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Comparing the effects of two neurotoxins in cortical astrocytes obtained from rats of different ages: involvement of oxidative damage

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ABSTRACT: Oxidative stress has been recognized as a potential mediator of cell death. Astrocytes play an active role in brain physiology responding to harmful stimuli by activating astrogliosis, which in turn has been associated either with survival or degenerative events. The characterization of the mechanistic actions exerted by different toxins in astrocytes is essential to understand the brain function and pathology. As age plays a critical role in degenerative processes, the aim of this study was to determine whether the administration of equimolar concentrations of two neurotoxins evoking different toxic patterns can induce differential effects on primary astrocytes obtained either from newborn or adult rats, with particular emphasis on those events linked to oxidative stress as a potential source of damage. Primary cortical astrocyte cultures derived from rat brains were exposed to 1-methyl-4-phenylpyridinium (MPP+) or beta-amyloid peptide (β-amyloid). Mitochondrial functionality and cell viability were determined as physiological parameters, whereas lipid and protein oxidation were used as markers of oxidative damage. The results of these experiments pointed towards a higher vulnerability to MPP + over β-amyloid, on most of the tested markers. Hence, in order to allow a comprehensive evaluation of astrocytic responses against MPP + intoxication, a third astrocyte group was included for dose-response experiments: astrocytes derived from aged rats. The present data indicate that the differences associated with age were mainly found in astrocytes exposed to MPP + (25 and 50 μM) at 1-h treatment. Results are discussed in terms of the differential mechanisms involved in each model.

Keywords: β-amyloid peptide; astrocyte primary cultures; MPP+; oxidative stress; cytotoxicity

Introduction

Several neurodegenerative disorders in humans, including Alzheimer's and Parkinson's diseases (AD and PD, respectively), share an important feature: a particular vulnerability of neuronal cells to the noxious effects of reactive oxygen species (ROS) (Baillet *et al*., 2010; Simonian and Coyle, 1996). It is currently accepted that oxidative stress plays a critical role in the establishment of brain damage not only through the structural and functional alterations that ROS produce to cell biomolecules, but also because they are potential mediators of cell death by either necrosis or apoptosis (Friedlander, 2003).

It is also known that the central nervous system (CNS) is highly susceptible to ROS because: (i) neuronal cells possess high concentrations of lipids that are more vulnerable to oxidation (Adibhatla and Hatcher, 2010); (ii) the CNS exhibits an elevated aerobic metabolism relying on glucose oxidation, hence inducing an increased mitochondrial activity, which in turn is known to be responsible for high ROS production (Fernández-Checa *et al*., 2010); (iii) the CNS also presents low levels of antioxidant enzymes, in contrast with the high activity of some pro-oxidant enzymes, as well as a constant auto-oxidation of catecholamines and ascorbate.

Astrocytes are the most abundant glial population in the brain, representing more than 50% of the total cells in the cerebral cortex; they are known to play an active role in brain physiology, responding to neurotransmitters and modulating neuronal excitability and synaptic function (Volterra and Meldolesi, 2005). These cells are also essential in maintaining ion fluxes, pH and

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transmitter homeostasis at the synaptic interstitial fluid. In addition, they regulate blood flow and contribute to the energy metabolism (Sofroniew and Vinters, 2010). Noteworthy, it has also been reported that astrocytes protect normal functions of the CNS against oxidative stress (Takuma *et al*., 2004), mainly because they produce and secrete several antioxidant enzymes (Dringen, 2000). Moreover, astrocytes are involved in responses to damage and stress in a multifactorial inflammatory response called reactive astrogliosis (Ting *et al*., 2009). This response may be either harmful or beneficial, as reactive astrocytes can exert both pro- and anti-inflammatory effects. Of note, severe astrogliosis has been associated with a considerable number of neurological disorders (Sofroniew and Vinters, 2010; Zhang *et al*., 2010). Therefore, the physiological characterization of astrocytes and their related responses to damage are essential in understanding the brain function and pathology. The following toxins were chosen, upon the rationale that they mimic a variety of pathological events in the CNS:

(1) Beta-amyloid (β-amyloid), a peptide containing 39–43 amino acid residues generated after amyloid precursor protein (APP) cleavage through β- and γ-secretases activity. β-Amyloid is known to induce apoptotic cell death as a result of the unfolded protein response (UPR) (Lee *et al*., 2010), and can induce inflammatory responses. Another suggested mechanism for its toxic effects is through a persistent stimulation of NMDA receptor-mediated transmission, which in turn might account for the beginning of the pathological changes associated with Alzheimer's disease (Peña *et al*., 2006).
(2) MPTP, a byproduct generated during meperidin synthesis, is recognized as a lipophylic compound capable of crossing the blood–brain barrier to be oxidized, mainly in astrocytes, throughout the action of monoamine oxidase B (MAO-B). MPTP is readily converted into 1-methyl-4-phenylpyridinium (MPP+), a toxic ion released from astrocytes, and selectively incorporated by the dopaminergic neurons through dopamine transporters, further inhibiting mitochondrial complex I and favoring ROS generation and cell demise (Przedborski and Vila, 2003; Zhang *et al*., 2010). Here, we decided to employ MPP+ instead of MPTP because although the later can be easily converted into the first to further abandon cells, part of this newly formed MPP+ could also exert toxicity in astrocytes by itself, but little is known about its effects. As it is difficult to establish the exact quantity of MPTP converted into MPP+, as well as the amount needed to induce toxicity, accurate concentrations of MPP+ were used in primary cultured astrocytes.

Because it is known that an increased glial reactivity is a common outcome of aging, and antioxidant content in the brain has been reported to diminish with age (Kanwar and Nehru, 2007), the purpose of this study was to determine in the first instance if cortical astrocytes derived from adult animals are more susceptible to the toxic effects of the tested agents than astrocytes from newborns, with particular emphasis on those events linked with oxidative damage as a potential source of injury. In addition, we investigated whether the administration of neurotoxins with different toxic profiles can induce differential effects on primary astrocytes obtained either from newborn or adult rats. Our results indicate that astrocytes exhibited a higher susceptibility to MPP+ over β-amyloid in long-term experiments. The only difference associated with age was found in the mitochondrial functionality of cells exposed to MPP+. These results are discussed in terms of the differential mechanisms implicated in these two toxic models.

Materials and Methods

Chemicals

All chemicals and reagents were purchased from Sigma Chemical Co. (St. Louis, MO, USA). The reagents obtained from other sources are detailed throughout the text.

Animals

Astrocytes were isolated from the frontal cortex of neonatal (3 day old), adult (9 months old), and for some experiments, old (24 months old) albino Wistar rat brains (*Rattus norvegicus*), provided by the closed breeding colony of the Universidad Autónoma Metropolitana-Iztapalapa (UAM-I). Before being assigned to the experiments, adult and old animals were housed five per cage in polycarbonate cages and provided with a standard commercial rat diet (Harlan 2018S; Madison WI, USA) and water *ad libitum*. All the procedures carried out on animals were done strictly according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and the Principles of the Mexican Official Ethics Standard 062-ZOO-1999.

Cortical Astrocytes Isolation and Culture

Astrocyte primary cultures were obtained according to a protocol established in previous studies (Lin *et al*., 2007; McCarthy and de Vellis, 1980). For neonatal astrocytes, pools of three animal tissues were used, whereas tissues of four animals were pooled tougher for adult or old cultures. Cells were maintained routinely in minimum essential medium (MEM) supplemented with 10% fetal bovine serum, 0.01% glutamine, 0.15 % glucose and 1% penicillin–streptomycin. The medium was replaced every 3 days. Cells were grown up at 37 °C in 60-mm-diameter plates (Corning, Acton, MA, USA) in an atmosphere of 95% air and 5% CO_2. All described experiments were performed after 2 weeks under *in vitro* conditions.

