



## Research Paper

# Tert-butylhydroquinone pre-conditioning exerts dual effects in old female rats exposed to 3-nitropropionic acid



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## ABSTRACT

The brain is a very susceptible organ to structural and functional alterations caused by oxidative stress and its vulnerability increases with age. Understanding the antioxidant response activated by the transcription factor Nrf2 has become very important in the aging field in order to activate cellular protection. However, the role of Nrf2 inducers during old age has not been completely understood. Our aim was to activate the Nrf2 pathway by pre-treating old rats with a widely used Nrf2-inducer, tert-butylhydroquinone (tBHQ), prior to 3-nitropropionic acid (3-NP) insult, in order to evaluate its effects at a behavioral, morphological and biochemical levels. 3-NP has been used to reproduce the biochemical and pathophysiological characteristics of Huntington's disease due to an oxidative effect. Our results suggest that tBHQ confers an important protective effect against 3-NP toxicity; nevertheless, Nrf2 seems not to be the main protective pathway associated to neuroprotection. Hormetic responses include the activation of more than one transcription factor. Nrf2 and NFκB are known to simultaneously initiate different cellular responses against stress by triggering parallel mechanisms, therefore NFκB nuclear accumulation was also evaluated.

## 1. Introduction

Brain age-related deterioration has been observed at anatomical, cellular, molecular and functional levels [1]. Neurodegeneration is a condition that describes neuronal progressive structure and/or function loss during aging, and is considered as one of the risk factors for various neurodegenerative diseases development [2,3]. Aging is a degenerative process characterized by a molecular and cellular functional decline, which is accompanied by the progressive accumulation of oxidative damaged macromolecules [4,5] generating a condition of oxidative stress (OS). Therefore, the molecular mechanism that sense these damages and activates cellular responses and resilience, have a critical role in preventing old-age diseases occurrence.

The neurodegeneration model based on the use of 3-nitropropionic acid (3-NP) has been widely studied as a mimetic for Huntington's disease (HD) because it reproduces the neuropathology and the symptoms of this disorder [6]. Briefly, 3-NP inhibits the succinate dehydrogenase (SDH, or mitochondrial complex II) catalytic site; blocking the electron transport chain and the Krebs cycle, decreasing

ATP synthesis and promoting reactive oxygen species (ROS) formation, thus fomenting mitochondrial dysfunction [7] and neuronal death [8,9].

The nuclear factor erythroid type-2 (Nrf2) is one of the antioxidant-response master regulators, which constitutes a promising target to counteract OS deleterious effects through phase II detoxifying enzymes genes up-regulation, such as heme-oxygenase (HO-1), glutathione S-transferase (GST) and others [10]. Nrf2 transcriptional activity depends on the dissociation of its repressor, Keap1, who inhibits Nrf2 in the cytoplasm, orchestrating its ubiquitination and degradation [11]. Keap1 is meticulously regulated by the cellular redox state [12]. The gene expression dysfunction mechanisms downstream Nrf2 pathway could be responsible for the observed decrease in the antioxidant capacity during aging. One of the most studied Nrf2 inducers is tert-butylhydroquinone (tBHQ), a phenolic compound with intrinsic antioxidant properties [13] used in the food industry as an antioxidant and preservative. tBHQ chemical structure allows its rapid diffusion from the plasma membrane into the cytosol, acting as a ROS scavenger and inducing the dissociation of the Keap1/Nrf2 complex [14].

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There are few studies that have attempted to induce Nrf2 activation in aged animals and the results are controversial, suggesting that the decrease in the antioxidant response is caused, at least in part, by the decline in Nrf2 signaling efficiency with age. Suh et al. [15] showed that total and nuclear Nrf2 protein levels significantly decreased in old animals compared to adults. Paradoxically, others have reported that total and nuclear Nrf2 cerebellum levels increase in aged mice in correlation with the expression of its target genes [16]. Shih et al. [17] showed that tBHQ decreased the damage caused by 3-NP in Nrf2<sup>+/+</sup> mice but not in Nrf2<sup>-/-</sup>, confirming the specific action of the inducer in young animals. However, the role of tBHQ/Nrf2 and 3-NP in old animals has not yet been elucidated. In this regard, Alarcón-Aguilar et al. [18] reported that astrocytes isolated from aged rats pretreated with tBHQ activate Nrf2 as a protective mechanism against the damage caused by a neurotoxin. Therefore, our aim was to activate the transcription factor Nrf2 by pre-treating old rats with tBHQ prior to treatment with 3-NP in order to evaluate the effects of this hormetic model, and its consequences at a behavioral, physiological and biochemical levels, comparing them with the group of old rats treated only with 3-NP. Our results show that tBHQ pretreatment correlates with neuroprotection, however, the Nrf2 pathway might not be the principal response associated with it. The mechanisms that have been described for this kind of hormetic response include the activation of more than one transcription factor that is sensitive to changes in redox state [19]. In particular, Nrf2 and NFκB are known to simultaneously initiate different cellular response to stress by triggering parallel response mechanisms, since both of them can regulate the expression of antioxidant enzymes and survival proteins [20–23]. Furthermore, NFκB has also been related to an inflammatory response, which is something that cannot be put aside when study cellular responses during aging, and therefore NFκB nuclear accumulation was also evaluated.

Old tBHQ pre-treated rats showed an improvement in the physiological and biochemical parameters, suggesting that old animals are able to activate an hormetic protective response against oxidative stress and toxic agents, but that response might not be modulated through the Nrf2 pathway.

## 2. Materials and methods

### 2.1. Reagents and antibodies

Tert-butylhydroquinone (tBHQ), 3-nitropropionic acid (3-NP) and the rest of chemicals and reagents were purchased from Sigma Chemical Co. (St. Louis, MO). Antibodies were obtained from different sources as follows: Nrf2 (sc-722), NFκB p65 (sc-8008), NFκB p50 (sc-8414), glutathione S-transferase (GST, sc-138), superoxide dismutase (SOD-1, sc-271014), tubulin-β (sc-58888), and secondary rabbit and mouse antibodies conjugated with horseradish peroxidase IgG were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Heme-oxygenase-1 (HO-1, #3391) from BioVision (Milpitas, CA), Lamin-A (ab26300) and GFAP (ab7260) from Abcam (Cambridge, MA). Chemiluminescent detection system and FluoroJade B (AG3010) were obtained from Millipore (Bedford, MA).

### 2.2. Animals

Adult (9 months-old) and old (24 months-old) albino Wistar female rats (*Rattus norvegicus*), provided by the closed breeding colony at the Autonomous Metropolitan University-Iztapalapa, were used in this study. A total of 63 adult and 36 old rats were employed. Before they were assigned to the experiments, adult animals were housed five-percentage in polycarbonate cages and provided with standard commercial rat diet (Harlan 2018S, USA) and water *ad libitum*. All procedures with animals were strictly carried out according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and the

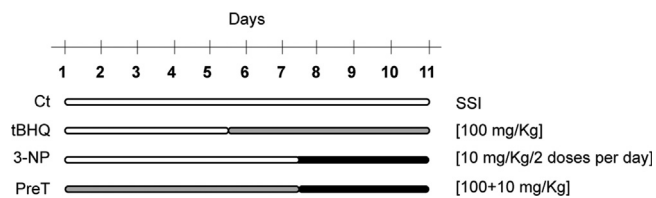


Fig. 1. Schematic representation of the treatments administered to adults and old animals. tBHQ treatments consisted of 7 i.p. injections (100 mg/kg/day) in total, given as a daily dose; 3-NP treatment (10 mg/kg) was injected *via* i.p. for 4 days, 2 doses a day. It consisted of 16 injections in total, with 8 h between each administration (two doses per day). The pre-treated (PreT) group received injections for 11 days in total (7 + 4) under the same schemes than tBHQ and 3-NP groups (100 mg/kg/10 mg/kg, respectively). A total of 64 adult and 36 old rats were used.

Principles of the Mexican Official Ethics Standard 062-ZOO-1999 and the Standard for the disposal of biological waste (NOM-087-ECOL-1995).

### 2.3. Experimental groups

Animals were randomly selected and divided into 4 experimental groups (Fig. 1):

1. *Control group*, rats treated with isotonic saline solution (ISS) as a vehicle.
2. *tBHQ group*, administered with tBHQ (100 mg/kg/day) intraperitoneal (i.p.) for 7 days.
3. *3-NP group*, administered with 3-NP (10 mg/kg) *via* i.p. for 4 days, 2 doses a day. The rationale for 3-NP dosage and administration schedule is based on previous reports demonstrating striatal toxicity [26,43,44].
4. *Pre-conditioning/pre-treatment group (tBHQ + 3-NP)*, administered with both compounds (100 mg/kg/10 mg/kg, respectively) *via* i.p. for 11 days.

At the end of each treatment, the animals were sacrificed and brain tissues were obtained. The doses used for 3-NP and tBHQ were determined on the basis of a dose-response curve performed for tBHQ and 3-NP independently in adult animals (Supplementary material Figs. 1 and 2) and the range of concentrations used was chosen from the reported literature [24–27].

