAPK was not activated in the striatum of $(PhTe)_2$ exposed pups. On the other hand, the cerebellum of pups exposed to $(PhTe)_2$ presented activated/phosphorylated Erk1/2 on PND 15 and 21 as well as activated/phosphorylated p38MAPK on PND 21, while JNK was not activated. Western blot assays showed that both in the striatum and in the cerebellum of $(PhTe)_2$ exposed pups, the immunocontent of the catalytic subunit of PKA (PKAcα) was increased on PND 15. Western blot showed that the phosphorylation level of NF-L Ser55 and NF-M/NF-H KSP repeats was increased in the striatum and cerebellum of both 15- and 21-day-old pups exposed to $(PhTe)_2$. Diphenyl diselenide $(PhSe)_2$, the selenium analog of $(PhTe)_2$, prevented $(PhTe)_2$-induced hyperphosphorylation of striatal intermediate filament (IF) proteins but it failed to prevent the action of $(PhTe)_2$ in cerebellum. Western blot assay showed that the $(PhSe)_2$ prevented activation/phosphorylation of Erk1/2, JNK and PKAcα but did not prevent the stimulatory effect of $(PhTe)_2$ on p38MAPK in cerebellum at PND 21. In conclusion, this study demonstrated that dam exposure to low doses of $(PhTe)_2$ can alter cellular signaling targeting the cytoskeleton of striatum and cerebellum in the offspring in a spatiotemporal manner, which can be related to the neurotoxic effects of $(PhTe)_2$.

1. Introduction

The neuronal cytoskeleton comprises a protein network formed mainly by microtubules (MT) and neurofilaments (NF), the intermediate filaments (IFs) of neurons. Neurofilaments are composed of three different polypeptides whose approximate molecular masses are 200, 160, and 68 kDa, and are commonly referred to as heavy (NF-H), medium (NF-M) and light (NF-L) neurofilament subunits (Ackerley et al., 2000). The assembly of the three NF subunits forms a typical NF, in which NF-L is known to polymerize on its own, whilst NF-M and NFH form lateral side-arms (Petzold, 2005). Glial fibrillary acidic protein (GFAP) is the IF of mature astrocytes (Eng et al., 2000) and vimentin (Vim) is the IF of cells of mesenchymal origin (Alberts et al., 2008).

The IF proteins are important phosphoproteins whose phosphorylation is a dynamic process mediated by the action of several protein kinases and phosphatases. The phosphorylation level of IFs provides the cells a mechanism to reorganize the filaments contributing to the maintenance of their homeostasis (Chang and Goldman, 2004). In particular, physiological levels of phosphorylation of NFs promote their integration into a cytoskeleton lattice, controlling the axonal caliber and stabilizing the axon. Therefore the physiological phosphorylation of IF proteins plays a major role on the cellular dynamics and this is dependent on the activation of

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several signaling pathways involved in phosphorylating specific sites on IF subunits in response to intra and extracellular signals (Sihag et al., 2007).

The major sites of phosphorylation of NF-L and NF-M subunits were identified as Ser-55, which is phosphorylated by protein kinase A (PKA); Ser-57, which is phosphorylated by Ca^{2+}/calmodulin-dependent protein kinase II (PKCaMII); Ser-51, by protein kinase C (PKC) (Gill et al., 1990; Heins et al., 1993); and Ser-23, by PKA and protein kinase C (PKC), respectively (Daile et al., 1975; Kemp et al., 1975). On the other hand, most of the phosphorylation sites on NF-M and NF-H are located on multiple lysine-serine-proline (KSP) repeat motifs abundant in the carboxyl-terminal tail domain of these NF subunits (Geisler et al., 1987; Lee et al., 1988; Xu et al., 1992). It is now evident that proline-directed kinases, such as cyclin-dependent kinase 5 (Cdk5) and mitogen-dependent protein kinase (MAPK) are the main kinases that phosphorylate Ser residues on the KSP repeats (Jaffe et al., 1998; Sun et al., 1996; Veeranna et al., 1998).

Phosphorylation of the amino-terminal head domain sites on GFAP and NF proteins plays a key role in the assembly/disassembly of IF subunits into 10 nm filaments and influences the phosphorylation of sites on the carboxyl-terminal tail domain (Sihag et al., 2007). Otherwise, the C-terminal regions of NF-H and NF-M protrude laterally from the filament backbone when phosphorylated (Sihag et al., 2007) and a considerable body of evidence supports the notion that phosphorylation of C-terminal side arms, in particular those of NF-H, regulates NF axonal transport (Shea and Chan, 2008).

The importance of types III and IV IFs, including GFAP and NF subunits, on cellular function is evident from the fact that perturbation of their function accounts for several genetically determined protein misfolding/aggregation diseases (Arbustini et al., 2006; Green et al., 2005). In this scenario, studies showing increased axonal accumulation of NFs in transgenic mice or in mice expressing mutant NF subunit have shown that aberrant organization or assembly of NFs is sufficient to cause disease arising from selective dysfunction and degeneration of neurons (Beaulieu et al., 1999; Julien et al., 1995). In fact, perikaryal accumulation/aggregation of aberrantly phosphorylated neurofilaments is a pathological feature of several human neurodegenerative diseases, such as Alzheimer's disease, motor neuron diseases and Parkinson's disease (Grant and Pant, 2000; Lariviere and Julien, 2004; Nixon, 1993; Sasaki et al., 2006).

Although the tellurium (Te) element rarely occurs in the free state in nature, metallic Te is known to be present in plant material, particularly in members of the Allium family, such as garlic (Larner, 1995). A number of studies have shown that trace amounts of Te are present in body fluids, such as blood and urine (Newman et al., 1989; Siddik and Newman, 1988). Neurotoxicity of tellurium has been reported in the literature. In this context, inorganic tellurium treatment was found to cause significant impairment in retention of the spatial learning task (Widy-Tyszkiewicz et al., 2002). But to date, no telluroproteins have been identified in animal cells. Nowadays, two cases of toxicity in young children from ingestion of metal-oxidizing solutions that contained substantial concentrations of Te were reported in the literature (Yarema and Curry, 2005). Clinical features of acute Te toxicity include a metallic taste, nausea, blackened oral mucosa and skin and garlic odor of the breath (Muller et al., 1989; Taylor, 1996).

Our laboratory have obtained persuasive evidence indicating that diphenyl ditelluride $(PhTe)_2$ is a neurotoxic compound for rats, disrupting the homeostasis of the cytoskeleton. In this context, cytoskeletal proteins from different brain regions of rats constitute important molecular targets of $(PhTe)_2$ both *in vivo* and *in vitro*. We reported that $(PhTe)_2$ induced *in vitro* hyperphosphorylation of GFAP, vimentin and NF subunits in hippocampus of PND 21 rats. This action showed a significant cross-talk among signaling pathways elicited by $(PhTe)_2$, connecting glutamate metabotropic cascade with activation of Ca^{2+} channels (Heimfarth et al., 2011). Nonetheless, $(PhTe)_2$ induced hypophosphorylation of GFAP and NF subunits only in cerebral cortex (not in hippocampus) of 9- and 15-day-old animals through Ca^{2+}-mediated mechanisms (Heimfarth et al., 2012).

In contrast to $(PhTe)_2$, diphenyl diselenide $(PhSe)_2$ exhibits neuroprotective and anti-inflammatory activities in different *in vivo* and *in vitro* models, including against the toxicity of $(PhTe)_2$ (Moretto et al., 2005; Funchal et al., 2006; Nogueira and Rocha, 2011). Accordingly, data from our laboratory showed that $(PhSe)_2$ prevented the *in vitro* effects of $(PhTe)_2$ on the phosphorylating levels of IF proteins in slices of cerebral cortex of 17-day-old rats (Funchal et al., 2006; Moretto et al., 2005). Most importantly, the *in vivo* hyperphosphorylation of cortical IF proteins, induced by a subcutaneous injection of $(PheTe)_2$, was totally reversed by a single injection of $(PheSe)_2$ 24 h after $(PheTe)_2$ administration (Heimfarth et al., 2008).

