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ORIGINAL ARTICLE

On the Role of DT-Diaphorase Inhibition in Aminochrome-Induced Neurotoxicity In Vivo

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Abstract Dopamine oxidation in the pathway leading to neuromelanin formation generates the *ortho*-quinone aminochrome, which is potentially neurotoxic but normally rapidly converted by DT-diaphorase to nontoxic leukoaminochrome. However, when administered exogenously into rat striatum, aminochrome is able to produce damage to dopaminergic neurons. Because of a recent report that substantia nigra pars compacta (SNpc) tyrosine hydroxylase (T-OH) levels were unaltered by aminochrome when there was cell shrinkage of dopaminergic neurons along with a reduction in striatal dopamine release, the following study was conducted to more accurately determine the role of DT-diaphorase in aminochrome neurotoxicity. In this study, a low dose of aminochrome (0.8 nmol) with or without the DT-diaphorase inhibitor dicoumarol (0.2 nmol) was injected into the left striatum of rats. Intrastriatal 6-hydroxydopamine (6-OHDA, 32 nmol) was used as a positive neurotoxin control in other rats. Two weeks later, there was significant loss in numbers of T-OH immunoreactive fibers in SNpc, also a loss in cell density in SNpc, and prominent apomorphine (0.5 mg/kg sc)-induced contralateral rotations in rats that had been treated with aminochrome, with aminochrome/dicoumarol, or with 6-OHDA. Findings demonstrate that neurotoxic aminochrome is able to exert neurotoxicity only when DT-diaphorase is suppressed—implying that DT-diaphorase is vital in normally suppressing toxicity of in vivo aminochrome, generated in the pathway towards neuromelanin formation.

Keywords Aminochrome · DT-diaphorase · Neurotoxicity · Substantia nigra · Dopamine · Neurodegeneration · Neuroprotection

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Introduction

Aminochrome is the most stable product of dopamine oxidation in the pathway leading to neuromelanin formation (Herrera et al. 2017). Aminochrome is a normal product since dopaminergic neurons containing neuromelanin in the substantia nigra, pars compacta (SNpc), and pars lateralis remain intact in healthy individuals. However, these neurons are lost in patients with Parkinson's disease (Segura-Aguilar et al. 2014; Zecca et al. 2002). Aminochrome induces mitochondrial dysfunction (Aguirre et al. 2012; Arriagada et al. 2004; Muñoz et al. 2012a; Paris et al. 2011), oxidative stress (Arriagada et al. 2004; Segura-Aguilar et al. 1998), protein degradation dysfunction of both lysosomal and proteasomal systems (Zafar et al. 2006; Huenchuguala et al. 2014; Muñoz et al. 2012b), the formation of neurotoxic alpha-synuclein oligomers (Muñoz et al. 2015), and endoplasmic reticulum stress (Xiong et al. 2014). However, aminochrome neurotoxicity is prevented when aminochrome is two-electron reduced by DT-diaphorase (Lozano et al. 2010; Segura-Aguilar et al. 2014, Segura-Aguilar et al. 2016a).

Recently, it was reported that aminochrome induced apomorphine-induced contralateral rotational behavior in rats that had previously been given a unilateral injection of aminochrome in the striatum despite the fact that there was

not a massive loss of dopaminergic neurons. The contralateral behavior can be explained by a significant decrease in dopamine release, owing to aminochrome-induced mitochondria dysfunction with resultant lowered ATP levels, and reduction of synaptic vesicle exocytosis. Interestingly, both the axonal transport of vesicles and dopamine release are dependent of ATP. Aminochrome induces a neuronal dysfunction as a consequence of an imbalance between dopamine and GABA and aminochrome-induced acute neurotoxicity (Herrera et al. 2016). These experiments were performed with a high concentration of aminochrome (1.6 nmol), surpassing the protective capacity of DT-diaphorase to divert aminochrome to an inactive leukoaminochrome product. The aim of the current study was to demonstrate in vivo the neuroprotective role of DT-diaphorase against aminochrome-dependent neurotoxicity, subsequent to a small unilateral intrastriatal dose of aminochrome (0.8 nmol) to induce aminochrome-dependent neuronal dysfunction when DT-diaphorase is inhibited by dicoumarol.