### 2.4. Evaluation of motor activity

Motor activity for the 3-NP model was estimated in a *Versamax Animal Activity Monitor and Analyzer* (Versadat version 3.02) open field (Accuscan Instruments Inc., Columbus, Ohio), as previously reported [28]. Animals of all groups were deposited in the open sensor device and monitored for 15 min. The horizontal and vertical activities, as well as total distance walked, were analyzed. During motor activity assay, different conditions were controlled following strict specifications described in specialized reports [29]. The room temperature (25 ± 2 °C) and constant cleaning of the open field arena were revised constantly. In addition, all animals belonged to the same age (9 or 24 months-old) and gender (female), and behavioral estimations were carried out during the morning time (7:00–10:00 a.m.). Outer distractors were kept at a minimum level as the evaluation room was kept isolated, and free of noise and movements. The room in which motor activity was recorded remained isolated and each rat was acclimated in its cage minimally for 5 min before recording activity. At the beginning of the test, animals were placed in the center of the chamber. Once into the motor activity device, each sensor line crossed by rats was scored as one unit of activity. Results were expressed as individual records collected throughout the entire test. At this point, however, it is pertinent to address that this is a simple design for measurement of

motor activity consisting of recording of a spontaneous single measure. Despite this design offers a simple, rapid and mostly reliable approach to motor activity estimations, it may present some limitations if contrasted with more strict and controlled designs [30].

### 2.5. Histological analysis

For the histological analysis, three animals from each experimental group were anesthetized i.p. with 500  $\mu$ l of sodium pentobarbital, and transcardially perfused with ISS, followed by *p*-formaldehyde 4% (w/v) at 4 °C. All brains were removed, then fixed for 48 h and embedded in paraffin as previously reported [31]. Briefly, fixed tissues were serially sectioned with a microtome *Histostat 820* (American Instrument Exchange Inc. Haverhill, MA). Striatal sections (100  $\mu$ m width by 5  $\mu$ m thick) were obtained. Sections were stained with hematoxylin-eosin (H & E) to visualize cell bodies using an IM100 image analyzer (Leica, Cambridge UK). Three sections were obtained from each brain and 5 different fields were analyzed in each section. The general criteria used to assess the neurons included preserved cores, pyknotic nuclei and cytoplasmic vacuolation. The number of preserved, damaged and total cells was obtained as an average of five randomly selected three sections of striatal fields per animal; data were expressed as cell damage per field.

### 2.6. Immunohistochemistry for glial fibrillary acidic protein (GFAP)

The immunohistochemical assay for glial fibrillary acidic protein (GFAP) was conducted for 3 animals of each experimental group. To prevent antigen degradation, sections were stored at 4 °C prior to assay. Briefly, brains were fixed in 4% (w/v) *p*-formaldehyde for 48 h, embedded in paraffin and sliced into 5-micrometer sections. Sections were deparaffined and boiled in the presence of 3% (w/v) sodium citrate with 0.2% (v/v) Triton X-100 to unmask antigen sites. Endogenous peroxidase activity was quenched with 1% (v/v) H<sub>2</sub>O<sub>2</sub>. Non-specific binding was avoided using a bovine serum albumin solution 1% (w/v). Sections were incubated with anti-GFAP at 1:5000 in PBS overnight at room temperature and then incubated with an anti-rabbit secondary antibody conjugated to HRP (DAKO kit, Dakocytomation). Finally, all sections were incubated with diaminobenzidine and co-stained with hematoxylin. Three sections were obtained from each brain and 5 different fields were analyzed in each section. Sections were analyzed field-by-field and determined with an image analyzer IM100 (Leica Cambridge, UK) [32]. All images correspond to dorsal striatum.

### 2.7. Neurodegeneration using stain detection of Fluoro Jade B (FJ)

Brain sections were collected from 3 animals of each experimental group and processed according to previous reports [32,33]. All sections were immersed in 1% (w/v) NaOH and 80% (v/v) ethanol for 5 min, followed by 2 min in 70% (v/v) ethanol. Slides were transferred to 0.06% (w/v) potassium permanganate (KMnO<sub>4</sub>) for 10 min on a shaker table for assessing background suppression consistent between sections, and rinsed with distilled water for 2 min. Then, the collected sections were stained with 0.0004% (w/v) FJ solution for 20 min. The slides were washed three times with distilled water for 1 min in each step. Excess water was removed and immersed in xylene for at least 1 min before covering with non-aqueous and non-fluorescent mounting. Three sections were obtained from each brain and 5 different fields were analyzed in each section. Immunofluorescence was visualized with a Cytation 3 cell imaging multi-mode reader (BioTek, USA), using a green filter FITC (excitation 480 nm and emission 525 nm peak).

### 2.8. Total, cytosolic and nuclear protein isolation

After each experimental protocol, the brain tissue (100 mg) was

obtained. Tissue was pulverized in a mortar previously frozen with liquid nitrogen, then, dissolved in buffer cold containing Tris-HCl 50 mM, NaCl 120 mM, Nonidet-P40 at 0.5% (w/w), 100 mM NaF, pH 8.0, a 1:3 w/v to obtain total protein. Homogenates were centrifuged at 900g for 10 min at 4 °C, the supernatant was recovered and placed in 1.5 ml Eppendorf tubes. Cytosolic and nuclear extracts were obtained according a manufacturer's instructions using the NE-PER<sup>®</sup> kit (Thermo Fisher Scientific, Waltham, MA). Protein concentration was determined in the supernatant by method of Lowry [34], stored at -70 °C for future determinations.

### 2.9. Western blot analysis

Proteins (60  $\mu$ g) were separated on 10% SDS-PAGE, transferred to polyvinylidene difluoride (PVDF) membranes (Immobilon<sup>®</sup>-P, Millipore) and incubated at room temperature for 1.5 h in TBS-T buffer (20 mM Tris-HCl, pH 7.6, 137 mM NaCl, 0.1% (v/v) Tween-20) containing 5% (w/v) defat milk. After incubation with specific primary antibodies against Nrf2 (1:200), GST (1:200), SOD-1 (1:500), HO-1 (0.5  $\mu$ g/ml), NF $\kappa$ B p-65 (1:200) and NF $\kappa$ B p50 (1:200) in TBS-T with 3% (w/v) defat milk at 4 °C, the membranes were washed 3 times with TBS-T buffer for 10 min and incubated with horseradish peroxidase (HRP) conjugated with anti-rabbit or anti-mouse IgG (1:15,000) during 1.5 h after 3 times washed in TBS-T for 10 min, the immunoblotted proteins were visualized using a chemiluminescent detection system (Millipore, Billerica, MA). Each membrane was stripped (Glycine 15 g, SDS 1 g and 10 ml Tween-20, pH 2.2) and incubated with their respective load control (markers of cytosolic and/or nuclear fractions, actin/tubulin<sup>®</sup> and lamin-A, respectively). All images were analyzed by the intensity of pixels quantified using Image Studio Lite Software (Li-Cor Biosciences, Lincoln, Nebraska USA).

### 2.10. Protein carbonyl measurement by DNP-protein immunoblotting

In order to evaluate oxidative stress in the adult and senile brains from all experimental groups, protein oxidation was measured according to the Oxyblot protein oxidation detection kit (OxiBlot<sup>™</sup>; Millipore, Billerica, MA), as described by Alarcón-Aguilar et al. [35]. Brain homogenates (20  $\mu$ g protein) were derivatized to 2,4-dinitrophenylhydrazine (DNP) with 2,4-dinitrophenylhydrazine (DNPH). Samples were neutralized, separated by SDS-PAGE at 12%, transferred to PVDF membranes, and incubated with rabbit anti-DNP antibodies in TBS-T. Horseradish peroxidase-conjugated secondary antibody and a chemiluminescent reagent were used for signal detection (Immobilon<sup>®</sup>-P, Millipore) and quantification of pixel intensity using the Image Studio Lite Software (Li-Cor Biosciences, Lincoln, Nebraska USA).

### 2.11. Lipid peroxidation assay

Thiobarbituric Acid Reactive Substances (TBARS) were used for screening lipid peroxidation in brain homogenates according to Persky et al. [36], with some modifications. Brain tissue homogenates were heated for 20 min at 37 °C before adding 12.5g of trichloroacetic acid (TAC) in hydrochloric acid (HCl) 1 M. The samples were incubated with thiobarbituric acid (TBA) 1% (w/v) for 15 min in boiling water (90 °C) and then, placed on ice and centrifuged 500g for 10 min at 4 °C the formed MDA-TBA adduct supernatant was detected at 532 nm in Beckman DU-65 Spectrophotometer (Brea, California, USA). The obtained values were compared with a standard curve of 1,1,3,3-tetramethoxypropane (TMPO) and results were expressed as TBARS nmol/mg protein. Protein concentration was determined in the supernatant by method of Lowry [34].

### 2.12. Isolation of mitochondria from brain tissues of adult and senile animals

The collected brains were placed in working solution (250 mM sucrose and 10 mM EDTA, pH 7.4) and cut into small pieces using surgical scissors. The suspension was homogenized using a Polytron at 4 °C and centrifuged at 700g for 15 min. Subsequently, the supernatants were re-centrifuged at 800g for 15 min; the pellets were resuspended in the same solution and centrifuged again for 15 min at 12,000 rpm. The pellets were resuspended again in the working solution at 4 °C. Protein concentration was determined using the commercial agent Bradford (Bio-Rad, Hercules, CA) [37].