To ensure that the isolated cells were indeed astrocytes, cells were immunostained using polyclonal antibodies against glial fibrillary acidic protein (GFAP). Under these conditions, cultures are known (and were confirmed) to contain more than 90% cells positive to GFAP. For immunofluorescence experiments, the cells were washed with phosphate-buffered saline (PBS) and fixed with 4% paraformaldehyde for 30 min. Immediately thereafter, the cells were incubated in blocking buffer [2% bovine serum albumin (BSA), 0.2% non-fat milk, 0.4% Triton X-100 in PBS] for 1 h at room temperature. The cells were then washed and incubated for one more hour with the primary antibody anti-GFAP (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The cells were washed three times more with PBS-Tween 0.2% and incubated with the secondary antibody (Alexa Fluor 594 anti-rabbit dilution 1:200). After four more washes, the cells were incubated with Hoechst stain (1 ug ml^{-1}) for 5 min to stain DNA and mark the nucleus. The cells were washed again twice and mounted with fluorescent mounting medium (Dako Cytomation, Glostrup, Denmark). Images were obtained with a confocal microscope OLYMPUS BX-51 W1 imaging at 30× with the Mercury Lamp, and

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two filters: U-MWU2 330-385 nm excitation and 420 nm emission for HOECHST, and U-MRFPHQ 535-555 excitation and 570–625 nm emission for ALEXA FLUOR 594.

For Western blot analysis, 5×10^5 cells were seeded, trypsinized and resuspended in lysis buffer (50 mM Tris-HCl pH 8.0, 120 mM NaCl, 0.5% NP40, 100 mM NaF, 0.2 mM $NaVO_3$, 1 μg ml^{-1} aprotinin, 1 mM PMSF and 1 μg ml^{-1} leupeptine). Cell homogenates were incubated at 4 °C for 5–10 min, and centrifuged at 22 000 *g* at 4 °C for 20 min. The protein concentration in the supernatants was determined using a commercial Bradford reagent (Bio-Rad, Hercules CA, USA) (Bradford, 1976). Cell lysates were separated in 13% SDS–PAGE and transferred to polyvinylidene difluoride (PVDF) membranes (Invitrogen) for further incubation with anti-GFAP (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Membranes were washed three times with TBS-Tween and incubated with a horseradish peroxidase-conjugated α-mouse IgG secondary antibody (Pierce, Rockford, IL, USA) for 1 h. After three more washes, blots were revealed using a commercial chemiluminescent reagent (Supersignal®; Pierce).

Cytotoxic Treatments

After being preserved for 2 weeks under *in vitro* conditions, astrocytes were reseeded at a cell density of 3×10^5 cells per well on a 24-well multichamber plate (Corning, Acton, MA, USA), and 50 μM soluble β-amyloid (25–35 fragment) (Croce *et al*., 2011; Ren *et al*., 2011) or 50 μM MPP^+ (Hazell *et al*., 1997) were added to the culture media at 37 °C and 5% CO_2. The concentrations used for β-amyloid and MPP^+ were originally established on the basis of previous observations of other groups (Ferrera *et al*., 2008). The experiments were performed in primary astrocytes derived from newborn or adult animals exposed to the toxins either for 5 or 72 h, with the aim of determining the short- and long-term responses for each age group. Previous reports have demonstrated that aged astrocytes are viable and serve for different experimental purposes (Lin *et al*., 2007). In addition, H_2O_2 was used as a positive control for oxidative toxicity. To determine the optimum concentration of this agent, a series of pilot assays were carried out in primary astrocytes from newborn animals and treated with 100, 200, 300 or 400 μM H_2O_2 for 1 h.

Cell Viability

To determine cell viability, astrocytes were trypsinized and a 20-μl aliquot was stained with an equal volume of a 0.4% trypan blue physiological solution (trypan blue exclusion). The number of living cells in 10 μl of this suspension was scored using five fields of a hemocytometer under a phase-contrast optical microscope.

Mitochondrial Functionality

The MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay (Mosmann, 1983) has been reported to quantify mitochondrial succinate dehydrogenase activity. This assay is commonly used to determine mitochondrial fitness and functionality (Cillero-Pastor *et al*., 2011; Golomb *et al*., 2012), and was therefore chosen as an indicator of cellular energy and metabolic status, although it is also often accepted as a marker of cell viability. MTT is a pale yellow substrate that produces a dark blue formazan product when the tetrazolium ring is cleaved in active mitochondria, mostly by the succinate dehydrogenase. It has been shown that the amount of formazan generated per cell depends on the level of mitochondrial energetic metabolism, and that activated cells produce more formazan than the resting ones, supporting the idea that active mitochondria are required to generate a strong signal (Sánchez and Konigsberg, 2006). Briefly, cells were incubated with minimal essential medium (MEM)-fetal bovine serum (FBS) containing 0.5 mg ml^{-1} MTT for 4 h at 37 °C. The medium was discarded and 800 μl of extracting solution (HCl 0.04 M in isopropanol) was added to each well to dissolve the water-insoluble formazan salt. After 15 min of incubation at room temperature, the colored formazan salt was determined at 570 nm using a Beckman DU 650 spectrophotometer.

Lipid Peroxidation Assay

Specific concentrations of the toxins were used in order to determine the oxidative damage to lipids and proteins. For these assays, cells were seeded at a cell density of 5×10^6 cells per 60-mm-diameter plate (Corning). After 24 h, cells were treated either with 50 μM β-amyloid or 50 μM MPP^+ for 5 or 72 h, or with 100 μM H_2O_2 for 1 h as a positive control.

Lipid peroxidation (LPX) was assessed in cells treated with different toxins by the assay of thiobarbituric acid-reactive substances (TBARS) formation, according to a previous report (Zentella de Piña *et al*., 2007). Immediately after being incubated, cells were scraped and pooled in 500 μl of the TBA reagent (containing TBA 0.125 g + trichloroacetic acid 375 ml + HCl 513 μl, in a 25-ml final volume), and re-incubated in a boiling water bath (94 °C) for 20 min. Samples were then kept on ice for 5 min and centrifuged at 3000 *g* for 15 min. The optical density was estimated in supernatants at a wavelength of 532 nm in a Beckmann DU 650 Spectrophotometer. The protein concentration was determined using a commercial Bradford reagent (Bio-Rad Hercules, CA, USA) (Bradford, 1976). The concentrations of malondialdehyde (MDA), expressed as nmol of MDA per mg of protein, were calculated by interpolation on a standard curve, normalized with protein content per sample, and expressed as a percent of MDA formed vs. control.

Production of the DNP Derivative of Oxidized Proteins

After being exposed to toxic treatments, cells were incubated with a 5 μl SDS (120 g l^{-1}) solution. Five min later, 10 μl of the DNPH solution (10 mmol l^{-1}) in HCl (2 mol l^{-1}) was added and the reaction was allowed to continue for 25 min, followed by the addition of 5 μl of a neutralizing solution, according to the Oxyblot protein oxidation detection kit described below.