Material and Methods

Chemicals

Dopamine, CM-Sephadex C50-100, DEAE Sephadex 50-120, Sephadex G-25, and tyrosinase (EC 1.14.18.1) from mushroom were from Sigma-Aldrich (St. Louis, MO, USA); apomorphine was from Apo-Go, Genes Pharmaceutical; and antibodies for tyrosine hydroxylase (T-OH) were from Santa Cruz Biotechnology Inc. (TH (A-1): sc-374047).

Synthesis and Purification of Aminochrome

Aminochrome was synthesized as described previously (Paris et al. 2010) by oxidizing 5 mM dopamine with 10 ng of tyrosinase in 1 ml of 2-(*N*-morpholino)ethanesulfonic acid hemisodium salt (MES) buffer 25 mM, pH 6.0, for 10 min at room temperature. Incubation solution was loaded onto a column of 3 × 0.4 cm resin CM-Sephadex C-50-120 which was eluted with 3 ml of 25 mM MES buffer pH 6.0. The concentration of aminochrome was determined by using the molar extinction coefficient 3058 M^{-1} cm^{-1} (Segura-Aguilar and Lind 1989).

Animals

Experiments with animals were conducted as described previously (Herrera et al. 2016). Adult Sprague-Dawley rats weighing 220–250 g at the start of the experiments, and 250–350 g at the time of behavioral experiments, were unilaterally injected into the left striatum with 0.8 nmol aminochrome, then housed in groups of two to three animals per cage in a well-ventilated, temperature-controlled environment (22 ± 1 °C), under a 12:12 light–dark cycle with free access to food and water. The minimum number of animals and duration of observation required to obtain consistent data was employed (n = 4). All experiments were conducted in accordance with international standards of animal welfare recommended by the Society for Neuroscience (*Handbook for the Use of Animals in Neuroscience Research*, 1997). The local Animal Care and Use Committee of Universidad de Chile approved the experimental protocols in accordance with the Animal Experiments and Ethics Committee of the Maastricht University.

Stereotaxic Injection

Following a prior protocol described (Herrera et al. 2016) for the left striatal injection, rats were anesthetized with sodium pentobarbital (30 mg/kg) and placed in a David Kopf stereotaxic frame. Stereotaxic coordinates relative to bregma for left striatum were as follows: AP = −0.48 mm, L = −2.5 mm, and V = −4.5 mm according to the atlas of Paxinos and Watson (2007). For the group dicoumarol + aminochrome, a total of 0.8 nmol aminochrome in 4 μl solution was injected into the left striatum with a rate of 4 μl/min using a 5-μl Hamilton microsyringe, subsequent to 2 nmol dicoumarol (10 min, same rate as above). To minimize the possibility of back flow, the needle remained in place for five additional minutes before aminochrome injection. After surgery, rats were allowed to recover for 7 days before conducting the rotational behavior test, induced by 0.5 mg/kg s.c. apomorhine. 6-Hydroxydopamine (6-OHDA; 32 nmol in saline-ascorbic acid, 0.1%)-treated rats comprised the positive control (C+) group. The intact control group (C) was injected with buffer (25 mM MES pH 6.0), the vehicle for aminochrome.

Rotational Behavior

Rotational behavior, induced by 0.5 mg/kg s.c. apomorhine treatment of rats, was conducted during 60 min, at 14 days after intrastriatal injection of 0.8 nmol aminochrome, 2 nmol dicoumarol alone, and dicoumarol combined with aminochrome. Activity was determined in a LE 902 Rotometer (Panlab, Barcelona, Spain) connected to a LE 3806 multicounter (Panlab, Barcelona, Spain). Results were expressed as the mean ± SEM total number of complete 360° turns during the entire period of observation.

Tissue Collection

Rats were terminated after the behavioral test at 14 days with an overdoses of sodium pentobarbital (60 mg/kg), then perfused intracardially with cold Tyrode's solution (NaCl 8 g, KCl 0.2 g, $MgCl_2 \cdot 6H_2O$ 0.05 g, $NaHCO_3$ 1.0 g, $NaH_2PO_4 \cdot H_2O$ 0.04 g, D(+)-glucose monohydrate 1.0 g) followed by cold 4% paraformaldehyde (PFA) for brain fixation. Brains were removed, post-fixed in 4% PFA overnight, and transferred to 10–20% and 30% of sucrose solution in three consecutive days for cryoprotection before cutting. Hereafter, brains were cut free-floating in 10 serial sections of coronal 30 μM thickness on a cryostat (Leica CM 3050, Leica Microsystems, Buffalo, NY) and stored at 4 °C in PBS 1X with 0.2% azide until further processing for immunohistochemistry.