Taking into account the importance of $(PhTe)_2$ as an intermediate in organic synthesis, the increasing evidence of its neurotoxicity, the high lipophilicity and the increasing possibility of occupational exposure to this compound, the present study evaluated the toxicity of $(PhTe)_2$ transmitted *via* maternal milk on the homeostasis of the cytoskeleton of pups during lactation as well as the ability of $(PhSe)_2$ in preventing these effects induced by low levels of exposure to $(PhTe)_2$. The purpose of these experiments was to define lactation as an important *via* of intoxication with Te and the susceptibility of specific brain structures to $(PhTe)_2$ during a period of intense brain development. In fact, during lactation intense biochemical and morphological changes make brain more susceptible to disruption by neurotoxic agents.

Considering the lipophylicity of this compound, we can suppose that it is excreted in milk like other hydrophobic toxicants, for instance, polychlorinated biphenyls (Nar et al., 2012). Data about the metabolism of $(PhTe)_2$ are also not available in the literature. However, the transformation of part of $(PhTe)_2$ to inorganic Te(IV), which is extremely reactive and could bind to milk proteins, cannot be ruled out. In fact, the determination of tellurium speciation in mothers and pups will be highly needed and, consequently, analytical methodologies must be developed to allow such type of toxicological studies.

We have chosen to study the effects of $(PhTe)_2$ in the cerebellum since the development of this brain structure is mainly postnatal and the vulnerability during this phase of rapid growth has been largely described (Dobbing et al., 1970; Dobbing and Sands, 1973; Dobbing, 1974). Similarly, important developmental events are described in the striatum during the first postnatal weeks (Chesselet et al., 2007; Pérez-Navarro et al., 1993). Therefore, considering striatum and cerebellum, elucidation of the biochemical steps leading to $(PhTe)_2$-induced neurotoxicity in this developmental period provide us new clues to the mechanisms underlying the actions of this neurotoxin in these two brain structures. Therefore, in the present report we describe the effects of dam exposure to $(PhTe)_2$ and/or $(PhSe)_2$ on the cellular signaling targeting the cytoskeleton of striatum and cerebellum in their offspring.

2. Materials and methods

2.1. Radiochemical and compounds

$[^{32}P]Na_2HPO_4$ was purchased from CNEN, São Paulo, Brazil. Benzamidine, leupeptin, antipain, pepstatin, chymostatin, acrylamide and bis-acrylamide and anti-PKAcα, anti-GFAP (G3893), anti-NF-L (N5264), anti-NF-M (N2787) and anti-NF-H (N0142) antibodies were obtained from Sigma (St. Louis, MO, USA). The chemiluminescence ECL kit peroxidase and the conjugated anti-rabbit IgG (A0545) were obtained from Amersham (Oakville, Ontario, Canada). Anti-ERK (#9102), anti-pERK (#3371), anti- anti-SAP/JNK (#4671S), anti-pSAP/JNK (#4671), anti-actin (#4967), anti-PKA (#4782), anti-KSP repeat (#MAB1592) antibodies were obtained from Cell Signaling Technology (USA) and anti-pSer55NF-L (sc12965-R) p38MAPK (sc7972), anti-phospho p38MAPK (sc17852R), were obtained from Santa Cruz Biotechnology Inc. The organochalcogenides $(PhSe)_2$ and $(PhTe)_2$ were synthesized using the method described by Paulmier (1986) and Petragnami (1994), respectively. Analysis of the 1H NMR and ^{13}C NMR spectra showed that the compound obtained presented analytical and spectroscopic data in full agreement with their assigned structures. The purity of the compounds were assayed by high resonance mass spectroscopy (HRMS) and was higher that 99.9%. $(PhTe)_2$ was dissolved in dimethylsulfoxide (DMSO) just before use. The final concentration of DMSO was adjusted to 0.1%. Solvent controls attested that at this concentration DMSO did not interfere with the phosphorylation measurement. All other chemicals were of analytical grade and were purchased from standard commercial supplier.

2.2. Animals

Adult female Wistar rats (200–250 g) and their offspring were obtained from our breeding stock. Rats were maintained on a 12-h light/12-h dark cycle in a constant temperature (22 °C) colony room. On the day of birth the litter size was culled to seven–eight pups. Litters smaller than seven pups were not included in the experiments. Water and a 20% (w/w) protein commercial chow were provided *ad libitum*. The experimental protocol followed the ‘‘Principles of Laboratory Animal Care’’ (NIH publication 85-23, revised 1985) and was approved by the Ethics Committee for Animal Research of the Federal University of Rio Grande do Sul.

2.3. Exposure to diphenyl ditelluride

Animal exposure to $(PhTe)_2$ was carried out as described by Stangherlin et al. (2006). Briefly, sexually naive female rats were mated with males previously tested as fertile (three females and one male in each cage). The onset of pregnancy was confirmed by the presence of sperm in vaginal smears (day 0 of pregnancy) and pregnant dams were immediately housed in individual cages. At birth, the dams received $(PhTe)_2$ (0.01 mg/kg, experimental group) or canola oil (1 ml/kg, control group) *via* subcutaneous (s.c.) injection once daily during the first 14 days of lactational period (sub-chronic exposure). At birth, all litters were culled to seven-eight pups. On PND 15, 21, 30 or 45 the animals from an entire litter were killed by decapitation without anesthesia, the brain was removed and cerebral structures – striatum and cerebellum – were separated. In the experiments with 30 or 45-day-old animals, pups from entire litters were weaned on PND 21 and placed on *ad libitum* standard rat chow diets until sacrifice. In the experiments designed to study prevention of $(PhTe)_2$ effects, animals were treated with a subcutaneous injection of $(PhSe)_2$ (1 mg/kg body weight) 30 min before each $(PhTe)_2$ administration. Rats were sacrificed on PND 21.

2.4. Preparation and labeling of slices

Rats were killed by decapitation, striatum and cerebellum were dissected onto Petri dishes placed on ice and cut into 400 μm thick slices with a McIlwain chopper.

2.5. Preincubation

Tissue slices were initially preincubated at 30 °C for 20 min in a Krebs–Hepes medium containing 124 mM NaCl, 4 mM KCl, 1.2 mM $MgSO_4$, 25 mM Na–HEPES (pH 7.4), 12 mM glucose, 1 mM $CaCl_2$, and the following protease inhibitors: 1 mM benzamidine, 0.1 μM leupeptin, 0.7 μM antipain, 0.7 μM pepstatin and 0.7 μM chymostatin.

2.6. In vitro ^{32}P incorporation experiments

After preincubation, the medium was changed and incubation was carried out at 30 °C with 100 μl of the basic medium containing 80 μCi of [^{32}P] orthophosphate. The labeling reaction was normally allowed to proceed for 30 min at 30 °C and stopped with 1 ml of cold stop buffer (150 mM NaF, 5 mM, EDTA, 5 mM EGTA, Tris–HCl 50 mM, pH 6.5), and the protease inhibitors described above. Slices were then washed twice with stop buffer to remove excess radioactivity.

2.7. Preparation of the high salt-Triton insoluble cytoskeletal fraction from tissue slices

After treatment, IF-enriched cytoskeletal fractions were obtained from striatum and cerebellum of 15-, 21-, 30- or 45-day-old rats as described by Funchal et al. (2003). Briefly, after the labeling reaction, slices were homogenized in 400 μl of ice-cold high salt buffer containing 5 mM KH_2PO_4 (pH 7.1), 600 mM KCl, 10 mM $MgCl_2$, 2 mM EGTA, 1 mM EDTA, 1% Triton X-100 and the protease inhibitors described above. The homogenate was centrifuged at 14,000 × g for 10 min at 4 °C, in Eppendorf centrifuge, the supernatant was discarded and the pellet homogenized with the same volume of the high salt medium. The suspended pellet was centrifuged as described and the