### 2.13. Mitochondrial complex II activity determination

The activity of the mitochondrial respiratory chain complex II was determined spectrophotometrically at 600 nm, using the technique based on the reduction of phenazine methosulfonate (PMS) by succinate dehydrogenase (SDH). The reduced PMS is immediately re-oxidized by 2,6-dichlorophenolindophenol (DCPIP), resulting in a decrease in the absorbance of the last dye [38]. Isolated mitochondria (40 µg/ml) were incubated in 10 mM KH<sub>2</sub>PO<sub>4</sub>, pH 7.3 and 1 mM succinate (pH 7.4). Complex II was activated by pre-incubation of mitochondria in the presence of 5 mM PMS and DCPIP (6.3 mM). The decrease in absorbance was recorded for 5 min [37].

### 2.14. Statistical analysis

Data are expressed as mean ± SD (standard deviation) of at least three independent experiments per group performed by triplicate. To compare the data, an analysis of variance (ANOVA) followed by *post hoc* analysis using the Tukey's multiple comparison test was performed with significance levels of  $p < 0.05$ ,  $p < 0.01$ ,  $p < 0.001$ . While to compare the data in the mitochondrial complex II activity, an ANOVA followed by *post-hoc* analysis using the Bonferroni's multiple comparison test was performed with the same significance levels above, using the statistical program GraphPad Prism version 6.0. For the NFκB densitometry analysis, the Kruskal–Wallis variance analysis followed by Dunn multiple comparison test were performed using the statistical program NCSS version PASS 15.

## 3. Results

### 3.1. tBHQ pretreatment improved motor activity in old animals exposed to 3-NP

Under our experimental conditions (Fig. 1), when the adult rats were treated with 3-NP, an hypoactive effect was observed, evidenced by a decrease in the number of vertical movements (VM) and in the TDW ( $p < 0.05$ ), representing the hypokinetic activity which is observed in late stages of Huntington's disease (HD) (Fig. 2B and C); on the contrary, animals treated with tBHQ showed an hyperactive pattern in the total distance walked (TDW) ( $p < 0.05$ ) (Fig. 2C). Old rats improved horizontal movements (HM) (Fig. 2A) and VM (Fig. 2B) with tBHQ pre-treatment (PreT) ( $p < 0.05$ ), compared with animals that were treated only with 3-NP and which showed a prominent VM hypoactive pattern ( $p < 0.05$ ). The Versamax Accuscan device employed here can record horizontal, vertical and ambulatory activities, displays maps of trajectories and also offers parameters such as total time in movement, total resting time, time spent in the center and stereotypies. Thus, we presented in this manuscript only those parameters showing changes or minimally important trends. No changes in stereotypies were found. The method used here is simple, but represents a real spontaneous activity measure, specially considering that the possible artefactual variables -such as fear and/or anxiety- were considerably reduced and strictly controlled prior to the assay.

### 3.2. tBHQ pretreatment preserved the striatal cellular architecture of old 3-NP-treated animals

Striatal morphology in both age groups after all treatments was evaluated. In contrast to the well-preserved architecture observed in the control (Ct) groups, animals treated with 3-NP showed considerable cellular damage, revealing an extensive cellular loss throughout the caudate-putamen, accompanied by abundant pyknotic nuclei, small cell size and striosome loss (Fig. 3A and B). Damaged cells represented 70% in adult rats ( $p < 0.01$ ) (Fig. 3A) and 60% ( $p < 0.05$ ) in old rats, compared with control (Fig. 3B). tBHQ PreT animals showed a striatal tissue normal appearance similar to Ct. Importantly, tBHQ did not induce cell death in striatal tissue in both age groups as quantified in Fig. 3C. These results indicate that 3-NP induced a significant loss of GABAergic neurons in the striatal tissue, whose effect was prevented by tBHQ PreT.

### 3.3. tBHQ pretreatment decreased reactive astrogliosis in senile animals treated with 3-NP

Reactive astrogliosis is known to occur in response to different stressors and increases during the normal aging process, as shown in Fig. 4, where levels of glial fibrillary acidic protein (GFAP) increased 4-fold in control old animals (Fig. 4B) when compared to control adults (Fig. 4A) ( $p < 0.01$ ). Interestingly, senile animals were more susceptible than adults to 3-NP toxicity, which induced about 40% GFAP immunopositive cells ( $p < 0.001$ ). Paradoxically, tBHQ considerably increased astrogliosis in adult rats ( $p < 0.001$ ) compared to aged rats, which decreased gliosis (Fig. 4A and B). PreT also decreased significantly the astrogliosis in old animals ( $p < 0.001$ ) (Fig. 4C), suggesting that a survival pathway might have an important participation in mediating this neuroprotection.

### 3.4. tBHQ pretreatment did not protect against 3-NP neurodegeneration in old animals

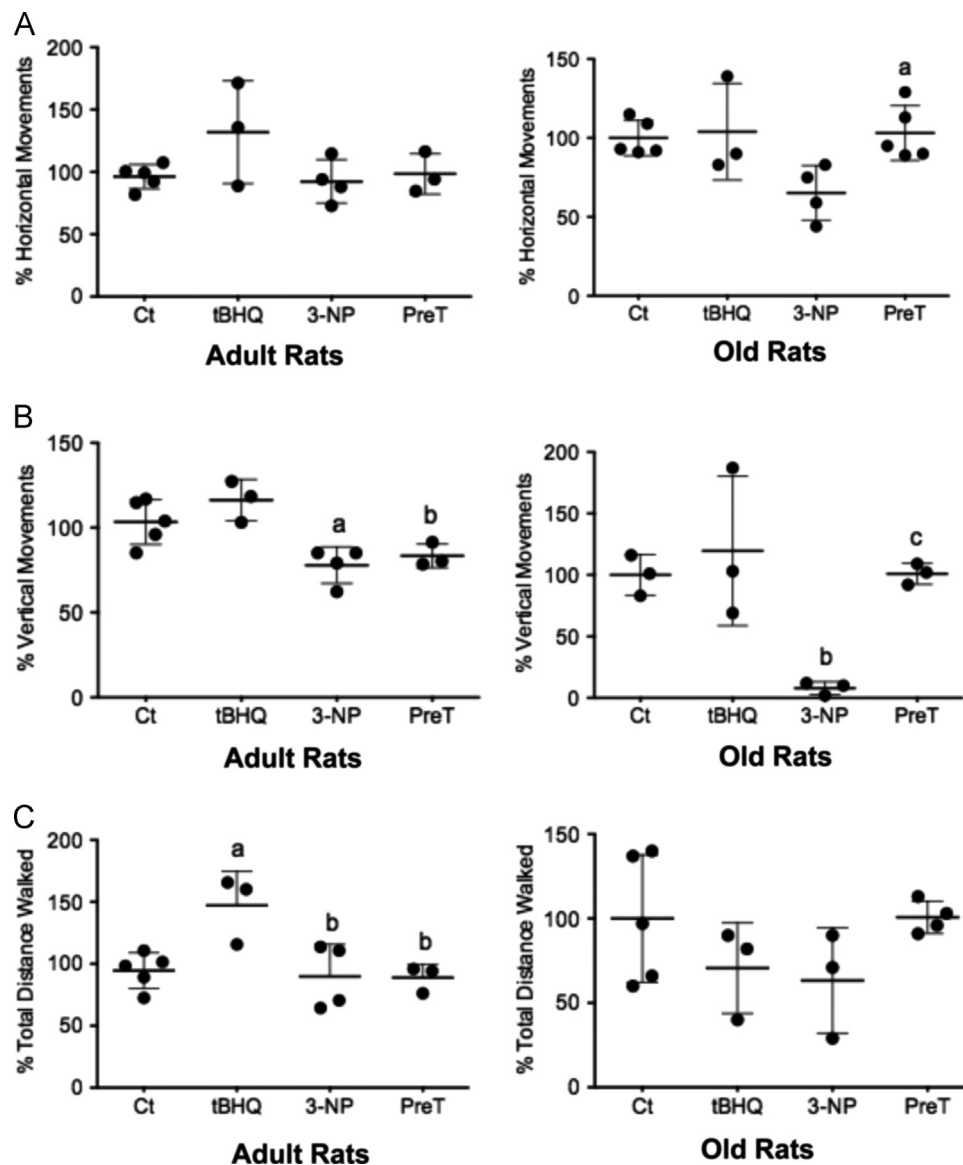
FluoroJade staining was used to evaluate neuronal degeneration in treated animals brains. Fig. 5A shows that adult rats treated only with 3-NP presented extensive degeneration along the striatum, and this effect was prevented with tBHQ PreT. Interestingly, in old animals the neurodegenerative pattern was maintained during all the treatments, suggesting that damage might be associated to increased age (Fig. 5B).