Measurement of Protein Carbonyl by Immunoblotting of DNP Protein

Twenty-five micrograms of the DNP derivative of protein were separated by SDS–PAGE (12%) followed by Western blotting, using a polyvinylidene difluoride membrane, according to the protocol established for the Oxyblot protein oxidation detection kit (Catalog no. S7150; Millipore, Billerica, MA, USA). Membranes were then incubated with the primary antibody, specific to the DNP moiety. This step was followed by incubation with a secondary antibody conjugated with horseradish peroxidase (goat anti-rabbit IgG) and directed against the primary antibody.

Blots were revealed using. Immobilon Western chemiluminiscent HRP substrate (Millipore, Billerica, MA, USA).

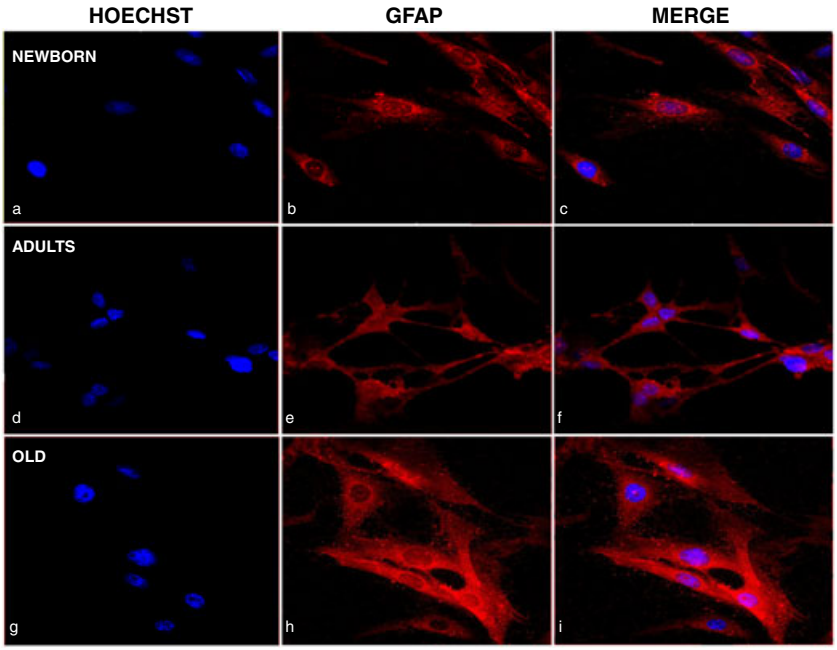
Statistical Analysis

Data are reported as mean values ± standard deviation (SD) of at least three independent experiments per group performed by triplicate. A multivariate linear model was used to establish differences among treatments, markers, ages, times and concentrations of exposure tested. After running the corresponding hypothesis tests, comparisons using Bonferroni's method were applied. Values of $P < 0.05$ were considered as statistically significant.

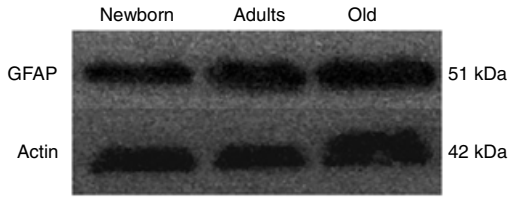
Results

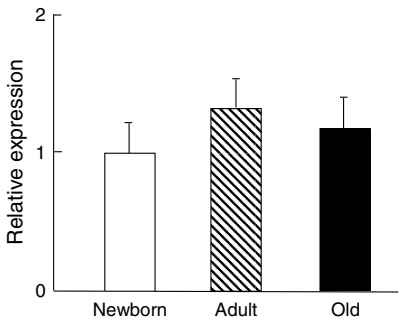
Immunocytochemistry and Immunoblotting for the Assessment of Functional Astrocytes in Cell Cultures

The presence of functional cortical astrocytes in cultures from newborn, adult, and in some experiments, old Wistar rats, was assessed by immunocytochemical and immunoblotting assays (Fig. 1). Immunocytochemical images revealed the presence of active astrocytes for GFAP production, and the proportion of these cells was more or less similar in cultures from all ages (Fig. 1A). More than 90% of the cultured cells were indeed astrocytes. These



A





B

Figure 1. Representative astrocyte staining and immunodetection in cultures from newborn and adult rats. (A) Confocal microscopic images of astrocyte primary cultures from 3-day-old newborn (a, b, c), 9-month-old adult rats (d, e, f) and 24-month-old aged rats (g, h, i). In a, d and g, Hoescht staining (blue); in b, e and h, GFAP immunostaining (red); in c, f and i, merge. (B) Immunoblot analysis and densitometry showing the expression of GFAP protein in astrocyte primary cultures from newborn (3 days old), adult (9 months old) and old (24 months old) rats.

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observations were confirmed by Western blot assays, demonstrating that the proportion of GFAP protein was the same in cultures from all ages (Fig. 1B).

H_2O_2 as a Toxic Positive Control

Figure 2 shows the results obtained when astrocytes were treated for 1 h with increasing concentrations of H_2O_2. Cell viability and mitochondrial functionality decreased in a dose-dependent manner; therefore, the lowest H_2O_2 concentration (100 μM) at which approximately 50% of the cells were viable and functional, was chosen and used as a positive control for further experiments.

Long-Term Cytotoxic Effects

To discern whether there is a differential effect when astrocytes are exposed to diverse toxic insults for a long period of time, cells were exposed to the toxins for 72 h. Both toxins tested were able to decrease cell viability and functionality in a significant manner (Fig. 3A, B). As expected, the effect observed in mitochondrial functionality was more evident than that of viability, but no differences were found between the two age groups.

Cell viability diminished by approximately 60% in response to β-amyloid treatment ($P < 0.01$), 80% in the MPP^+ treatment ($P < 0.01$) and 95% in the H_2O_2 treatment ($P < 0.001$) (Fig. 3A). Mitochondrial functionality was severely affected (Fig. 3B) by approximately 60–70% when astrocytes were exposed to β-amyloid treatment ($P < 0.05$), or completely lost in the MPP^+ and H_2O_2 treatments ($P < 0.01$).

Figure 3C shows the results obtained from the LPX assay. All toxins induced LPX after 72 h of treatment; however, once again, no differences were found between the two age groups. Interestingly, LPX levels were lower after 72 h than 5 h of treatment, probably because of a high mortality prevailing at the long-term condition.

A representative oxyblot is presented in Fig. 4 to contrast protein oxidation among toxins after 72 h of treatment in astrocytes from newborn (Fig. 4A) and adult (Fig. 4B) rats. It seems evident that proteins are more oxidized in MPP^+ and H_2O_2 treatments, in concordance to all other results presented here. No differences were found in protein damage between the two age groups.

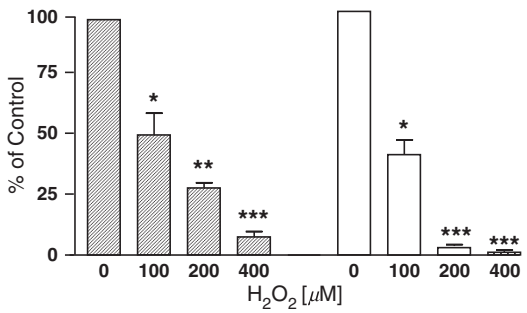

Figure 2. Cell viability and mitochondrial functionality in H_2O_2-treated astrocytes. Cellular viability (shredded bars) and mitochondrial functionality (white bars) were determined in primary cortical astrocytes from newborn animals at different H_2O_2 concentrations after 1-h exposure, as described in Materials and Methods, in order to determine the optimum concentration to use this agent as a control in further oxidative toxicity assays. Each point represents the mean ± standard deviation (SD) of nine determinations performed in three independent experiments. Statistical significance with respect to untreated cells: *($P < 0.05$), **($P < 0.01$), ***($P < 0.001$) were considered.