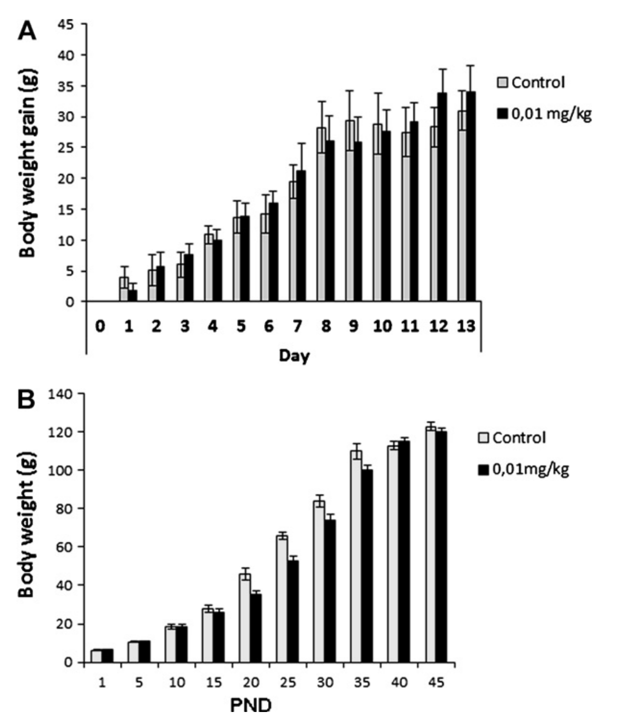

Fig. 1. Effects of $(PhTe)_2$ administered to dams during lactation period on the gain of weight of the dams (A) and on the body weight of their pups (B). Body weight was obtained daily after $(PhTe)_2$ administration. Data were analyzed by a two-way ANOVA (2 treatments × 8 dams or 24 pups weight determinations) with the last factor treated as a repeated measure. Data are reported as means ± SEM of 8–16 animals and expressed in grams.

supernatant was discarded. The final Triton-insoluble IF-enriched pellet, containing NF subunits, Vim and GFAP, was dissolved in 1% SDS and protein concentration was determined (Lowry et al., 1951).

2.8. Polyacrylamide gel electrophoresis (SDS-PAGE)

The cytoskeletal fraction was prepared as described above. Equal protein concentrations were loaded onto 10% polyacrylamide gels and analyzed by SDS-PAGE according to the discontinuous system of Laemmli (1970). After drying, the gels were exposed to X-ray films (Kodak T-Mat) at −70 °C with intensifying screens and finally the autoradiograph was obtained. Cytoskeletal proteins were quantified by scanning the films with a Hewlett-Packard Scanjet 6100C scanner and determining optical densities with an Optiquant version 02.00 software (Packard Instrument Company). Density values were obtained for the studied proteins.

2.9. Preparation of total protein homogenate

Tissue slices were homogenized in 100 μl of a lysis solution containing 2 mM EDTA, 50 mM Tris–HCl, pH 6.8, 4% (w/v) SDS. For electrophoresis analysis, samples were dissolved in 25% (v/v) of solution containing 40% glycerol, 5% mercaptoethanol, 50 mM Tris–HCl, pH 6.8 and boiled for 3 min.

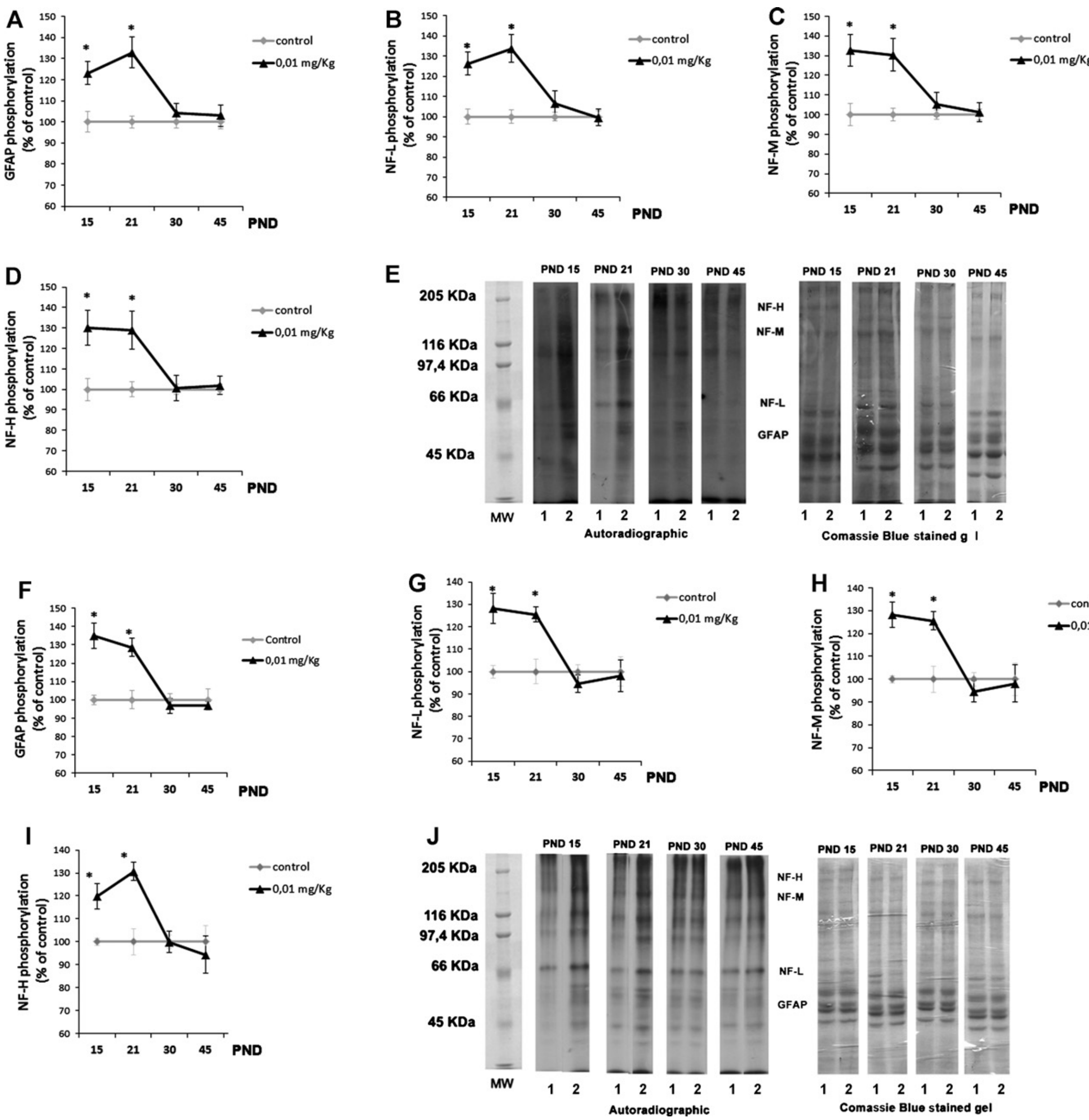

Fig. 2. Effects of $(PhTe)_2$ administered to dams during lactation period on the *in vitro* phosphorylation of IF proteins in striatum (A, B, C, D and E) and cerebellum (F, G, H, I and J) of their pups. At birth, dams received $(PhTe)_2$ (0.01 mg/kg, experimental group) or canola oil (1 ml/kg, control group) *via* subcutaneous injection once daily during the first 14 days of lactational period. On PND 15, 21, 30 or 45 the animals were killed by decapitation without anesthesia, the brain was removed, striatum and cerebellum were isolated and the *in vitro* phosphorylation of IF proteins in the striatum (A, B, C, D and E) and cerebellum (F, G, H, I and J) of the pups were determined. NF-H, high molecular weight neurofilament; NF-M, middle molecular weight neurofilament subunit; NF-L, low molecular weight neurofilament subunit and GFAP, glial fibrillary acidic protein. In Fig. 2E and G: 1 = control; 2 = $(PhTe)_2$. Representative stained gel and autoradiographs of the proteins studied are shown (E, striatum; J, cerebellum). Data are reported as means ± SEM of 10–12 animals and expressed as percent of control. Statistically significant differences from canola oil-treated rats, as determined by one-way ANOVA followed by Tukey–Kramer test are indicated: $^{*}P < 0.05$.