Immunohistochemistry

To evaluate the number of dopaminergic cells in SNpc and the expression levels of T-OH in the striatum, one series of sections from each animal was processed for T-OH immunostaining. Free-floating sections from striatum and SN were incubated overnight at 4 °C with the primary antibody mouse anti-T-OH, diluted 1:3000 (T-OH), in TBS 1X. After washing, sections were incubated with 1:400 dilution of biotinylated donkey anti-mouse secondary antibody (Vector laboratories, Burlingame, CA, USA) during 1.5 h followed by an incubation with avidin–biotin–peroxidase complex diluted 1/800 during 1.5 h (Vectastain, ABC kit, Vector laboratories). Finally, the substrate H_2O_2 30% was added in the presence of DAB (1 mg/ml) to visualize the antigen–antibody complex. Slides were washed, dehydrated, and coverslipped using Pertex (Histolab Products ab, Göteborg, Sweden).

Estimation of T-OH-Immunopositive Cell Numbers in the SNpc

One serial section (300 μm between sections) from each animal per treatment group was used for quantification of T-OH-immunoreactivity (TH-ir) and cell density (cells/mm^3). An optical dissector method (West et al. 1991) was used to estimate T-OH-immunopositive cells in SNpc using the optical fractionator workflow in Stereo Investigator software (MBF Bioscience, Wilmington, VT) (Faunes et al. 2015). Briefly, after tracing the contours of the left and right SNpc at ×4 magnification (Nikon Eclipse Ci-L; N.A. 0.75), the number of neurons TH-ir positive was calculated with the optical fractionator (×40 magnification); height of the dissector box 10 μm with a guard zone of 2 μm. The average section thickness after tissue processing was 15 ± 1.5 μm. The area of each dissector box was 100 × 100 μm, using a grid size of 100%. These parameters yielded a Gundersen coefficient of error (CE) ranging between 0.04 and 0.1, which are values acceptable for stereological analyses (Faunes et al. 2013; Gutiérrez-Ibañez et al. 2012; Henny et al. 2012). Since measurements were made on every tenth serial section, separated by 300 μm, it was unlikely that each neuron would be counted twice. A minimum of three sections per animal was analyzed for one serial section. All the estimates were performed by one researcher.

Data Expression and Statistical Analysis

Statistical analysis was performed using the GraphPad Prism 5.0 (GraphPad Software, Inc., Chicago, IL). For behavior analysis among the treatment groups, a one-way ANOVA was used followed by the Bonferroni multiple comparison test to detect differences between groups and days of treatment. For TH-ir(+) cell counts and density measurement, the unpaired Student *t* test was used to compare between groups. Values are expressed as mean ± SEM, and $^{*}P < 0.05$, $^{**}P < 0.001$, ***, and $^{\#\#\#}P < 0.0001$ are considered statistically significant.

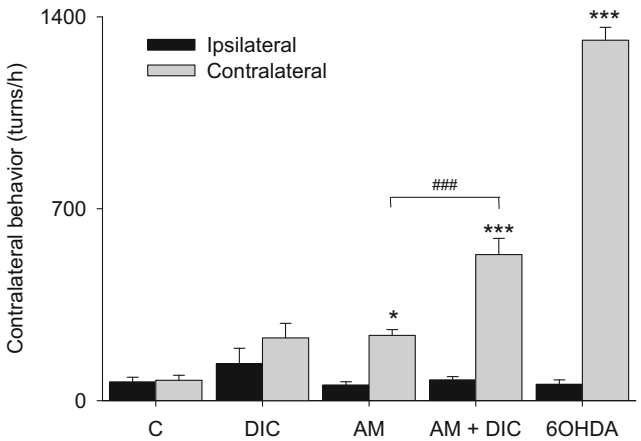

Fig. 1 Effect of unilateral injection of aminochrome in the presence of dicoumarol on apomorphine-induced rotational behavior. The rotational behavior test induced by apomorphine (0.5 mg/kg. s.c.) was determined 14 days after stereotaxic injection into left striatum of MES buffer alone (*C*), 2 nmol dicoumarol (*DIC*), 0.8 nmol aminochrome (*AM*), 0.8 nmol aminochrome plus 2 nmol dicoumarol (*AM + DIC*), and as positive control, 32 nmol 6-OHDA. Aminochrome alone induced an increase in the number of contralateral rotations versus the control group ($^{*}P < 0.05$). Aminochrome plus dicoumarol, also 6-OHDA, induced a greater increase in contralateral rotations in comparison to the control group (*C*) ($^{***}P < 0.0001$). Also, there is a significant difference between aminochrome alone versus the group receiving aminochrome plus dicoumarol (*AM + DIC*), $^{\#\#\#}P < 0.0001$. Values are expressed as mean ± SEM ($n \leq 4$). One-way ANOVA followed by a Bonferroni post hoc multi-comparison test was used for detect differences