### 3.5. Adult and old animals pretreated with tBHQ did not increase Nrf2 nuclear translocation

Nrf2 distribution in nuclear and cytosolic fractions was evaluated in the brains from old and adult treated animals. Differential effects in both age groups were observed (Fig. 6A). The old animals slightly increased Nrf2 nuclear translocation with tBHQ while in increased Nrf2 was found in the nuclear fraction of adult animals treated with 3-NP alone, ( $p < 0.01$ ) when compared to the control, tBHQ and PreT treated animals. In contrast, nuclear Nrf2 accumulation in old animals was only observed (10%,  $p < 0.01$ ) in tBHQ treated rats. Both animal groups, adult and old, decreased Nrf2 nuclear accumulation after tBHQ PreT (Fig. 6A). On the contrary, tBHQ PreT induced a greater cytoplasmic Nrf2 accumulation in adult animals (60%,  $p < 0.01$ ), while in old animals Nrf2 levels decreased ( $p < 0.05$ ) compared to the 3-NP group, which did not modify the Nrf2 cytosolic content (Fig. 6B). Our data indicate that Nrf2 is not efficiently translocated to the nucleus after tBHQ PreT in both age groups striatum.

In line with the above, the content of three proteins regulated by this transcription factor were evaluated (Fig. 6C). Our results demonstrated that tBHQ as well as 3-NP-treatment significantly increased heme-oxygenase-1 (HO-1) and Glutathione S-transferase (GST) ( $p < 0.001$ ) levels in adult animals, while the content of superoxide





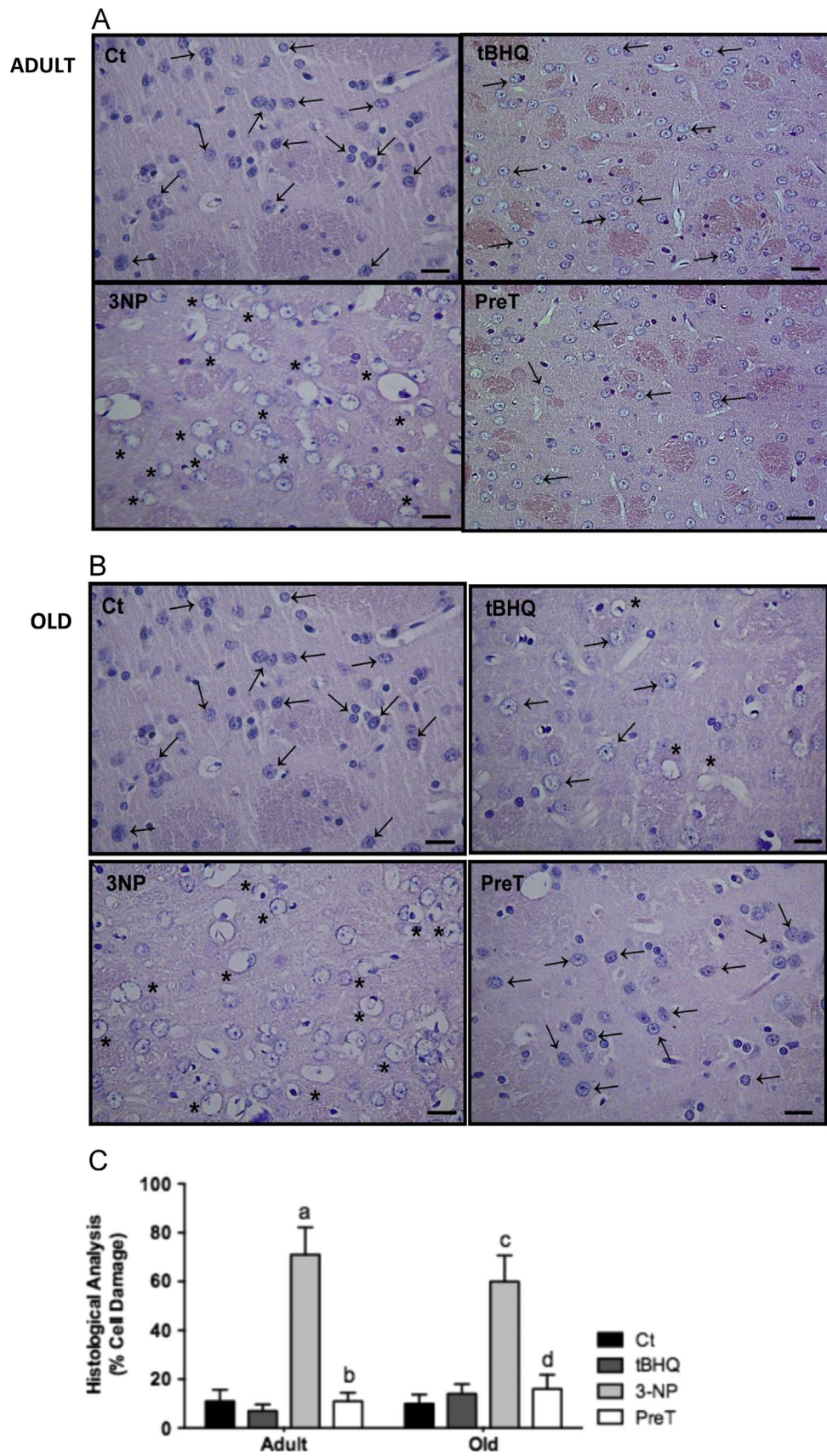
**Fig. 2.** tBHQ pretreatment preventive effect on motor changes induced by 3-NP. Parameters of motor activity were determined as described in Section 2. A. Percentage of horizontal movements determined for adult and old rats. B. Percentage of vertical movements determined for adult and old rats. C. Percentage of total distance walked determined for adult and old rats. Black circles represent the number of independent experiments carried out in each treatment in both groups of animals ( $n=3-5$ ) and represent the mean  $\pm$  SD. The significance level for adult animals is expressed as follows: Adult <sup>a</sup> $p < 0.01$  vs Ct, <sup>b</sup> $p < 0.01$  vs tBHQ and the old animals <sup>a</sup> $p < 0.05$  vs 3-NP, <sup>b</sup> $p < 0.05$  vs Ct, <sup>c</sup> $p < 0.05$  vs 3-NP.

dismutase-1 (SOD-1) decreased with tBHQ ( $p < 0.05$ ) (Fig. 6C). Conversely, in old animals treated only with tBHQ, a significant decrease was observed only in HO-1 levels ( $p < 0.001$ ) while GST and SOD-1 decreased with 3-NP treatment ( $p < 0.001$ ,  $p < 0.05$ ). Paradoxically, tBHQ PreT drastically reduced the content of the three enzymes regulated by Nrf2 in adult animals up to 90% ( $p < 0.001$ ) with respect to the neurotoxin 3NP. Old rats pretreated with tBHQ, recovered SOD-1 levels ( $p < 0.05$ ), while GST levels decreased even more with the PreT (40%,  $p < 0.001$ ) compared to 3NP (Fig. 6C).

### 3.6. tBHQ pretreatment protected protein against oxidation but increased lipid oxidation in old rats

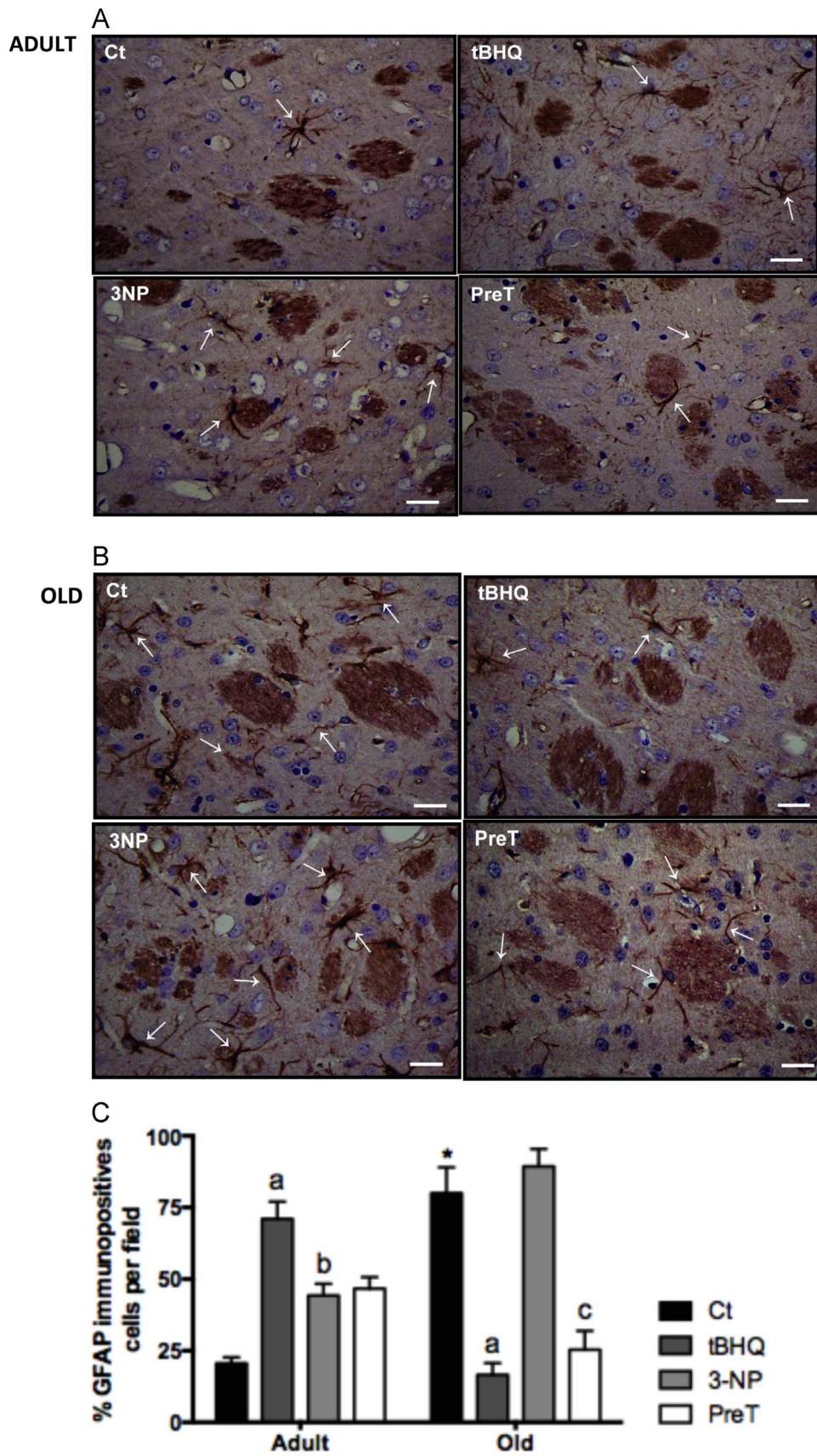
The classical markers of oxidative damage, lipid and protein oxidation, were evaluated in the rats striatum. Old animals were more susceptible to the 3-NP treatment than adult and increased protein oxidation up to 30% ( $p < 0.01$ ) above control, an effect that was