2.10. Western blot assay

Cytoskeletal fractions (50 μg) or homogenate (80 μg) were separated by SDS-PAGE and transferred to nitrocellulose membranes (Trans-blot SD semi-dry transfer cell, BioRad) for 1 h at 15 V in transfer buffer (48 mM Trizma, 39 mM glycine, 20% methanol and 0.25% SDS). The nitrocellulose membranes were washed for 10 min in Tris–buffered saline (TBS; 0.5 M NaCl, 20 mM Trizma, pH 7.5), followed by 2 h incubation in blocking solution (TBS plus 5% defatted dried milk). After incubation, the blot was washed twice for 5 min with TBS plus 0.05% Tween-20 (T-TBS), and then incubated overnight at 4 °C in blocking solution containing the following monoclonal antibodies: anti-NF-H (clone N52), diluted 1:1000, anti-NF-150 (clone NN-18) diluted 1:500, anti-NF-68 (clone NR-4) diluted 1:1000, anti-GFAP (clone G-A-5) diluted 1:400, anti-ERK diluted 1:1000, anti-pERK diluted 1:1000, anti-SAP/JNK (clone 98F2) diluted 1:1000, anti-pSAP/JNK, diluted 1:1000, anti-p38MAPK (A-12) diluted 1:1000, anti-phospho p38, diluted 1:1000, anti-PKAcα, diluted 1:1000, anti-KSP repeats diluted 1:1000 or anti-pSer55NF-L diluted 1:800. The blot was then washed twice for 5 min with T-TBS and incubated for 2 h in blocking solution containing peroxidase conjugated anti-rabbit IgG diluted 1:2000 or peroxidase conjugated anti-mouse IgG diluted 1:2000. The blot was washed twice again for 5 min with T-TBS and twice for 5 min with TBS. The blot was then developed using a chemiluminescence ECL kit. Immunoblots were quantified by scanning the films as described above. Optical density values were obtained for the studied proteins.

2.11. Protein determination

The protein concentration was determined by the method of Lowry et al. (1951) using serum bovine albumin as the standard.

2.12. Statistical analysis

Data were statistically analyzed by one-way analysis of variance (ANOVA) followed by the Tukey–Kramer multiple comparison test when the *F*-test was significant. All analyses were performed using the SPSS software program on an IBM-PC compatible computer.

3. Results

In the present report we attempted to analyze the *in vivo* effects of $(PhTe)_2$ (0.01 mg/kg of body weight) administered to dams during lactation on the homeostasis of the cytoskeleton of their pups. We therefore analyzed the phosphorylating system associated with the IF proteins of striatum and cerebellum of pups on PND 15, 21, 30 and 45. To access the systemic toxicity of the neurotoxin, the body weight of dams and their offspring were initially recorded during the experimental period. Results showed that $(PhTe)_2$ did not reduce body weight of dams during the first 14 days of lactation period, when compared with non-exposed control dams (Fig. 1A). Also, the body weight of offspring from $(PhTe)_2$-injected dams was not altered until PND 45 when compared with control pups (Fig. 1B).

Slices from striatum and cerebellum of pups were incubated with ^{32}P-orthophosphate and the phosphorylation pattern of astrocyte (GFAP) as well as neuron (NF-L, NF-M and NF-H) IF proteins recovered in the cytoskeletal fraction was evaluated during development. As depicted in Fig. 2, we found that $(PhTe)_2$ induced hyperphosphorylation of all the IF proteins studied in the striatum (Fig. 2A–D) and cerebellum (Fig. 2F–I) at PND 15 and 21, recovering control values at PND 30 and 45. Protein levels evaluated by Western blot assay showed increased immunocontent of the GFAP, NF-L, NF-M and NF-H in the cerebellum of 15-day-old pups (Fig. 3B), while in the striatum $(PhTe)_2$ failed to alter the immunocontent of the proteins studied (Fig. 3A).

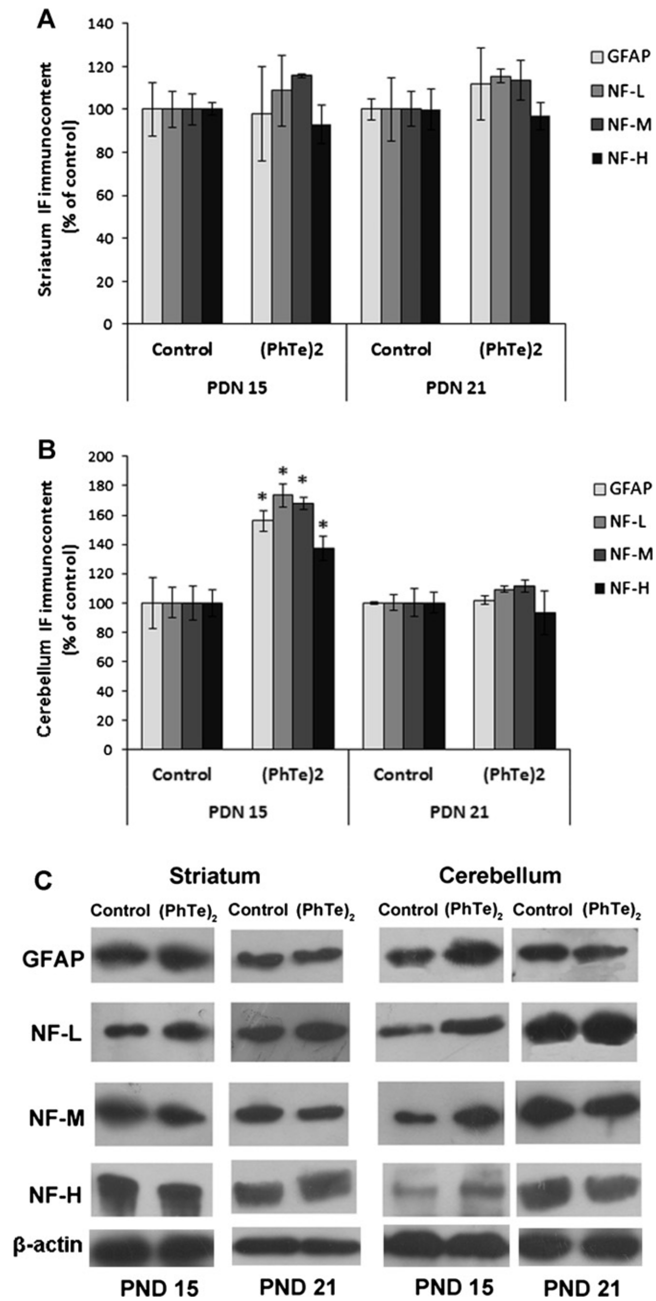

Fig. 3. Effect of $(PhTe)_2$ administered to dams during lactation on the immunoreactivity of IFs in the cytoskeletal fraction from striatum (A) and cerebellum (B) of pups on PND 15 and 21. The IF immunocontent was measured by Western blot assay, as described in Section 2.10, using specific antibodies. Representative blots are shown in (C). β-actin was used as loading control. Data are reported as means ± SEM of 10–12 animals and expressed as percent of control. Statistically significant differences from canola oil-treated rats, as determined by one-way ANOVA followed by Tukey–Kramer test are indicated: $^{*}P < 0.05$. NF-H, High molecular weight neurofilament subunit; NF-M, middle molecular weight neurofilament subunit; NF-L, low molecular weight neurofilament subunit and GFAP, glial fibrillary acidic protein.

Next, we investigated the potential participation of the second messenger-independent protein kinases, which phosphorylate sites located on the carboxyl-terminal tail domain and second messenger-dependent protein kinases, described to target residues on the amino-terminal head domains of the IF subunits (Grant and Pant, 2000) in the $(PhTe)_2$-induced hyperphosphorylation of the IF proteins from striatum and cerebellum of pups.

Western blot assays using specific antibodies against total and phosphorylated forms of MAPKs in the striatum showed activation/phosphorylation of Erk1/2 on PND 21 (Fig. 4A) and activation/phosphorylation of JNK on PND 15 (Fig. 4B). Otherwise, p38MAPK was not activated in the striatum of $(PhTe)_2$ exposed pups (Fig. 4C). On the other hand, the cerebellum of pups exposed to $(PhTe)_2$ presented activated/phosphorylated Erk1/2 at PND 15 and 21 (Fig. 4D) as well as activated/phosphorylated p38MAPK on PND 21 (Fig. 4F), while JNK was not activated (Fig. 4E). In addition, Western blot assays showed that either in the striatum or in the cerebellum of $(PhTe)_2$ exposed pups, the immunocontent of the catalytic subunit of PKA (PKAcα) was increased on PND 15 and 21 (Fig. 5A and B).