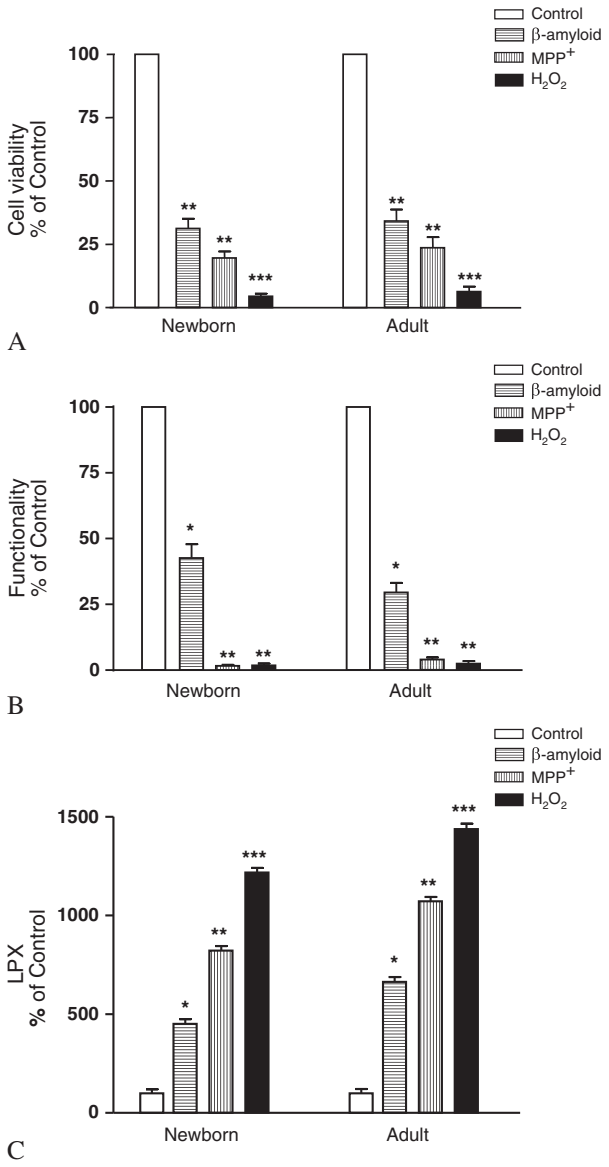

Figure 3. Long-term cytotoxic effects of β-amyloid and 1-methyl-4-phenylpyridinium (MPP+) in rat cultured astrocytes. Cellular viability (A), mitochondrial functionality (B) and lipid peroxidation (C) were determined in primary astrocytes derived from newborn and adult animals after 72-h treatment with each toxin, as described in Materials and Methods. Each point represents the mean ± standard deviation (SD) of nine determinations performed in three independent experiments. Statistical significance with respect to untreated cells: *($P < 0.05$), **($P < 0.01$), ***($P < 0.001$) were considered.

Short-Term Cytotoxic Effects

To determine if a more evident effect could be observed between the age groups, the same experiments were performed at a shorter time. Figure 5 illustrates the data obtained after 5 h of exposure to toxic treatments. In regard to the effect of β-amyloid, this toxin significantly diminished cell viability and functionality by approximately 40% and 60–70% (respectively), when compared with untreated control cells (Fig. 5A, B); however,

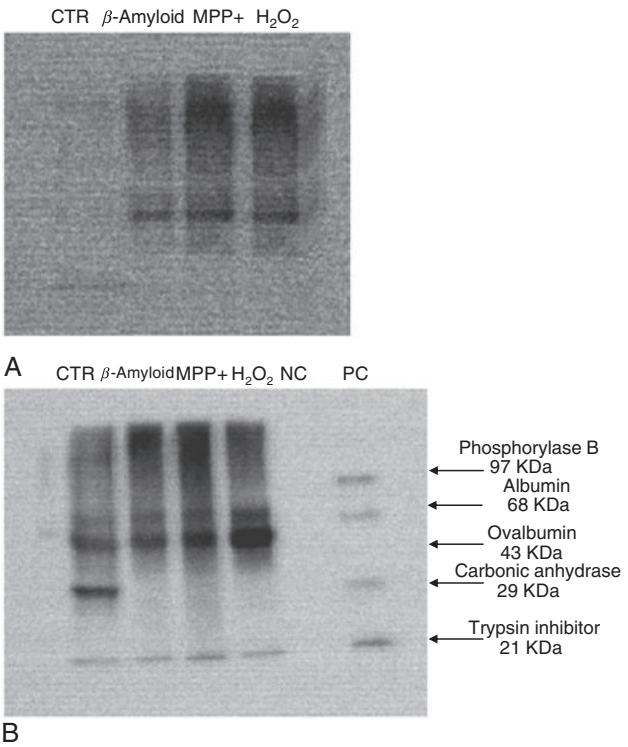

Figure 4. Protein oxidation estimated after long-term treatments with β-amyloid and 1-methyl-4-phenylpyridinium (MPP+) to rat-cultured astrocytes. Representative oxyblots obtained of primary astrocytes from newborn (A) and adult (B) animals after a 72-h exposure to the toxins β-amyloid, MPP + and H_2O_2 (as a positive control for oxidative toxicity). The negative control (NC) and positive control (PC) are internal standards provided by the commercial kit. The oxyblots were performed two times in three independent experiments, as described in Materials and Methods. Statistical significance with respect to untreated cells: *($P < 0.05$), **($P < 0.01$), ***($P < 0.001$) were considered.