significantly prevented with tBHQ PreT (40%;  $p < 0.001$ ) (Fig. 7A and B). Protein oxidation in adult rats was not modified in any of the treatments (Fig. 7B). In the case of lipid peroxidation, TBARS levels increased only 10% in adult animals treated with tBHQ ( $p < 0.05$ ); paradoxically 3-NP did not induce damage to lipids, and as expected, no damage was found in the tBHQ PreT group (Fig. 7C). In contrast, tBHQ PreT displayed more susceptible to lipid oxidation in the old group ( $p < 0.05$ ) compared to the 3-NP group, and contrary to adult groups, where tBHQ did not induce an increase in TBARS content ( $p < 0.01$ ). Interestingly, tBHQ decreased lipid oxidation in aged rats ( $p < 0.05$ ) (Fig. 7C). Since 3-NP is known to induce SDH irreversible inhibition, SDH activity was evaluated. The results in Fig. 7D show that adult animals treated with 3-NP effectively decreased complex II activity ( $p < 0.05$ ), which was prevented with tBHQ PreT ( $p < 0.05$ ). Interestingly, a lower activity was determined in the aged animals prior to the treatments ( $p < 0.01$ ), which decreased even more with 3-NP treatment ( $p < 0.05$ ), and was prevented with tBHQ PreT ( $p < 0.05$ ).

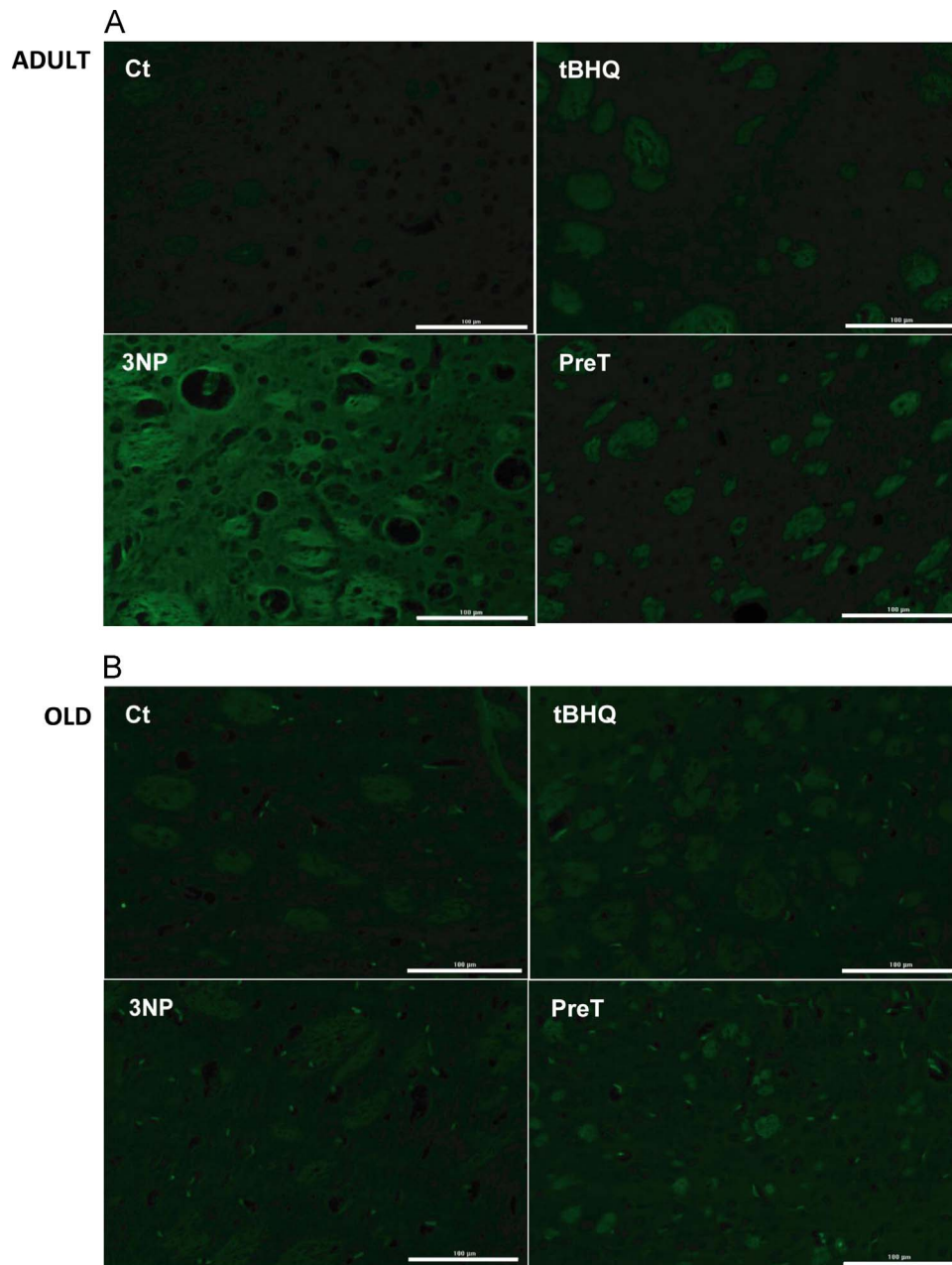


**Fig. 3.** Striatum cytoarchitecture determination in adult and old rats treated with 3-NP after tBHQ pretreatment. **A.** Adult and **B.** old animals were treated as described in Fig. 1 and samples were processed according to the Section 2. Morphological changes were analyzed by microscopy (40×); normal cells (arrows) and pyknotic cells (asterisks) are shown. **C.** Quantitative analysis was performed by counting the number of death cells in histological striatum sections obtained from adult and old treated rats. Similar results were obtained from three independent experiments. All data are presented as mean ± SD (n=3 in each group). Significance levels is expressed as follows <sup>a</sup>p < 0.01, <sup>b</sup>p < 0.05 vs Ct, <sup>c</sup>p < 0.01, <sup>d</sup>p < 0.05 vs 3-NP.





**Fig. 4.** Glial fibrillary acidic protein (GFAP) in adult and old animals striatum. GFAP was used as a reactive astrogliosis marker. Morphological details of adult (A) and old (B) rats after the different treatments are shown. Samples were processed according to the Section 2. Morphological changes were analyzed by microscopy (40×); the GFAP immunopositive astrocytes are shown with arrows. (C). Quantitative analysis of GFAP presence in adult and old striatum. The results were obtained from three independent experiments. All data are represented as mean ± SD (n=3 in each group). <sup>a</sup>*p* < 0.001, <sup>b</sup>*p* < 0.01 vs Ct, <sup>c</sup>*p* < 0.001 vs 3-NP. The asterisks (\*) indicate *p* < 0.05 vs Adult Ct.



**Fig. 5.** Higher susceptibility to neurodegeneration in aged rats stained with FluoroJade B. Cellular degeneration was analyzed with FluoroJade B staining in adult (A) and old (B) rats striatal sections. Samples were processed according to the Section 2; cells were detected by epifluorescence: 480 nm excitation and 525 nm emission fluorescence peaks, at 100 μm. Similar results were obtained from three independent experiments (n = 3 in each group).