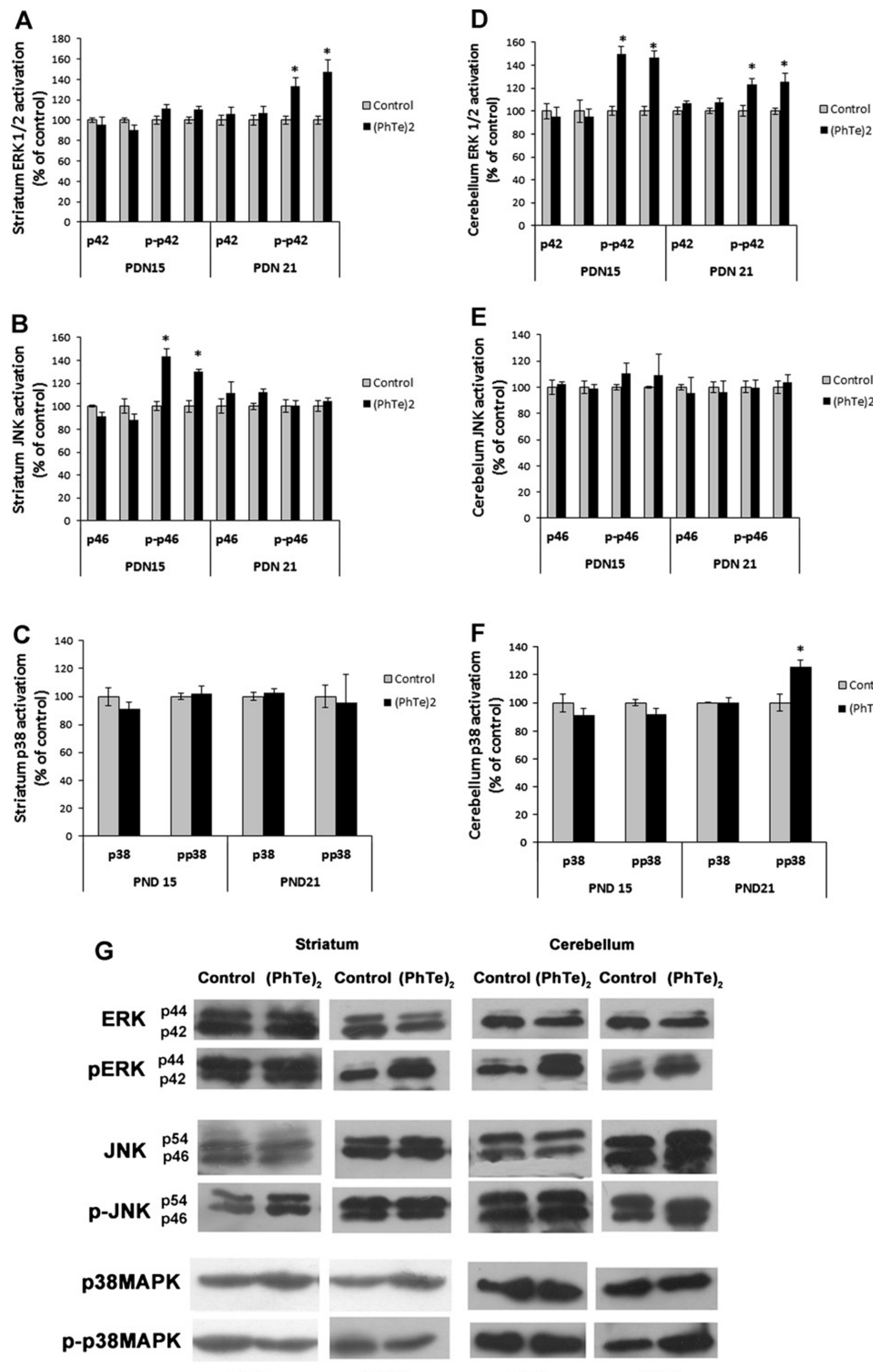

Fig. 4. Effect of $(PhTe)_2$ administered to dams during lactation on MAPK pathways of their pups on PND 15 and 21. Western blot assay of total and phosphorylated forms of ERK1/2 (A, D), JNK (B, E) and p38MAPK (C, F) of striatum (A, B, C) and cerebellum (D, E, F) were carried out as described in Section 2.10. Representative blots are shown (G). β-Actin was used as loading control. Data are reported as means ± SEM of 10–12 animals and expressed as percent of control. Statistically significant differences from canola oil-treated rats, as determined by one-way ANOVA followed by Tukey–Kramer test are indicated: *$P < 0.05$.

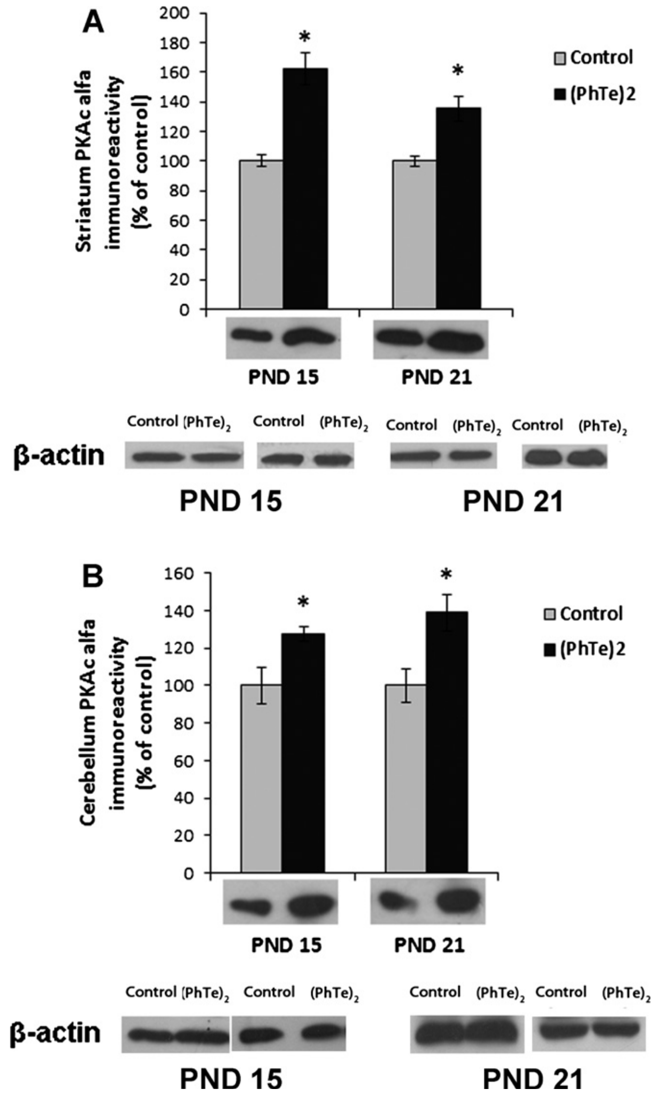

Fig. 5. Effect of $(PhTe)_2$ administered to dams during lactation on PKAc-α immunoreactivity in the striatum (A) and cerebellum (B) of their pups on PND 15 and 21. Western blot assay of PKAc-α was carried out as described in Section 2.10. Representative blots are shown. β-Actin was used as loading control. Data are reported as means ± SEM of 10–12 animals and expressed as percent of control. Statistically significant differences from canola oil-treated rats, as determined by one-way ANOVA followed by Tukey–Kramer test are indicated: $^{*}P < 0.05$.

In an attempt to identify the phosphorylating sites targeted by the protein kinases PKA and MAPK, we assayed NF-LSer55, the main phosphorylating site targeted by PKA on NF-L, as well as KSP repeats, targeted by MAPKs (Heimfarth et al., 2011) on NF-M/NF-H, respectively. Western blot assay using anti-phosphoSer55 antibody and anti-NF-M/NF-H KSP repeats showed that the phosphorylation level of NF-M/NF-H KSP repeats and NF-LSer55 was increased in striatum (Fig. 6A and B) and cerebellum (Fig. 6C and D) of both 15- and 21-day-old pups exposed to $(PhTe)_2$. These findings are in line with the evidence that activated MAPKs and PKA target phosphorylating sites on IFs in the cerebral structures of lactating rats whose dams were injected with $(PhTe)_2$.