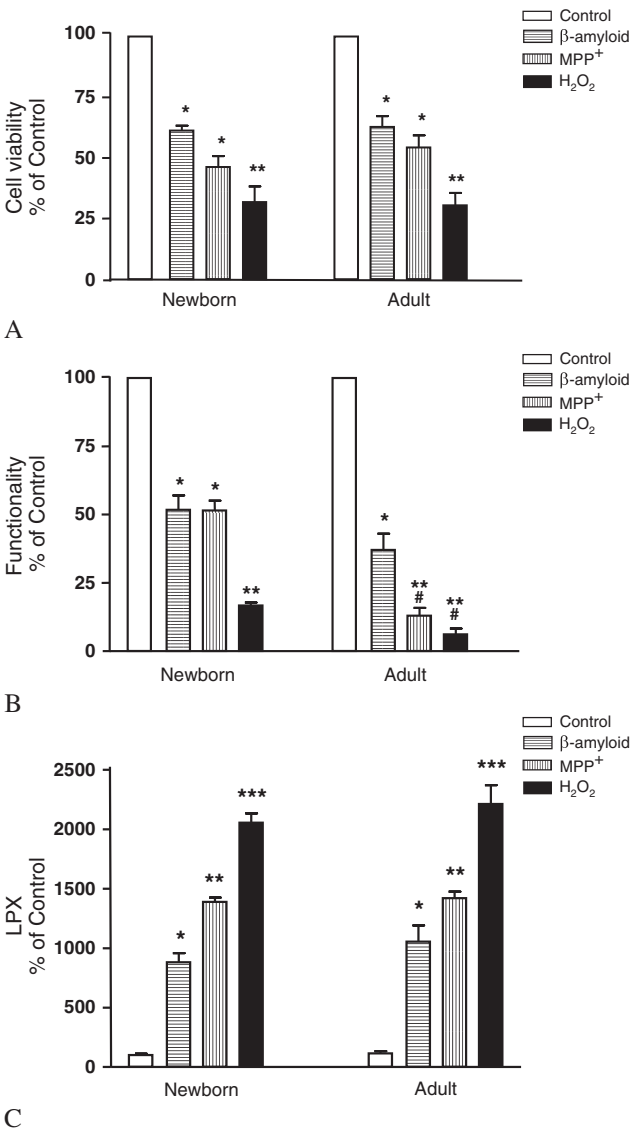

Figure 5. Short-term cytotoxic effects of β-amyloid and 1-methyl-4-phenylpyridinium (MPP+) on rat cultured astrocytes. Cellular viability (A), mitochondrial functionality (B) and lipid peroxidation (C) were determined in primary cortical astrocytes derived from newborn and adult animals after 5-h treatment with each toxin, as described in Materials and Methods. Each point represents the mean ± standard deviation (SD) of nine determinations performed in three independent experiments. Statistical significance with respect to untreated cells: *($P < 0.05$), **($P < 0.01$), ***($P < 0.001$) were considered. Statistical difference between newborn and adult derived astrocytes was designated as #.

when cells derived from adult animals were compared with cells from newborns, no statistical differences were found.

A significant decrease in cell viability was found in astrocytes isolated from the two age groups when treated with MPP^+ and compared with the control (50–55), but when compared with each other, no differences were observed (Fig. 5A). Interestingly, when mitochondrial functionality was analyzed, a significant decrease was found between MPP^+-treated and control cells, as well as among the two age groups. Astrocytes derived from newborns decreased their functionality by approximately 50%, whereas cells derived from adults declined this marker by approximately 88% (Fig. 5B). A similar result was observed for H_2O_2-treated cells, where the fall in mitochondrial functionality was more evident in cells derived from adults.

Figure 5C shows the results of LPX assessed after the exposure of astrocytes to the different toxicants. All toxins tested produced significant oxidative damage to lipids when compared with control cells: β-amyloid ($P < 0.05$), MPP^+ ($P < 0.01$) and H_2O_2 ($P < 0.001$).

The images presented in Fig. 6 illustrate protein oxidation. In order to compare the oxidative damage between ages, proteins obtained from newborn and adult astrocytes were assessed in parallel and run in the same gel. To better appreciate differences owing to the toxic model used, protein extracts were analyzed and grouped to compare the oxidative effect of the toxins at 5 h in astrocytes from newborn (Fig. 6A) and adult (Fig. 6B) rats. In agreement with those data obtained from LPX assay, astrocytes treated with MPP^+ and H_2O_2 showed the highest oxidative damage, whereas β-amyloid-treated cells presented only a moderate effect. Also, in general terms, astrocytes derived from adult animals were more affected than those from newborn rats, both during short- and long-term exposures, indicating that there is a higher oxidative damage related to age.

In summary, even although it is clear that alterations in astrocytes are different when they are exposed to dissimilar toxic insults, the only variation associated with age found in response to the toxins used was observed as a higher susceptibility to protein oxidation in astrocytes from adult animals, accompanied by a decreased mitochondrial functionality in the MPP^+ and H_2O_2 conditions (Fig. 6).

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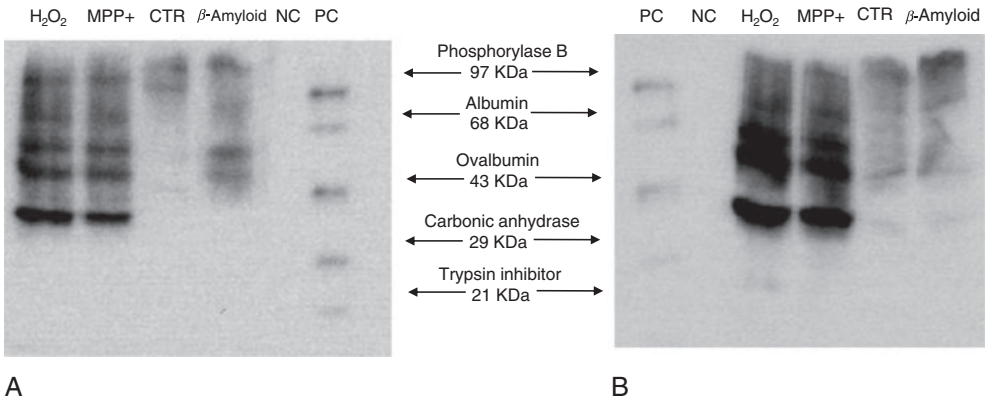

Figure 6. Protein oxidation estimated after short-term treatments with β-amyloid and 1-methyl-4-phenylpyridinium (MPP+) to rat-cultured astrocytes. Representative oxyblots obtained of primary astrocytes from newborn (A) and adult (B) animals after a 5-h exposure to the toxins β-amyloid, MPP + and H_2O_2 (as a positive control for oxidative toxicity). The negative control (NC) and positive control (PC) are internal standards provided by the commercial kit. The oxyblots were performed two times in three independent experiments, as described in Materials and Methods. Statistical significance with respect to untreated cells: *($P < 0.05$), **($P < 0.01$), ***($P < 0.001$) were considered. Statistical difference between newborn and adult derived astrocytes was designated as #.

MPP+ Dose-response Studies Introducing Astrocytes Derived from Aged Rats

So far, our results showed that MPP+ reduced cellular viability and functionality notably more than β-amyloid, and this effect showed a significant difference between age groups only at the short-term exposure (5 h), suggesting that at long-term exposure (72 h), the cells were so damaged that no difference could be observed between them. Therefore, in order to determine if a major susceptibility of older astrocytes could became evident, we decided to perform dose-response studies at lower concentrations (10, 25, 50 and 100 μM), reducing the exposure time (5, 3 and 1 h).

To further allow a comprehensive evaluation of astrocytic responses, it was worth introducing a third astrocyte group: astrocytes derived from aged rats (24 months) (Fig. 1A, B). As these experiments were performed with three age groups, from now on, astrocytes derived from old rats will be abbreviated as OA, whereas astrocytes from adult and newborn rats will be called as AA and NA, respectively.

Our data show that at 5 and 3 h, most AA and OA died, thus making it very difficult to observe differences between the ages (Fig. 7A, B). Specifically, at 5 h (Fig. 7A), even with the lower MPP + concentration (10 μM), 63% of the OA, and 57% of the AA and NA died. These were more evident at higher concentrations (50 and 100 μM), where more than 95% of OA and AA died, and only 23% (50 μM) and 11% (100 μM) of NA survived. At 3 h, the only difference between the groups was observed at the higher concentrations. At 50 μM, 74% of the OA died whereas AA and NA resisted more (62% of deaths). Finally at 100 μM, 88% of OA did not survive, and 78% of AA and NA died.

Interestingly, only at 1 h were the differences detectable, and cellular viability significantly decreased in all age groups of cultured astrocytes. At 1 h, OA exhibited a higher sensibility to the toxin, even at the lower concentrations tested (10 and 25 μM), while a significant difference between AA and NA was only observed at 50 μM MPP + treatment. Specifically, the 25 μM MPP + concentration decreased cellular viability by 22% for NA, 35% for AA and 45% for OA ($P < 0.05$), whereas the 50 μM concentration decreased by 30% for NA, 40% for AA and 60% for OA ($P < 0.05$) (Fig. 7C).