### 3.7. Old animals pretreated with tBHQ significantly increase NFκB p65 and p50 subunits

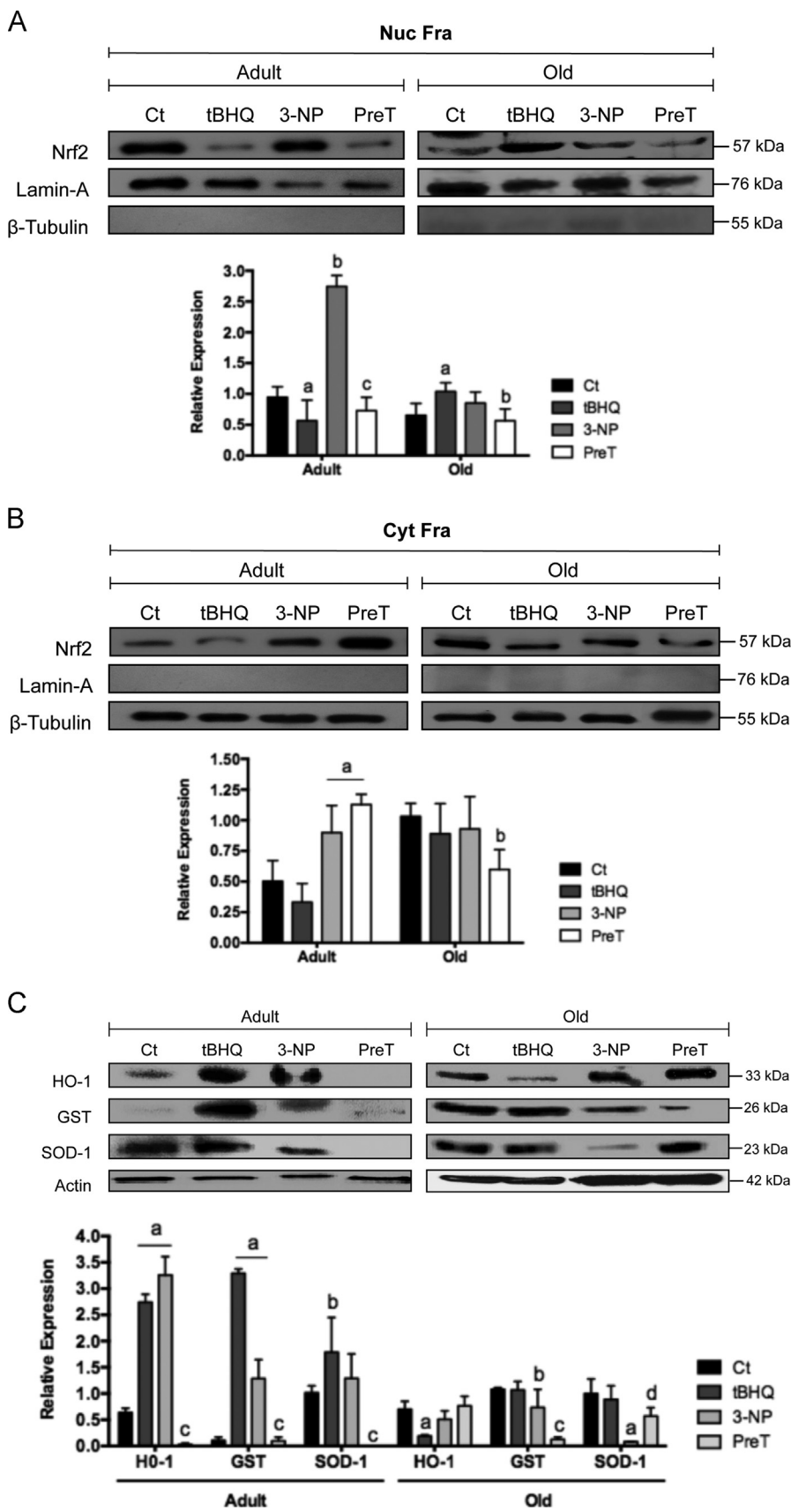
NFκB content in the nucleus during the different treatments in adult and old rats was determined (Fig. 8). Surprisingly, our results indicated that old tBHQ PreT animals had an increased amount of p65 and p50 subunits in the nucleus compared to the controls (over 50%,  $p < 0.05$ ) (Fig. 8B). Paradoxically, in the group of adult animals under the same treatment, p50 decreased ( $p < 0.051$ ) with the PreT and p65 increased ( $p < 0.05$ ) only with tBHQ (Fig. 8A).

## 4. Discussion

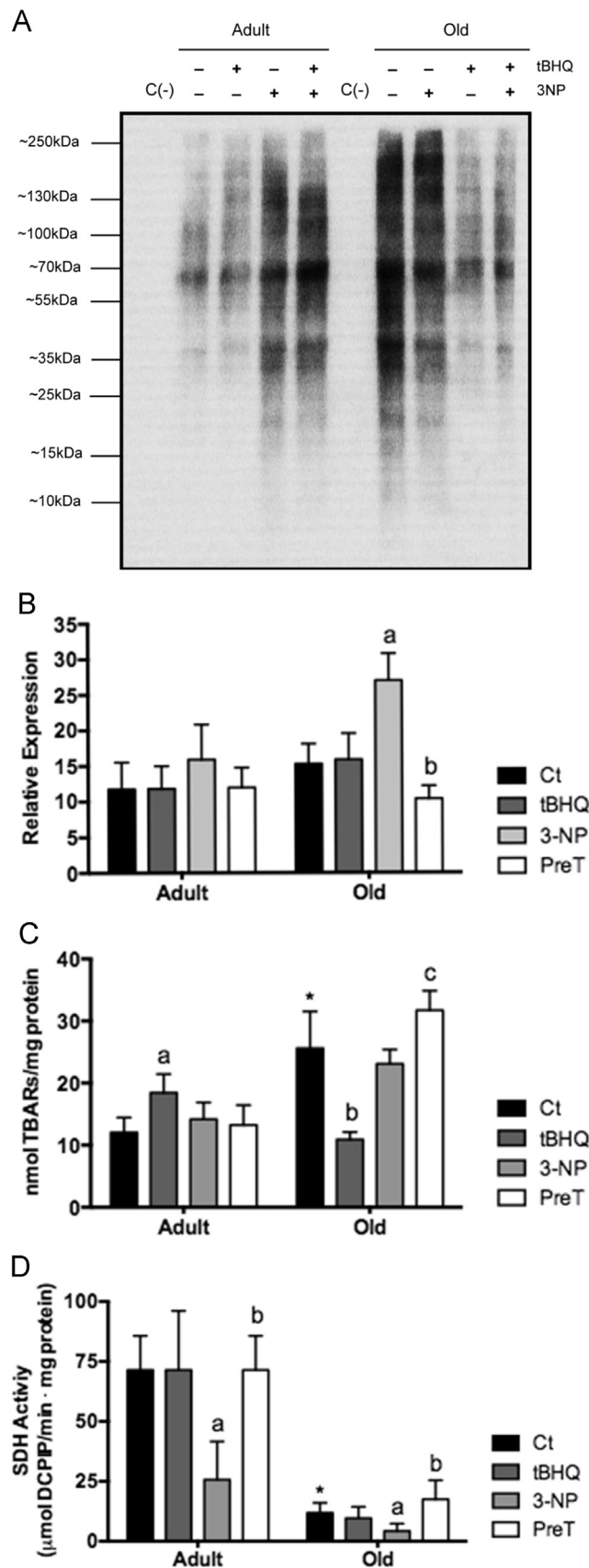
In recent years, the study of the aging process has gained special attention, since it is now considered a risk factor for the development of multiples pathologies [3]. Particularly, brain aging is associated with a

progressive imbalance between intracellular ROS levels and antioxidant defense [39]. Neuronal susceptibility to damage has been associated with the activation of Nrf2 pathway, however, little is known about the physiological signals that regulate this neuroprotective pathway in the central nervous system during aging [40]. In the present study we showed that old animals are more susceptible to the 3-NP toxicity, which induced cell death and reactive astrogliosis, and decreased their motion capabilities. Interestingly, these alterations were partially prevented with the tBHQ PreT, as observed in other disease models (besides HD), such as Parkinson's disease [41]. This fact is relevant because it confirms the usefulness of 3-NP to mimic the early and late stages of neurodegenerative diseases, depending on the age [42–45]. It has been reported that Nrf2<sup>-/-</sup> mice treated with 3-NP present motor deficits, homologating the late stage of HD [46], according to the classification described by Gabrielson et al. [41,47]. The use of certain molecules with antioxidant properties, such as S-allylcysteine (SAC),