To access the ability of $(PhSe)_2$ to prevent the action of $(PhTe)_2$ on the phosphorylating system associated with the cytoskeleton, dams were injected with the organic selenium (1 mg/kg body weight) 30 min before each $(PhTe)_2$ administration. Interestingly, we found that $(PhSe)_2$ prevented hyperphosphorylation of striatal IF proteins from astrocytes and neurons, but it failed to prevent the action of $(PhTe)_2$ in the cerebellum, as demonstrated in 21-day-old pups (Fig. 7A and B).

Next, we intended to identify some protein kinases involved in the ability of $(PhSe)_2$ to prevent the action of $(PhTe)_2$ on the cytoskeletal proteins. Therefore, we evaluated the effects of that compound on MAPKs and PKAcα activities. Western blot assays using specific antibodies against total and phosphorylated forms of Erk1/2 showed that the Se compound prevented activation of this protein kinase either in striatum or in cerebellum of 21-day-old pups (Fig. 8A and B). Similarly, Western blot assay using anti-PKAcα antibody showed that in the presence of $(PhSe)_2$ the level of the active form of the enzyme was not different from control levels in both striatum and cerebellum of PND 21 pups (Fig. 9A and B). Interestingly, we found that $(PhSe)_2$ failed to prevent the stimulatory effect of $(PhTe)_2$ on p38MAPK in cerebellum (Fig. 10).

4. Discussion

The suckling period in the rat represents a period of intense development of brain, particularly of neural components that will modulate synaptogenesis. Consequently, neurotoxicants that disrupt neural development during this critical period can cause permanent changes in brain biochemistry and behavior (Rice and Barone, 2000). In this context, the lactation in rats corresponds to a period of brain development ranging from the last gestational period to the onset of puberty in humans (Haut et al., 2004). Although extrapolation of conclusions from animal data to humans must be done with caution, the use of experimental animals of various developmental ages give us important clues about the evolution of neurotoxicant-induced brain damage and its possible consequences in humans. Therefore in the present study we used an experimental model of lactational intoxication with $(PhTe)_2$ to determine potential changes in IF phosphorylation in rat brain. We demonstrate that exposure to $(PhTe)_2$, *via* maternal milk lead to altered homeostasis of the cytoskeleton of striatum and cerebellum of PND 15 and 21 pups. In our experimental conditions we used a low dose of $(PhTe)_2$ (0.01 mg/kg of body weight) which did not provoke any significant specific overt sign of maternal intoxication, such as reduction of body weight, tremor, garlic odor and loss of hair. Also, pups presented a normal development and gain of body weight. However, despite the absence of an apparent systemic toxicity, we found altered protein kinase activities and disruption of the homeostasis of the cytoskeleton in neural cells of both striatum and cerebellum of these pups.

Although we cannot exclude the involvement of a systemic toxicity of $(PhTe)_2$ on the observed IF hyperphosphorylation in lactating pups, our previous data showing hyperphosphorylation induced by *in vitro* treatment with $(PhTe)_2$ (Heimfarth et al., 2011, 2012) strongly suggest that the effect of the neurotoxicant is mainly related to an action on signaling mechanisms upstream of the enzymatic activities targeting the cytoskeleton, rather than an indirect effect in organs other than the brain.

The neurotoxic effect of this compound was evidenced by hyperphosphorylation of IF proteins associated with the IF enriched cytoskeletal fraction of glial cells (mainly astrocytes) and neurons from the two brain structures studied on PND 15 and 21 pups. The treatment with $(PhTe)_2$ provoked activation of PKA and MAPKs such as Erk1/2, JNK and p38MAPK, targeting neuronal cytoskeletal proteins both on NF-LSer55 and on KSP repeats. Activation of the protein kinases is a spatiotemporally regulated event providing an interesting insight on the differential susceptibility of the protein kinases associated with the IF cytoskeleton of striatum and cerebellum at different developmental stages, in response to the injury induced by this neurotoxicant *via* maternal milk.

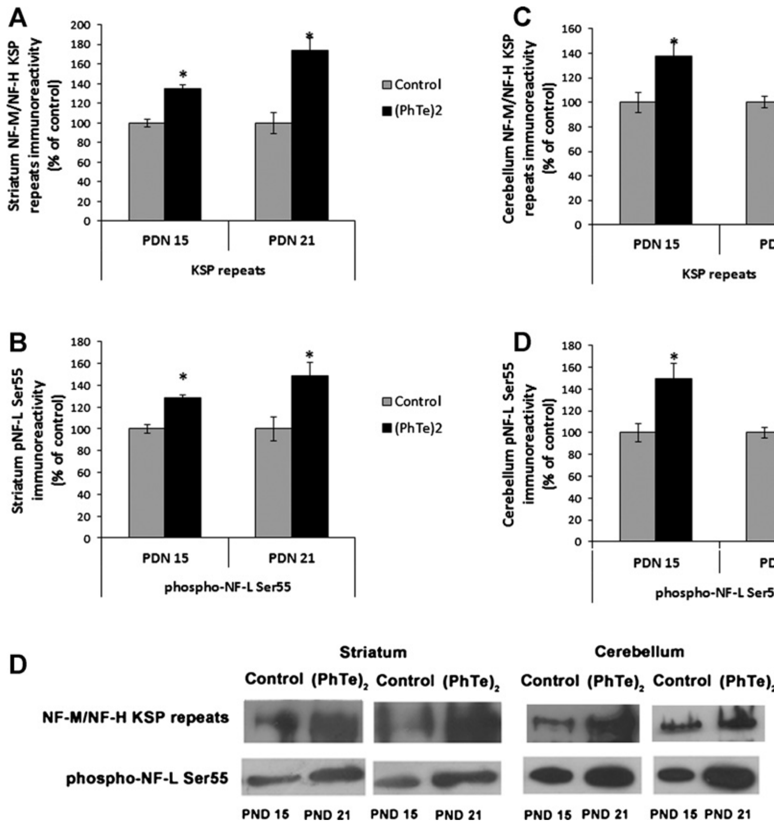

Fig. 6. Effect of $(PhTe)_2$ administered to dams during lactation on the immunocontent of phosphoNF-H KSP repeats (A and C) and phosphoNF-L Ser55 (B and D) of striatum (A and B) and cerebellum (C and D) of their pups on PND 15 and 21. Western blot assays were carried out as described in Section 2.10. Representative blots are shown in (E). Data are reported as means $\pm$ SEM of 10–12 animals and expressed as percent of control. Statistically significant differences from canola oil-treated rats, as determined by one-way ANOVA followed by Tukey–Kramer test are indicated: $^{*}P < 0.05$.

It is important to note that IF hyperphosphorylation was observed on PND 15 and 21, restoring control values afterwards. It is difficult to evaluate the molecular mechanisms leading to the disruption of cytoskeletal homeostasis until PND 21, however they could be related with the maturation program of these brain structures. In fact, during the suckling period, the brain of rats undergoes intensive morphological and biochemical modifications (Ben-Ari and Holmes, 2006). In this context, Tepper et al. (1998) showed that the postnatal third week is an intense period of morphological and electrophysiological changes in the striatum. Therefore, it is feasible that the most prominent susceptibility of striatum until the third postnatal week be related to the developmental events characteristic of this period. Moreover, in the cerebellum, the susceptibility to $(PhTe)_2$ could be related to the postnatal appearance of granule cells (Fonnum and Lock, 2000).

In the cerebellum of 15-day-old pups, the IF hyperphosphorylation was accompanied by an increased immunocontent of the astrocyte and neuron IF proteins. This is in line with previously reported data showing increased immunocontent of IF proteins in cerebral cortex of 15-day-old rats injected with $(PhTe)_2$ (0.3 mmol/kg body weight) (Heimfarth et al., 2008).