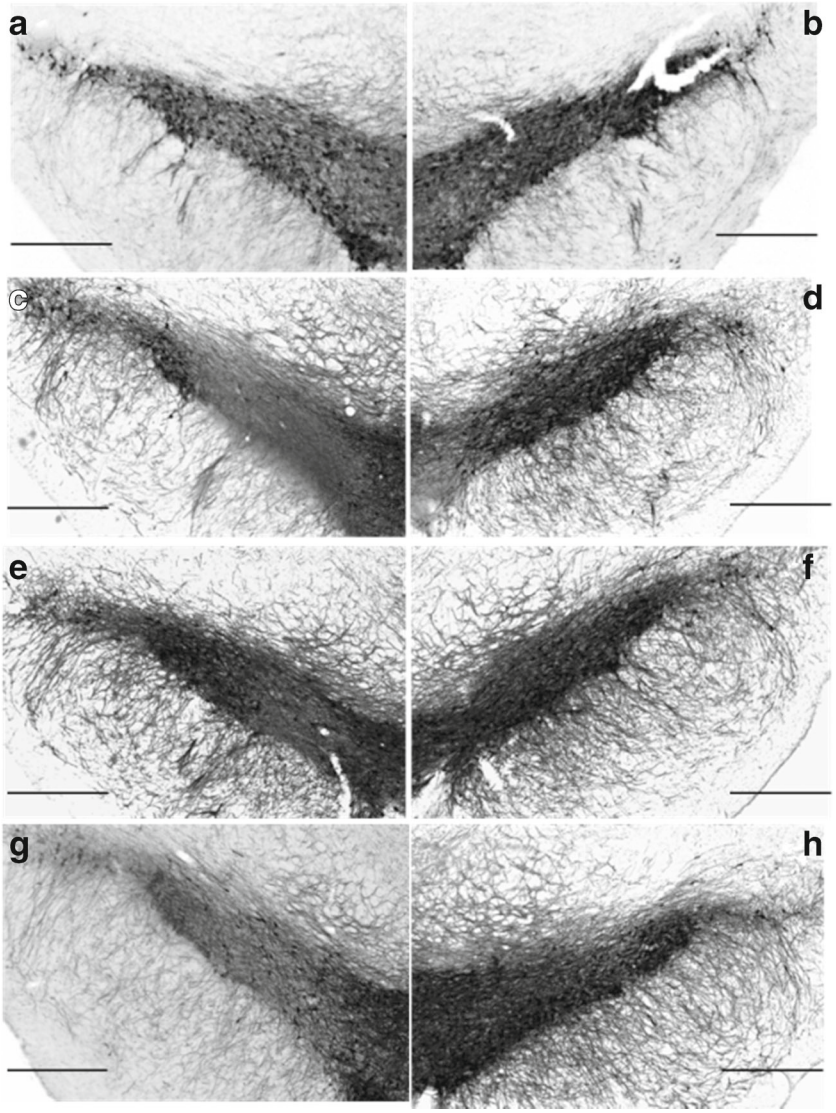

Fig. 2 Aminochrome effect on substantia nigra morphology. **a** T-OH-positive immunostaining in the SNpc revealed that 0.8 nmol aminochrome alone (**a** and **b**) does not induce significant cell loss in SN. However, aminochrome 0.8 nmol with 2 nmol dicoumarol induced cell loss in the SN (**c**, lesion side). As negative control rats were injected with dicoumarol alone (**e** and **f**), no changes were observed. As positive control, rats were injected with 32 nmol 6-OHDA, resulting in cell loss (**g**) compared to the control side of the injection (**h**). The *left panel* corresponds to the lesion side and the right to the control side of the injection. *Scale bar* of 200 μm

Results

Rats unilaterally injected in the striatum with 0.8 nmol aminochrome showed a significant increase in numbers of apomorphine (0.5 mg/kg sc)-induced contralateral turnings (238 ± 21 turns; $*P < 0.05$) versus MES controls or rats treated with 2 nmol dicoumarol alone. However, the inhibition of DT-diaphorase with 2 nmol dicoumarol in the presence of 0.8 nmol aminochrome induces a greater increase in contralateral rotations (533 ± 59 turns; $***P < 0.0001$) versus both the control group and aminochrome group alone. As expected, unilateral 6-OHDA (32 nmol) induced a significant increase in contralateral rotations versus the control MES group (1315 ± 47 turns; $***P < 0.0001$) (Fig. 1).