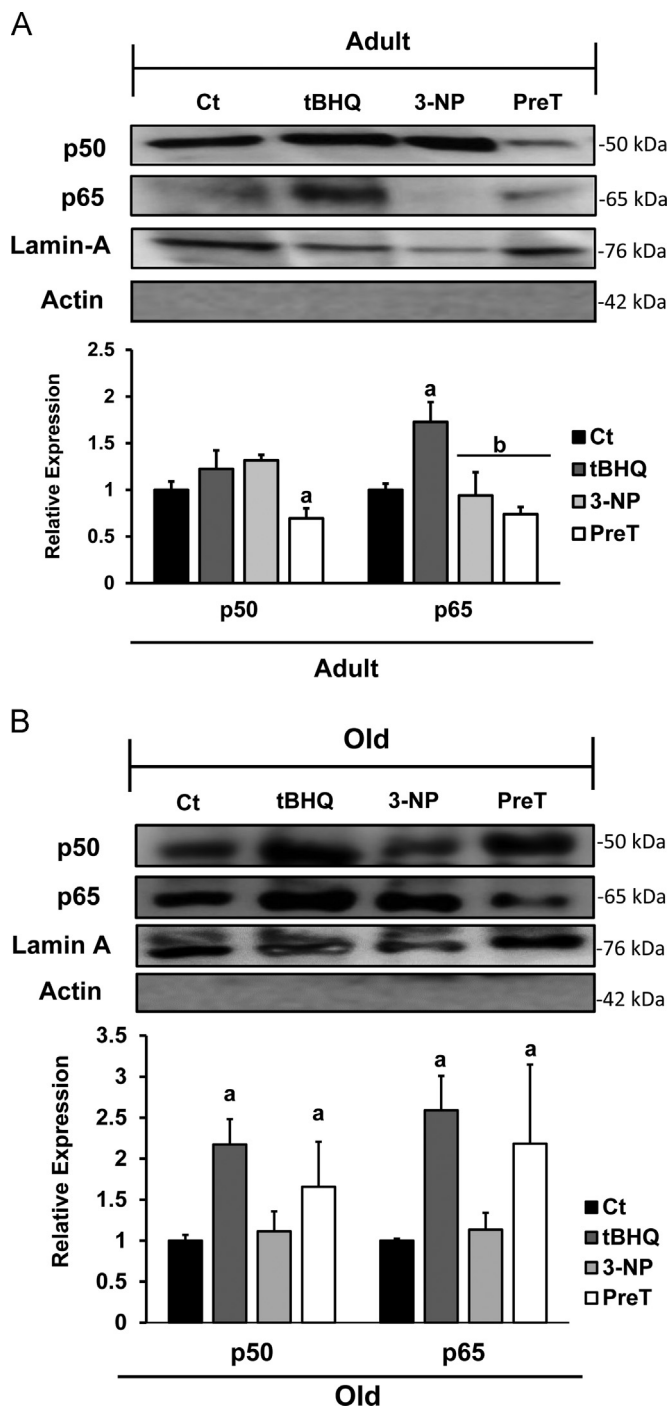




**Fig. 6.** Nrf2 nuclear and cytosolic levels. A. Representative images of Nrf2 nuclear accumulation (Nuc Fra) in adult and old animals after tBHQ PreT and densitometric analysis. For Adult, <sup>a</sup>*p* < 0.05, <sup>b</sup>*p* < 0.01 vs Ct, <sup>c</sup>*p* < 0.001 vs 3-NP. For Old, <sup>a</sup>*p* < 0.01 vs Ct, <sup>b</sup>*p* < 0.05 vs 3-NP. B. Representative images of Nrf2 content in the cytosolic fraction (Cyt Fra) in adult and old animals and densitometric analysis. <sup>a</sup>*p* < 0.01 vs Ct, <sup>b</sup>*p* < 0.05 vs 3NP. C. Representative images of antioxidant enzymes regulated by Nrf2 evaluated in the Cyt Fra in adult and old animals and densitometric analysis. <sup>a</sup>*p* < 0.001, <sup>b</sup>*p* < 0.05 vs Ct, <sup>c</sup>*p* < 0.001, <sup>d</sup>*p* < 0.05 vs 3-NP. Similar results were obtained from three independent experiments. All data are represented as mean ± SD (n=3 in each group).



**Fig. 7.** Oxidative stress markers in adult and old animals brain tissue. **A.** Representative image of protein oxidation obtained from adult and old brains homogenates using a commercial kit as described in Section 2. **B.** Densitometric analysis of protein oxidation. The following levels were considered: <sup>a</sup>*p* < 0.01 vs Ct; <sup>b</sup>*p* < 0.001 vs 3-NP. **C.** TBARs measurement as an indirect determination of lipid peroxidation in brain tissue homogenates. <sup>a</sup>*p* < 0.05, <sup>b</sup>*p* < 0.01 vs Ct; <sup>c</sup>*p* < 0.05 vs 3-NP. The asterisks indicate <sup>a</sup>*p* < 0.05 vs Adult Ct. **D.** Complex II activity in isolated mitochondria from brain tissue in both age groups under all treatment regimens. <sup>a</sup>*p* < 0.05 vs Ct; <sup>b</sup>*p* < 0.05 vs 3-NP. The asterisk indicate <sup>a</sup>*p* < 0.01 vs Adult Ct. Similar results were obtained from three independent experiments. All data are represented as mean ± SD (n = 3 in each group).



**Fig. 8.** Determination of the transcription factor NFκB in the brain of adult and old rats treated with 3-NP after tBHQ PreT. NFκB subunits p65 and p50 in brain tissue from adult (A) and old (B) animals after the different treatments. The Kruskal–Wallis variance analysis followed by Dunn multiple comparison test were done to compare the data using the statistical program NCSS version PASS 15, with a significance: <sup>a</sup> $p < 0.05$ , <sup>b</sup> $p < 0.01$  vs Ct. The results were obtained from four independent experiments and the data are expressed as mean  $\pm$  SE (Three animals in each group).

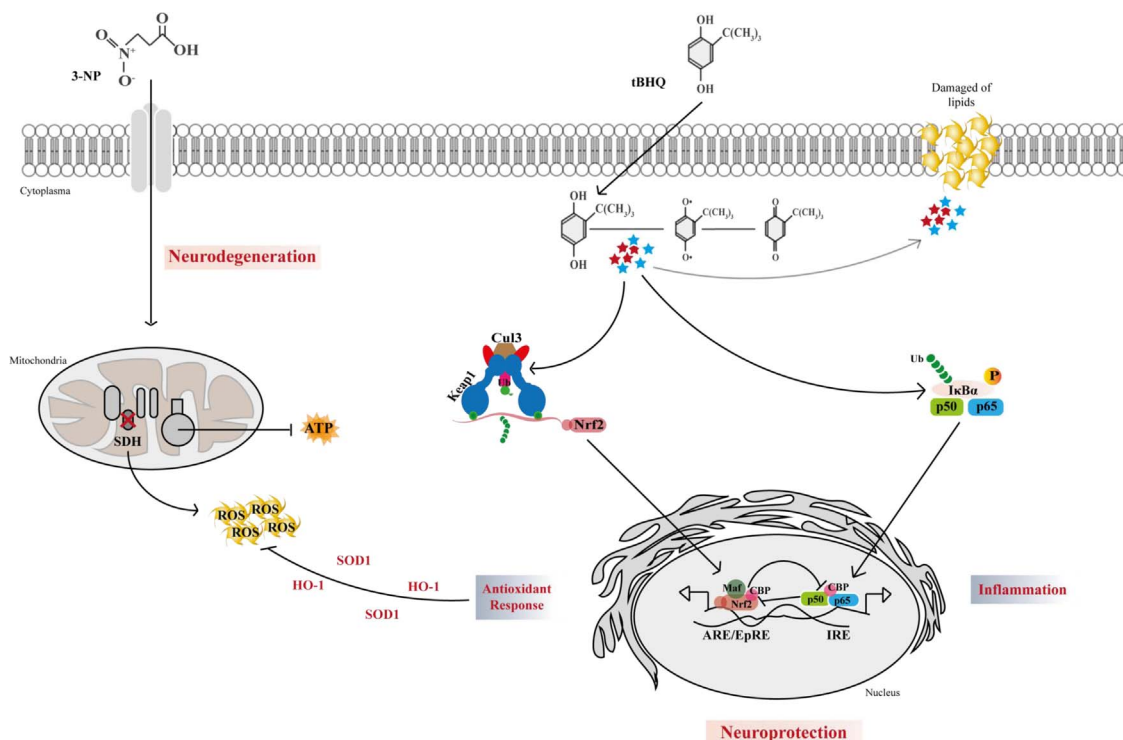
has been shown to reduce the effects of oxidative stress in adult animals and improve their motor behavior [28]. In relation to this, we demonstrate herein that tBHQ induces a hyperactivity pattern only in adult animals, without inducing a toxic process. A similar effect was observed after the acute administration of propofol to adult rats, which induced a short-term hyperactivity associated with the nitrenergic system [48]. Since our perspective, the transient hyperactive pattern produced by antioxidants, such as the observed herein, and in other studies,

might be linked with the non-toxic stimulation of general energy metabolism, thus resulting in improved movement and the stimulation of “searching” behavior; this issue deserves further investigation. However, unlike the protective effect that was found using astrocytes isolated from old rats [18], in the animal model, tBHQ pre-treatment presented a dual role. On one hand, it prevented the alterations in cytoarchitecture and cell death in both age groups; it avoided protein oxidation and improved motor activity and SDH activity, compared to the group treated with the neurotoxin. On the other hand, it increased in reactive astrogliosis and lipid peroxidation. This is interesting because tBHQ preT significantly enhanced lipid peroxidation in aged animals (predicting an important damage to neurons), but despite that damage, these animals showed a better performance at the behavioral tests. This discrepancy is difficult to explain, but the PreT might be activating an hormetic response in order to protect particular cellular pathways. This could also be related with NFκB or autophagy triggering as discussed later.

Part of our results suggests that the antioxidant effect of tBHQ *per se* is beneficial, exerting a positive role as other antioxidants [49], however it has been shown that at high concentrations, tBHQ is able to cause DNA damage and stimulate apoptosis and carcinogenesis *in vitro* [50,51] and *in vivo* [52]. High concentrations of tBHQ exert these cytotoxic effects because of its ability to generate ROS, as a consequence of its dual redox activity [53], which is very relevant when studying tBHQ effects during aging.