The IF organization in eukaryotic cells depends on the phosphorylation level of its constituent proteins which are controlled by the activity of the cytoskeletal-associated phosphorylating/dephosphorylating system (Sihag et al., 2007). In this context, aberrant phosphorylation/dephosphorylation of cytoskeletal proteins in response to different stressors could be a consequence of changes in the activity of IF-associated kinases or phosphatases and may have serious consequences for cellular function and structure (Loureiro et al., 2010, 2011; Pierozan et al., 2012). This evidence is supported by the present results, showing the action of $(PhTe)_2$ on the protein kinase activities which, in turn, disrupt the homeostasis of the cytoskeleton and this could be on the basis of the neurotoxicity of this compound. Aberrant phosphorylation of cytoskeletal proteins is thought to be related to neuronal damage and formation of aggregates of cytoskeletal elements in different cell compartments, which can be considered a common characteristic of some neurodegenerative diseases (Petzold, 2005). It is known that carboxyl-terminal phosphorylation of NF-H progressively restricts association of NF with kinesin, the axonal anterograde motor protein, and stimulates its interaction with dynein, the axonal retrograde motor protein (Motil et al., 2006). This event could represent one of the mechanisms by which carboxyl-terminal phosphorylation would slow NF axonal transport. Consistent with this, MAPK phosphorylates NF-M and NF-H tail domains (Chan et al., 2004; Li et al., 1999; Veeranna et al., 1998) and alters the association of neurofilaments with motor proteins (Yabe et al., 2000). Therefore, extensively phosphorylated NF-M and NF-H as well as MAPK activation could interfere with NF axonal transport and explain, at least in part, the consequent neural dysfunction associated with this intoxication. In astrocytes, the action of $(PhTe)_2$ induced hyperphosphorylation of GFAP, by PKA. It is of note that this protein kinase is implicated in the phosphorylation of sites in the head domain of GFAP, as well as NF-L in neurons (Pierozan et al., 2012). Phosphorylation of the head domain of these IF subunits is known to be important for filament assembly. Therefore, abnormal phosphorylation of the head domain sites of these IF proteins could lead to nonphysiological disassembly of IFs contributing to disruption of cell homeostasis (Gill et al., 1990; Heins et al., 1993).

Also, misregulation of the phosphorylating level of the cytoskeletal proteins in intoxicated pups could be related with

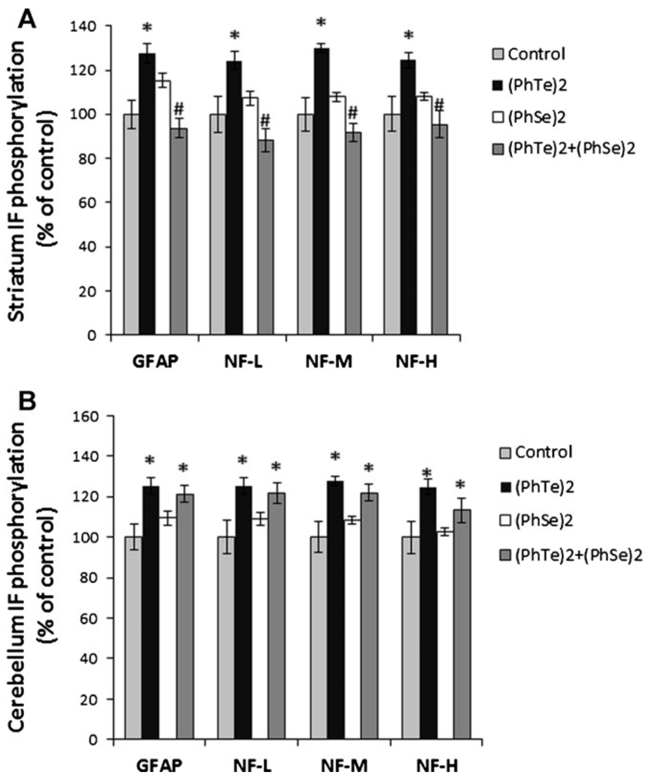

Fig. 7. Prevention of the effect of $(PhTe)_2$ on the phosphorylation of IF proteins by $(PhSe)_2$ on PND 21 pups. Striatum (A); cerebellum (B). Dams received $(PhSe)_2$ (1 mg/kg body weight) 30 min before each $(PhTe)_2$ or canola oil administration once daily during the first 14 days of lactation, as described in Section 2.3. NF-H, high molecular weight neurofilament; NF-M, middle molecular weight neurofilament subunit; NF-L, low molecular weight neurofilament subunit and GFAP, glial fibrillary acidic protein. Data are reported as means ± SEM of 10–12 animals and expressed as percent of control. Statistically significant differences from canola oil-treated rats, as determined by one-way ANOVA followed by Tukey–Kramer test are indicated: $^{*}P < 0.05$.

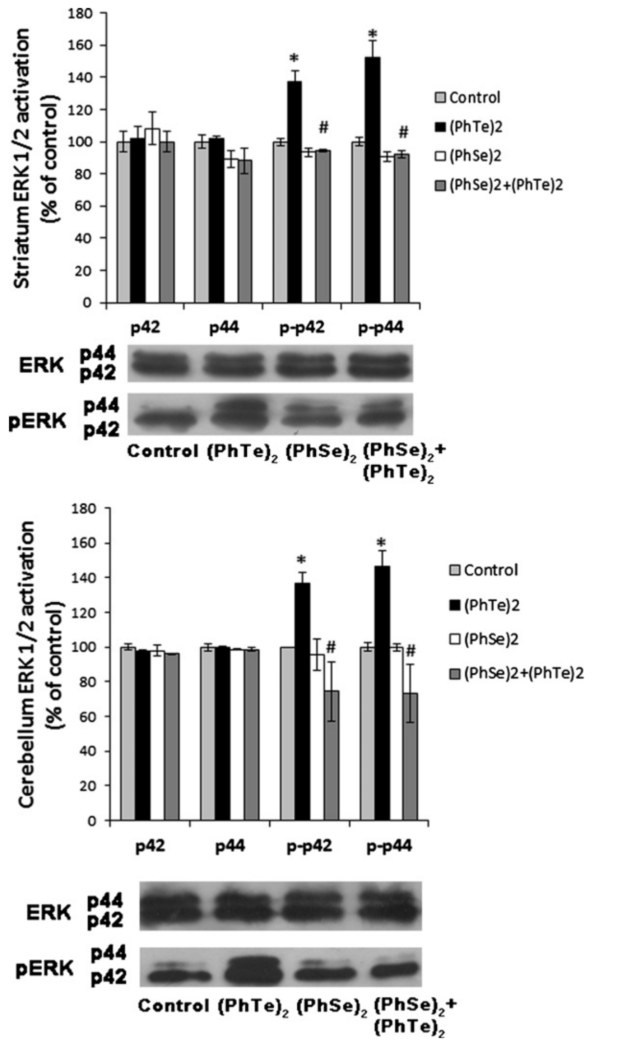

Fig. 8. Prevention of the effect of $(PhTe)_2$ on ERK1/2 MAPK by $(PhSe)_2$ on PND 21 pups. Dams received $(PhSe)_2$ (1 mg/kg body weight) 30 min before each $(PhTe)_2$ or canola oil administration once daily during the first 14 days of lactation, as described in Section 2.3. The immunocontent of ERK 1/2 and phospho-ERK 1/2 were determined by Western blot assay in striatum (A) and cerebellum (B) of their pups. Representative blots are shown. Data are reported as means ± SEM of 10–12 animals and expressed as percent of control. Statistically significant differences from canola oil-treated rats, as determined by one-way ANOVA followed by Tukey–Kramer test are indicated: $^{*}P < 0.05$.

the behavioral deficits reported in $(PhTe)_2$ injected rats (Widy-Tyszkiewicz et al., 2002). It is always expected that the deleterious effects of tellurium are preferentially expressed during development, since the intense plasticity underlying the developmental events (Xie et al., 2006; Tolias et al., 2011) are dependent on efficient remodeling of the cytoskeleton which, in turn, is dependent on the physiological phosphorylation of the cytoskeletal proteins. Improper developmental plasticity likely impedes information processing in the brain.

It is important to emphasize that the effect of $(PhTe)_2$ was not mimicked by its analogous selenium compound $(PhSe)_2$, since diselenide *per se* was unable to cause alterations in the phosphorylation level of the IF proteins. Nonetheless, exposure to $(PhTe)_2$ plus $(PhSe)_2$ *via* maternal milk prevented activation of Erk1/2 and PKA in the striatum on PND 21 pups, but failed to prevent activation of p38MAPK in the cerebellum at the same developmental stage. Considering that p38MAPK was phosphorylated/activated only in the cerebellum of PND 21, we are tempted to speculate that these findings support the inability of $(PhSe)_2$ to prevent hyperphosphorylation of the IF proteins of this cerebral structure.