In concordance with data about cell viability, not many differences were found in functionality among groups after the 3- and 5-h treatments (Fig. 8A, B). After 5 h of 10-μM treatment, OA showed a 43% decrease in functionality whereas AA and NA only decreased by 17% and 10%, respectively. While at the higher concentration (100 μM), OA and AA diminished their functionality in 75% and NA in 60%. In contrast, after the 1-h treatment, and with the 25 μM MPP + concentration, there was a significant decrease in cellular functionality among groups: 10% NA, 20% AA and 24% OA ($P < 0.001$) (Fig. 8C).

LPX was also assessed in order to determine oxidative damage at these MPP + concentrations and time points. Our results showed a significant increase in LPX, observed since the lowest concentration (Fig. 9). In agreement with previous data, differences among the age groups were found after 1-h treatment at 25 and 50 μM MPP+. Particularly, LPX increased 0.5 times in NA, 2.7 times in AA and 3 times in OA after 25 μM MPP+, when compared with untreated cells ($P < 0.001$). Astrocytes exposed to 50 μM MPP + increased lipid oxidative damage by 3 times in NA, 3.6 times in AA and 3.9 times in OA ($P < 0.001$) (Fig. 9C).

Discussion

Considering that the brain is highly dependent on a glucose and oxygen supply, and that it also contains elevated amounts of unsaturated lipids and low levels of antioxidant enzymes, it is not surprising for this organ to become susceptible to oxidative damage, and therefore, several neurological disorders have been related to oxidative stress (Baillet *et al*., 2010; Fernández-Checa *et al*., 2010).

Astrocytes are now being studied with a renewed interest as new features of their physiology and function are being discovered. Astrocytes regulate the synaptic transmission as part of the tripartite synapse (Halassa *et al*., 2009); they maintain the blood–brain barrier integrity (Sofroniew and Vinters, 2010), as well as the brain cholesterol levels (Chen *et al*., 2012) and copper homeostasis (Scheiber and Dringen, 2012). Another important facet of these cells is that they decrease their neuroprotective capacity during aging (Pertusa *et al*., 2007), thereby playing critical roles in neurodegenerative and neurodevelopmental diseases

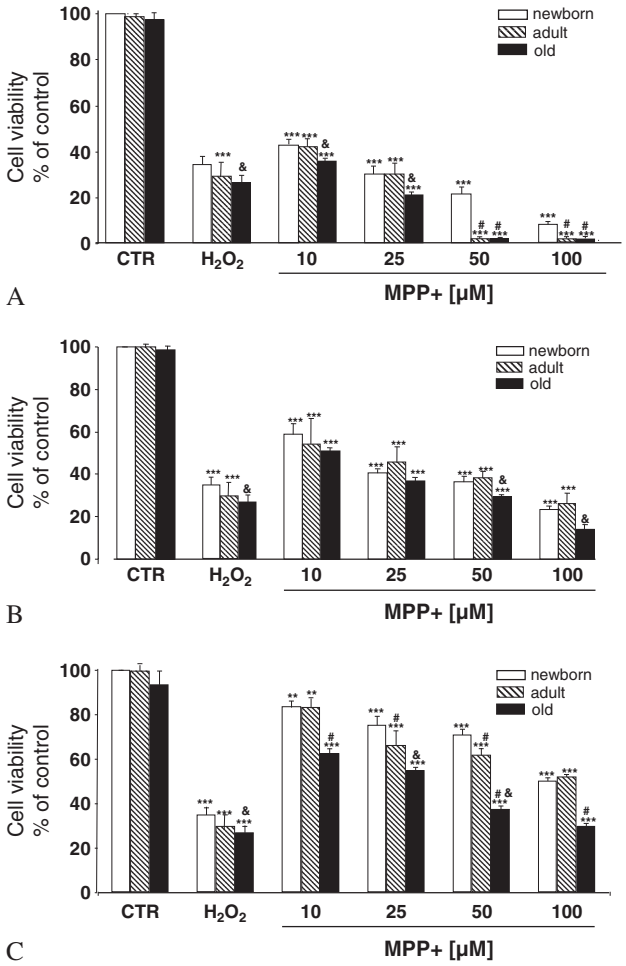

Figure 7. Cell viability in 1-methyl-4-phenylpyridinium (MPP+)-treated astrocytes: dose-response curve. Primary astrocytes derived from newborn, adult and old rats were treated with different MPP + concentrations (10, 25, 50 and 100 μM) for 5 (A), 3 (B) and 1 h (**C**). Cell viability was assessed as described in Materials and Methods, and was normalized against newborn astrocytes as a control, which was considered 100%. 100 μM H_2O_2 for 1 h H_2O_2 was used as a positive control for oxidative damage. Each point represents the mean ± standard deviation (SD) of nine determinations performed in three independent experiments. Statistical significance with respect to untreated cells: *($P < 0.05$), **($P < 0.01$), ***($P < 0.001$) were considered; i.e. treated newborn (NA), adult (AA) and old astrocytes (OA) vs. untreated NA. Statistical difference between AA and OA vs. NA treated with the same MPP + concentration was designated as & ($P < 0.05$); statistical difference between AA vs. OA treated with the same MPP + concentration was designated as # ($P < 0.05$).

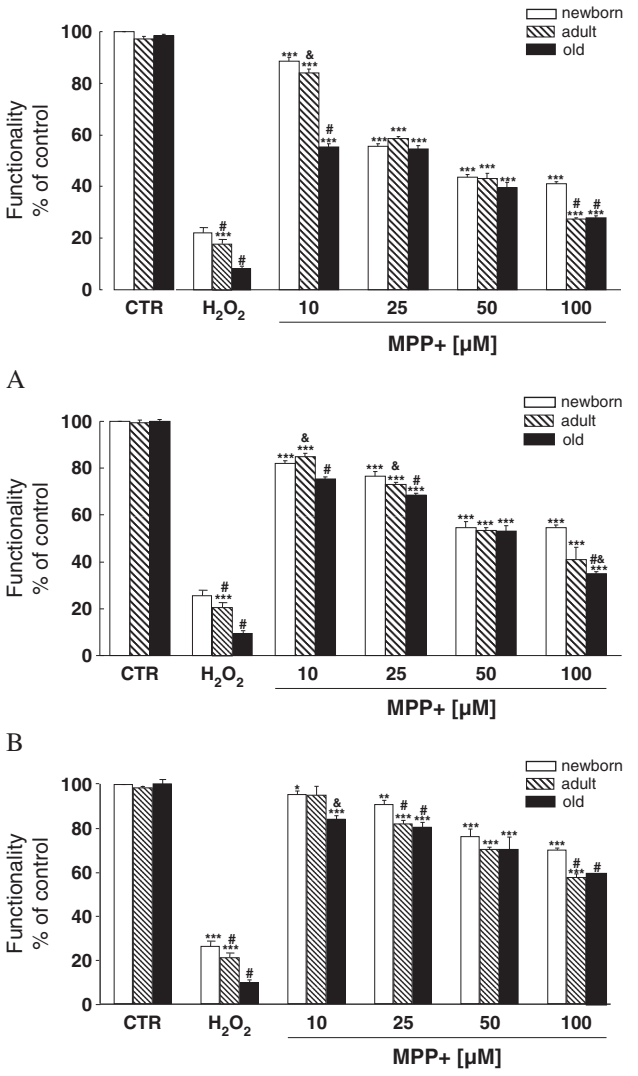

Figure 8. Functionality in 1-methyl-4-phenylpyridinium (MPP+)-treated astrocytes: dose-response curve. Primary astrocytes derived from newborn, adult and old rats were treated with different MPP + concentrations (10, 25, 50 and 100 μM), for 5 (A), 3 (B) and 1-h (**C**). Functionality was determined measuring MTT as described in Materials and Methods and was normalized against NA as the control, which was considered 100 %. 100 μM H_2O_2 for 1 h H_2O_2 was used as a positive control for oxidative damage. Each point represents the mean ± standard devation (SD) of nine determinations performed in three independent experiments. Statistical significance with respect to untreated cells: *($P < 0.05$), **($P < 0.01$), ***($P < 0.001$) were considered; i.e. treated NA, AA and OA vs. untreated NA. Statistical difference between AA and OA vs. NA treated with the same MPP + concentration was designated as & ($P < 0.05$); Statistical difference between AA vs. OA treated with the same MPP + concentration was designated as # ($P < 0.05$).