Neurodegeneration is associated with oxidative stress and mitochondrial dysfunction [54], this was confirmed here by experiments using the fluorescent marker FluoroJade B, which revealed striatal degeneration after the 3-NP insult and the possible neuroprotection given by tBHQ PreT. Our results demonstrated that adult rats exposed to this mitochondrial toxin, have increased susceptibility to neurodegeneration, an effect that was prevented with the tBHQ pre-treatment. Interestingly, it has been reported that not only 3-NP, but also other toxic treatments, increase the number of immunopositive cells to FluoroJade B in senescent animals [55], suggesting that neurodegeneration is age-dependent, and that damage intensity largely depends on the strain and age of the animals, as well as different doses and time of exposure [56,57]. In addition to the tBHQ antioxidant properties, it has been reported that tBHQ modifies the redox status and activates Nrf2. However, the neurons in the old animals brains might be too susceptible to those oxidative or redox changes, this might be sufficient to trigger neuronal cell death. On the contrary, it has been shown that tBHQ decreases neurodegeneration in intracerebral and subarachnoid hemorrhage models [58,59], through the stimulation of the PI3K/Akt signaling pathway, and by inhibiting the translocation and activity of transcription factor FoxO3 [60]. tBHQ also increases Nrf2 activity, which has been demonstrated to be important for protection against oxidative stress in several neurodegenerative models [61,62]. However, it is known that Nrf2 signaling decreases with age [15]; in this regard, we confirm that the old animals pretreated with tBHQ did not show Nrf2 nuclear accumulation. An unexpected effect was observed in adult animals that drastically reduced the antioxidant enzymes content, which could be related to the low Nrf2 nuclear translocation. In relation to this, Lee et al. [63] demonstrated that exercise decreased SOD levels, in contrast, pretreatment with quercetin increased a variety of antioxidant enzymes regulated by Nrf2 [64]. Recently, Alarcón-Aguilar et al. [18] showed that primary astrocytes survival under different tBHQ concentrations depended greatly on the age of the donor animal. In particular, the pre-conditioning with tBHQ (25 mM), was able to generate two conditions: Nrf2 immediate activation and survival, and on the other hand, it generated an oxidative conditioned hormetic response (HOC), thereby achieving high protection against a further oxidant insult [19]. Although the antioxidant responses are favorable in senile animals, this would culminate in a limited protection in terms of cell protection. Studies have shown an increase in oxidized proteins [65], proteasome inhibition [55], high-ubiquitinated proteins





**Fig. 9.** Nrf2-NFκB cross-talk during the tBHQ PreT model. Mitochondrial dysfunction caused by inhibition of succinate dehydrogenase (SDH) in the presence of 3-NP is characterized by ATP depletion along with ROS generation, causing striatal GABAergic cell death. tBHQ, once metabolized, plays a dual (antioxidant/pro-oxidant) role due by generation superoxide anion (O<sub>2</sub><sup>•-</sup>) and hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) (red and blue stars, respectively), who oxidize Keap-1 and allow Nrf2 nuclear translocation. Nrf2 along with small Maf binds to the ARE/EpRE (antioxidant response element/electrophiles), allowing the transcription of genes for antioxidants enzymes such as superoxide dismutase (SOD), exerting neuroprotection and maintaining cellular architecture, protected against neuronal cell death and reactive gliosis reactive, probably by decreasing ROS. The ROS produced by tBHQ metabolism also allow the activation of other transcription factors such as NFκB, which is sensitive to changes in redox state, and which initiates different cellular responses against stress in old animals. NFκB in the nucleus binds the IRE (inflammatory response element) permitting transcription of genes related to inflammatory response. Interestingly, ROS produced by tBHQ redox cycles might cause lipid damaged, thus playing a dual antioxidant/pro-oxidant role. The crosstalk mechanism between NFκB and Nrf2 illustrates the NFκB transcriptional activation and its putative interaction with Maf or other Nrf2 regulatory proteins. Conversely, Nrf2 signaling activation and consequent NFκB pathway inhibition has been demonstrated elsewhere [11]. Abbreviations: P, phosphorylation; Ub, ubiquitination; Maf, small Maf proteins; CBP, CREB-binding protein; Cul3, (Cullin3)-based ubiquitin E3 ligase complex, IκBα, regulatory subunit phosphorylates specific Ser residues. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

and S-nitrosylation [66], HNE (4-hydroxynonenal), MDA (malondialdehyde) and 8-OHdG increased levels in old organisms [67]. Therefore, it is important not only to modulate the antioxidant response, but also the activation of other signaling pathways involved in cellular protection.

Recently, p62, an adaptor protein related with autophagy, has been identified in several neurodegenerative disorders, because it directly interacts with Keap1 (Nrf2 specific inhibitor). Apparently, p62 leads Keap1 into the autophagosome, inhibiting Keap1 ubiquitination effect on Nrf2, therefore promoting its activation [68]. In this regard, accumulation in pathological conditions promotes Nrf2 hyperactivation [69]; other Nrf2 routes and selective autophagy are interconnected through p62 phosphorylation at Ser351 [70]. Li et al. [71] demonstrated that treatment with tBHQ protects hepatocytes *via* induction of autophagy, regardless of Nrf2 activation. However, the importance of autophagy induced by this non-canonical pathway (p62/Nrf2) to maintain longevity is not yet fully elucidated. It is known that macroautophagy [72], as well as chaperone-mediated autophagy (CMA) [73], decrease with age. However, the use of antioxidants, such as tBHQ, and its role in the p62/Nrf2 route and protection *via* autophagy in the elderly, has not yet been understood.

As mentioned before, mitochondrial dysfunction is related to the aging process, and one of the most widely used model to mimic Huntington's disease is the mitochondrial toxin 3-NP [74]. The administration of this mycotoxin regulates p53 expression, and therefore, its target genes such as Bax and proteins involved in the apoptosis and autophagy processes [75]. Furthermore, defects in mitochondrial complex II have been consistently reported in nonhuman primates

[76], rodents with mutated Htt [77] and senile mice [41]. Inhibition of SDH by 3-NP causes a decrease in its activity [78,79], excessive mitochondrial fission and subsequent neuronal cell death associated with a second cascade of ROS generation [80]. Several studies have suggested, including the results presented here, that the deficiency in complex II activity is directly proportional to increased age in various tissues [81–83]. However, controversial data have shown that treatment with 3-NP may vary and cause different alterations such as striatal injury, cerebral damage and behavioral changes, depending on various factors, including age [41,84]. Interestingly the pre-treatment with an antioxidant, such as tBHQ, preserved SDH activity in old animals, which so far has not been reported. The search for new pathways implicated in health improvement in old animal models is still an unresolved challenge.

Although we demonstrated herein that the Nrf2 did not increase in the nucleus, and it did not augmented antioxidant enzymes synthesis (with exception of SOD-1) a protective effect was observed, which might also be related with other pathways such as NFκB and autophagy activation. Furthermore, based on the cross-talk mechanism exerted between Nrf2 and NFκB, we evaluated the latter as another possible candidate who was participating in tBHQ hormetic response. We showed that subunits p50 and p65 are over-expressed during PreT condition in old animals. It has been inferred that receptor modulation 11-imidazole, and the NFκB can be considered drug targets in the experimental model 3-NP induced HG [85]. NFκB is known activate in response to oxidative, metabolic and excitotoxic stress in neurons, suggesting that glutamate induces activation NFκB through ionotropic receptors [86]. Interestingly, neuronal damage by the neurotoxin is

through to be related to excitotoxicity mechanisms. Besides this, p50<sup>-/-</sup> mice increased neuronal damage after 3-NP administration [87]. Jang and Cho, recently demonstrated that the pre-treatment with sulforaphane 30 min before 3-NP treatment in mice decreased striatal toxicity by activating the Keap1/Nrf2 pathway, but inhibiting NFκB [88]. Other aging studies using different experimental models have shown that molecules that activate NFκB, also favor the expression of pro-inflammatory genes [89,90]. Therefore, NFκB participation during tBHQ pre-treatment might be considered when understanding the protective and pro-survival effect, without underestimating its pro-inflammatory contribution in old animals. However, to fully understand NFκB participation in the gene regulation mechanism observed during tBHQ pre-treatment, it is necessary to determine the interactions that might occur among the different subunits that are able to conform NFκB (p65, p50, p52 Rel B and c-Rel), as well as NFκB functionality and activation.

In summary, Nrf2 could still be considered a good therapeutic target, which could be activated in order to maintain the antioxidant/pro-oxidant balance and therefore reduce the risk of developing diseases associated with old age, without excluding, the participation of other transcription factors such as NFκB (Fig. 9). Exploring these avenues would be interesting because it might suggest that despite oxidative damage, old animals are able to activate several important pathways in order to survive. However the cross-talk mechanisms and the side effects provoked by the potential Nrf2 inducers, need to be contemplated and studied before considering their use in the elderly.

## Conflicts of interest

The authors declare that they have not competing interests.

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## Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.redox.2017.03.029](https://doi.org/10.1016/j.redox.2017.03.029).

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