Supporting the relevance of maternal milk as *via* of exposure for the $(PhTe)_2$ toxicity, Stangherlin et al. (2009a) reported the effect of $(PhTe)_2$ (0.03 mg/kg of body weight) exposure to mothers on the cerebral oxidative status in hippocampus and striatum of their offspring. Also, the same concentration of $(PhTe)_2$ administered to dams caused cognitive impairment in pups intoxicated *via* maternal milk (Stangherlin et al., 2009b). Otherwise, higher doses of $(PhTe)_2$ (0.12 mg/kg of body weight) administered to dams provoked reduction of body weight gain of dams and teratogenic effects in fetuses (Stangherlin et al., 2005).

The neuroprotective effect of $(PhSe)_2$ against the neurotoxic effects of $(PhTe)_2$ can be related in part to the antioxidant and anti-inflammatory properties of the selenium compound (Nogueira and Rocha, 2011). Furthermore, $(PhSe)_2$ could also change the distribution of tellurium in the dam and pups. We could also propose that prevention of the toxic effects of $(PhTe)_2$ could be related to the fact that $(PhSe)_2$ is less reactive than $(PhTe)_2$, and consequently could interact with target proteins without interfering with the protein function.

Also, it is important to note that our group previously reported that young rats injected with $(PhTe)_2$ (0.3 μmol/kg body weight) presented hyperphosphorylation of NF subunits, GFAP and vimentin in cerebral cortex as well as GFAP and vimentin in hippocampus, reinforcing that one of the actions of the neurotoxicant *in vivo* is focused on the signaling mechanisms upstream of the homeostasis of the cytoskeleton of neural cells. Interestingly, these effects were totally reversed by a single subcutaneous injection of $(PhSe)_2$

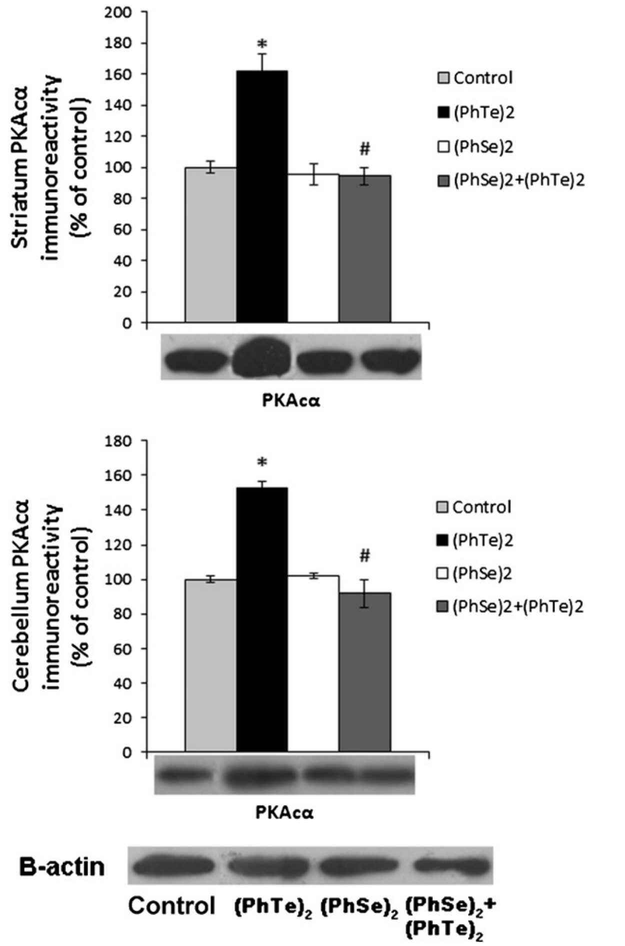

Fig. 9. Prevention of the effect of $(PhTe)_2$ on PKA activation by $(PhSe)_2$ on PND 21 pups. Striatum (A); cerebellum (B). Dams received $(PhSe)_2$ (1 mg/kg body weight) 30 min before each $(PhTe)_2$ or canola oil administration once daily during the first 14 days of lactation, as described in Section 2.3. The immunocontent of PKAcα was determined by Western blot assay. Representative blots are shown. β-Actin was used as loading control. Data are reported as means ± SEM of 10–12 animals and expressed as percent of control. Statistically significant differences from canola oil-treated rats, as determined by one-way ANOVA followed by Tukey–Kramer test are indicated: $^{*}P < 0.05$.

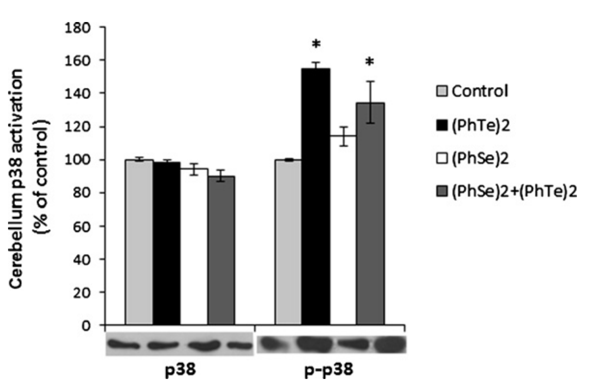

Fig. 10. Prevention of the effect of $(PhTe)_2$ on p38MAPK activation from cerebellum of 21-day-old pups by $(PhSe)_2$. Dams received $(PhSe)_2$ (1 mg/kg body weight) 30 min before each $(PhTe)_2$ or canola oil administration once daily during the first 14 days of lactation, as described in Section 2.3. The immunocontent of p38MAPK and phospho-p38MAPK was determined by Western blot assay. Representative blots are shown. Data are reported as means ± SEM of 10–12 animals and expressed as percent of control. Statistically significant differences from canola oil-treated rats, as determined by one-way ANOVA followed by Tukey–Kramer test are indicated: $^{*}P < 0.05$.

(5 μmol/kg body weight) (Heimfarth et al., 2008). Therefore, the present data together with evidence in the literature are in line with Stangherlin et al. (2006) who proposed that it seems plausible to assume that tellurium become bioavailable to suckling rats after exposure of their mothers to $(PhTe)_2$.

5. Conclusions

In conclusion, $(PhTe)_2$ injected to dams markedly activated MAPKs and PKA taking part of the phosphorylating system associated with the cytoskeleton in striatum and cerebellum of their offspring, reinforcing the relevance of maternal milk as transmission *via* for this neurotoxicant. This effect was spatiotemporally regulated, and apparently in lactating pups, the post-traductional mechanisms regulating the cytoskeleton from striatum and cerebellum in younger pups is more susceptible to the action of the neurotoxicant than in older ones. In fact, suckling rats can be considered extremely susceptible to $(PhTe)_2$-induced neurotoxicity, since the dose of $(PhTe)_2$ given to dams was extremely low. As corollary, the offspring of $(PhTe)_2$-treated dams is expected to be exposed to telluride levels much lower than that given to their mothers. Regarding to the ability of selenium compounds to protect against the tellurium toxicity toward the phosphorylating system associated with the cytoskeletal proteins, the present findings show a promising route to be exploited for a possible treatment of organic tellurium poisoning.

Taking into account the relevance of the signaling mechanisms targeting the cytoskeleton during early postnatal brain development (Guardiola-Diaz et al., 2011; Riederer, 1992), we presume that misregulation of the homeostasis of the cytoskeleton we evidenced can probably contribute to the deleterious action of $(PhTe)_2$ on the developing and adult brain, a fact that might explain at least in part the neurotoxicity of this compound, however these consequences need further investigation.

Although the exposure of pregnant humans to $(PhTe)_2$ is unlike, the extensive use of this compound in organic synthesis and, particularly, its high lipophilicity can determine it deposition in adipose tissue for a long time. Consequently, an occasional exposure to $(PhTe)_2$ in a period before pregnancy could lead to exposure to this compound during pregnancy and/or lactation, depending on the it mobilization from adipose tissue. The results presented here clearly indicate that manipulation and use of $(PhTe)_2$ must be done with caution in order to avoid contamination. This is more important to women in the reproductive period particularly in view of the neurotoxicity of very low doses of $(PhTe)_2$.

Conflict of interest statement

The authors declare no conflicts of interest.

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