such as Alzheimer's disease (Bhat *et al*., 2012) and Rett syndrome (Molofsky *et al*., 2012). Therefore, it is of paramount importance to understand their physiology and susceptibility to toxics.

In this study, we compared the effects of equimolar concentrations of two different neurotoxins that have been used to mimic some specific features observed in neurodegenerative diseases by enhancing free radical production and oxidative stress. For this purpose, we first validated our experiments through a positive control of cell damage, namely H_2O_2. This agent accurately induced damage to cells, which is traditionally linked with oxidative damage, as demonstrated throughout the results of the present study. Therefore, H_2O_2 was included in further experiments for comparative purposes as another toxic paradigm. In light of the consistent effects exhibited by this pro-oxidant compound, we considered all our experiments as satisfactorily accurate and reproducible.

Effects of β-Amyloid on Toxic Markers and Oxidative Damage in Astrocytes

With regard to the effects of β-amyloid, it is known that during the progression of AD, the neurological dysfunction is preceded

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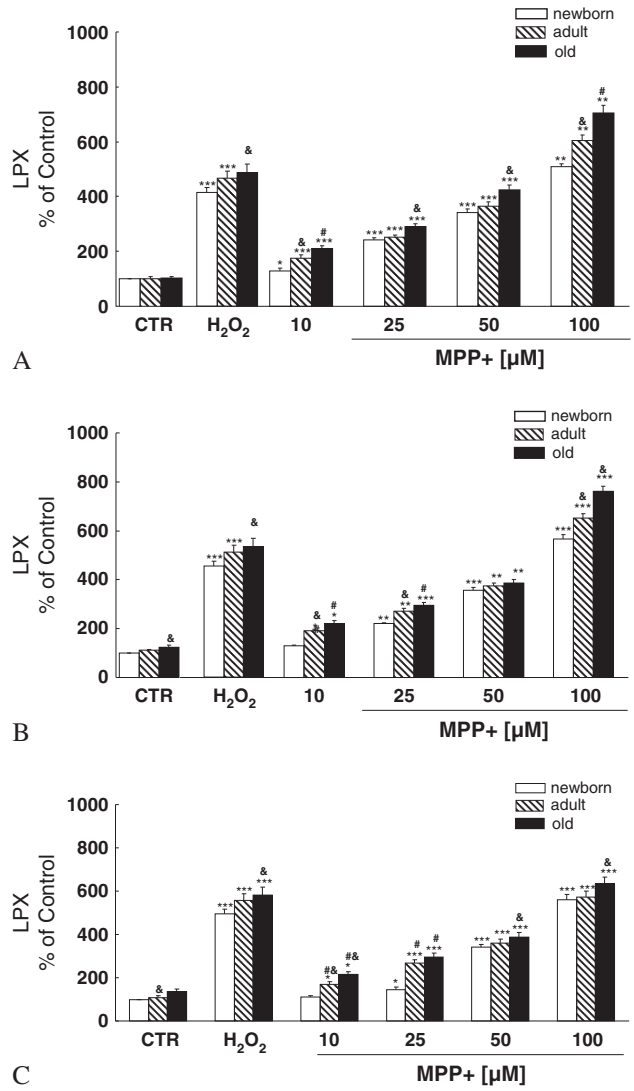

Figure 9. Lipid peroxidation in 1-methyl-4-phenylpyridinium (MPP+)-treated astrocytes: dose-response curve. LPX was determined in NA, AA and OA after MPP + treatment with different concentrations (10, 25, 50 and 100 μM), for 5 (A), 3 (B) and 1 h (**C**) as described in Materials and Methods. H_2O_2 (100 μM for 1 h) was used as a positive control for LPX. Each point represents the mean ± standard deviation (SD) of three independent experiments performed by triplicate. Statistical significance with respect to untreated cells: *($P < 0.05$), **($P < 0.01$), ***($P < 0.001$) were considered; i.e. treated NA, AA and OA vs. untreated NA. Statistical difference between AA and OA vs. NA treated with the same MPP + concentration was designated as & ($P < 0.05$); statistical difference between AA vs. OA treated with the same MPP + concentration was designated as # ($P < 0.05$).

by the development of fibrillar depositions of this peptide (Arnold *et al*., 1998). As mentioned previously, the induction of reactive astrocytes, or astrogliosis, is necessary to protect cells in the CNS; however, this response involves the release of pro-inflammatory cytokines, ROS, RNS, complement factors, etc., and all together are able to promote cellular damage (Reale *et al*., 2012). Previous studies under *in vivo* and *in vitro* conditions have shown that β-amyloid has a toxic effect on neurons, whereas it activates astroglia (Malchiodi-Albedi *et al*., 2001; Rogers *et al*., 2002). Of note, there are some reports where astrocytes are supposed to protect neurons against the harmful effects induced by β-amyloid (Giulian *et al*., 1993; Wilson, 1997), whereas others mention that astrocytes enhance β-amyloid toxicity (Hu *et al*., 1998; Johnstone *et al*., 1999; Malchiodi-Albedi *et al*., 2001). Our results suggest that the decrease in cell viability during the short- and long-terms of β-amyloid treatment is related to a mechanism that involves generation of oxidative stress expressed as lipid and protein oxidative damage.

Further considerations are needed for β-amyloid toxicity. For instance, calcium mishandling and glutathione depletion are probably mediating astrocytic damage in mixed astrocytes/neurons cultures (Abramov *et al*., 2004). Mitochondrial impairment and oxidative stress have also been implicated in cultured rat astrocytes as part of the toxic pattern exerted by β-amyloid (Bisaglia *et al*., 2004), and this was confirmed in this study. In turn, these two events might be related to the activation of NADPH oxidase, a pro-oxidant enzyme, as further suggested by Abravom *et al*. (2004). Moreover, although the direct actions of β-amyloid on NMDA receptors and further excitotoxicity have been readily reported (Peña *et al*., 2006), it is unlikely that this toxic mechanism could account for the peptide toxicity exerted on cultured astrocytes in our study as it has been demonstrated that functional NMDA receptors are not currently expressed by astrocytes in cell culture (revised by Matyash and Kettenmann, 2010).

We also found that astrocytes exposed to β-amyloid (fragment 25–35) exhibited mild resistance, being the less harmful among the toxic models tested with regard to the degree of damage exerted on these cells. This result may be as a result to a biphasic effect of β-amyloid already reported for astrocytes in culture, where these cells were shown to be resistant for short times to different fragments of the toxin (Pentreath and Mead, 2004). Although in our study astrocytes showed moderate resistance, they mostly suffered severe alterations, making them vulnerable to the toxin by mechanisms already described above. An additional consideration should be made with the findings of Paradisi *et al*. (2004), who found that astrocytes unexposed to β-amyloid might be protective for cultured neurons, but when exposed to β-amyloid, astrocytes not only suffered toxicity, but also contributed to neuronal cell damage. Thus, it seems crucial for astrocytic-associated resistance to deal with β-amyloid toxicity before they can protect other cell types. But, by which mechanism(s) astrocytes could eventually resist toxicity induced by β-amyloid or other toxins? It seems that, as long as these cells better preserve their antioxidant capacities through the activation of antioxidant enzymes, they could be more resistant (Jeong *et al*., 2005; Valles *et al*., 2010). Therefore, an antioxidant capacity might be the clue in this and other paradigms to counteract toxicity.

Effects of MPP^+ on Toxic Markers and Oxidative Damage in Astrocytes

While dopaminergic neurons are vulnerable to the toxic actions of MPTP owing to its ability to accumulate and retain MPP+, only a few reports suggest that MPP+ can directly exert damage on cultured astrocytes from rats. For instance, there is evidence showing that MPP+ causes impaired energy in astrocytes by affecting mitochondrial function, further altering glutamate clearance (Di Monte *et al*., 1999). In addition, it has been suggested that MPP+ toxicity in cultured astrocyes depends on oxidative and nitrergic stress (Tsai and Lee, 1998). This evidence was preceded by comparative studies demonstrating a differential ability to accumulate MPP+ and express toxicity between rats and mice, suggesting that cultured astrocytes from the first species accumulate less MPP+ while express toxicity at higher

concentrations (Tsai and Lee, 1994). This is relevant because, in contrast to mice astrocytes that accumulates more MPP+ and are more vulnerable (Wu *et al.*, 1992), it seems that the toxicant could be acting by different mechanisms in rat astrocytes. Altogether, this evidence demonstrates that astrocytes can be vulnerable to MPP+ owing to the limited ability of these cells to counteract the many toxic actions of this metabolite once it is available in the medium. The relevance of this study is that it provides evidence, for the first time, on the vulnerability of astrocytes obtained from rats of different ages. Our results related to astrocytes from newborns agree with those that have been reported by other groups, where the concentrations used are in the same range (10–100 μM) (Chen *et al.*, 2008; Chuang and Chen, 2004; Hu *et al.*, 2005). Similarly to β-amyloid, MPP^+ was able to induce oxidative damage to lipids and proteins, confirming that the patterns exerted by these two toxic agents are related to pro-oxidant mechanisms. Of note, the only significant difference found between newborn and adult mitochondrial dysfunction was observed for the MPP^+ treatment at 5 h (at 72 h almost all cells of both culture conditions were assumed to be dead). This result is relevant as MPP^+ is produced in astrocytes and released to the extracellular space, where it is selectively taken by the dopaminergic neurons, causing mitochondrial damage. However, here we demonstrated that, along with the often reported damage that is currently in progress in neuronal cells owing to MPTP transformation by astrocytes, these glial cells are also susceptible to be damaged by MPP+.

As we found a significant failure in mitochondrial function in response to MPP^+ treatment, it was interesting to perform a dose-response study at lower concentrations and shorter exposure times, because during the longer treatments, cells were so damage that no difference could be observed between them. To allow a comprehensive evaluation of astrocytic responses, astrocytes derived from aged rats were also used in these experiments. Our data show that the differences among ages were more evident after 1 h treatment at 25 and 50 μM; at this time point, it was clear that when an oxidant condition is induced with MPP+, astrocytes viability and functionality diminish significantly in direct proportion to the age of the group tested. Moreover, oxidative damage, defined as lipid peroxidation, also increased with age. These results may be explained according to Harman's free radicals theory of aging (1956), which postulates that the decrease in the biochemical and physiological functions associated with aging are because of an increase in accumulated oxidative damage. However, it would be interesting to further explore if the mechanism in astrocytes is the same as in neurons, especially in consideration of the differential susceptibility of adult astrocytes found in this study. Moreover, Chuang and Chen (2004) reported that the astrocytome cell line U373MG responds to oxidative stress by eliminating ROS mainly through the GSH antioxidant system, but this is just effective at low MPP^+ treatments.

For further consideration in regard to MPP^+ toxicity is the fact that it has been recently demonstrated that astrocytes from a human origin are capable of attenuating oxidative damage produced by this toxin when treated with the cruciferous nutraceutical 3H-1,2-dithiole-3-thione, which in turn is responsible for upregulation of phase 2 antioxidant enzymes and recovery of GSH levels (Jia *et al.*, 2009). Whether rat astrocytes could also be able to respond through these protective mechanisms remain to be elucidated. In the meantime, it should be emphasized that human and rat astrocytes are different, and so, comparative studies on their properties should be performed in the near future. Therefore, care must be taken when interpreting and extrapolating the results obtained with astrocytes from these two species. This is particularly relevant as mitochondrial and general functioning of astrocytes is known to be crucial for resistance against MPP^+ toxicity through different mechanisms, including an adequate reuptake of glutamate (Di Monte *et al.*, 1999) via II and III metabotropic receptors (Yao *et al.*, 2005).

Another original contribution of our work was the demonstration of an early toxic effect exerted by MPP+ in rat astrocytes. Several toxic conditions evoked by MPTP and MPP^+ have been tested in mouse astrocytes (Di Monte *et al.*, 1992; Mallajosyula *et al.*, 2008), but only a limited number of studies have brought attention to rat astrocytes, in spite of the fact that it has been shown that they undergo toxicity induced by these agents in a similar manner (Tsai and Lee, 1994). Therefore, intensive research in rat astrocytes challenged by these agents is still required in order to learn more about their responses and properties.

In summary, here we described for the first time an age-, time-, and treatment-dependent differential susceptibility of rat cultured astrocytes to toxic insults. The effects of two neurotoxic agents acting through different mechanisms served to establish these differential patterns. While astrocytes were consistently sensitive to a toxin that is known to produce various toxic effects (β-amyloid), including auto-aggregation, inflammation and excitotoxicity, a direct action of MPP+ on mitochondrial function was more intense in regard to its toxic potency. Therefore, it is likely that exerting a toxic action on mitochondria results in much more potent and sooner toxicity in astrocytes than moderately stimulating oxidative damage and activating the inflammatory processes. Nonetheless all of these models recruited oxidative damage as part of their toxic events, suggesting that this process is actively regulating astrocytic responses to toxic insults. By which precise means these differences are expressed between models? This is a question needing further clarification. In the meantime, and for further consideration, it can be considered that cultured astrocytes represent an interesting and promising biological tool for these comparative studies, although their main limitation lies in the fact that it does not involve the intricate relationships between neurons and glial cells, and so, the way astrocytes behave under these controlled experimental conditions might not completely represent a situation of a given human pathology. In this regard, mixed cultures will bring us more accurate information on these processes in the near future. In addition, it has been demonstrated that there are a number of regional morphological and functional differences between astrocytes under 'normal' and pathological conditions (including functional gap junctional coupling, expression of transmitter receptors, membrane currents and glutamate transporters), as well as among astrocytes from humans and rats (Matyash and Kettenmann, 2010), and together, these differences could limit the representativeness and usefulness of primary cultures of rat astrocytes to accurately mimic models of neurodegenerative disorders. Therefore, astrocytes remain to be fully explored in the near future.

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