Aminochrome (0.8 nmol) alone and dicoumarol (2 nmol) alone did not produce a decrease of T-OH-positive immunostainings in the SN at 14 days. However, a significant loss of TH positive neurons in the SNpc, specifically in the dorsal tier of the lesion side, was observed in the group treated with both aminochrome and dicoumarol (Fig. 2c). The 6-OHDA group did display a prominent reduction in T-OH-positive immunostaining (Fig. 2g). The loss of T-OH-positive immunostaining neurons induced by aminochrome together with dicoumarol was confirmed with the stereological analysis of neuron T-OH-positive immunostained neuronal fibers. A significant decrease was observed in the estimated number of neuron T-OH-positive immunostaining versus the control group. The loss in T-OH-positive immunostaining was also observed in the group injected with 6-OHDA (Fig. 3a).

The nucleus volume estimates and cell density estimates were done according the optical fractionator method and Cavielieri's principle in which the SNpc was identified according to the rat brain atlas (Paxinos and Watson, 2007; West et al. 1991). This measure revealed a significant difference in cell density between the aminochrome plus dicoumarol group (3019 ± 370 versus 6002 ± 514 cell/mm^3, respectively) (Fig. 3b; *$P < 0.05$). The estimation also revealed a significant difference for the 6-OHDA group (3138 ± 267 cell/mm^3, **$P < 0.001$), without any change observed in the volume of the SNpc. This revealed that the different treatment does not produce any alteration in the structure that could bias the estimation of cell density.

Discussion

Dopamine oxidation to neuromelanin is a benign pathway despite the formation of *o*-quinones, such as aminochrome. This is attributable to the protective action of DT-diaphorase, preventing aminochrome-induced neurotoxicity (Lozano et al. 2010; Arriagada et al. 2004; Zafar et al. 2006; Fuentes et al. 2007; Muñoz et al. 2012a, 2015; Paris et al. 2010, 2011; Huenchuguala et al. 2016, Fig. 4). However, DT-diaphorase capacity to prevent aminochrome neurotoxicity is not unlimited since a high aminochrome concentration (i.e., 1.6 nmol) surmounts the protective action of DT-diaphorase. Aminochrome at this concentration induces mitochondria dysfunction and progressive apomorphine-induced contralateral rotational behavior, suggesting that aminochrome induces a neurotoxic effect, resulting in an imbalance between dopamine and GABA, and cell shrinkage (Herrera et al. 2016). Therefore, aminochrome at low concentration (50%) in the presence of an inhibitor of DT-diaphorase induces a contralateral behavior with loss of dopaminergic neurons and decreased cellular T-OH in comparison to the control group without any change in the volume of the observed structure. These results support the protective role of DT-diaphorase against aminochrome neurotoxicity in vivo, explaining why dopamine oxidation to *o*-quinones is not always a detrimental event associated with dopaminergic neurotoxicity.

Aminochrome has been proposed as a novel neurotoxin for production of a rodent preclinical model for Parkinson's disease. This compound is completely different from exogenous neurotoxins such as 6-OHDA, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), and rotenone because (i) aminochrome is an endogenous species generated inside neuromelanin-dopaminergic neurons in contrast to 6-OHDA and MPTP which are not synthesized by the brain (Segura-Aguilar and Kostrzewa 2015). Also, (ii) aminochrome induces a very slow progressive chronic neurotoxicity versus the acute neurotoxicity of 6-OHDA and MPTP—resulting in massive and extremely rapid degeneration of the nigrostriatal system. MPTP induces a severe Parkinsonism in just 3 days, while the degenerative process and progression of Parkinson's disease take years (Williams, 1986). There is a long list of failed clinical studies based on successful results obtained with 6-OHDA, MPTP, and rotenone preclinical models (Olanow et al. 2015; Lindholm et al. 2015; Park et al. 2015; Athauda and Foltynie et al. 2015; Segura-Aguilar et al. 2016a, b, c; Muñoz et al. 2016; Muñoz and Segura-Aguilar, 2016, 2017). Therefore, aminochrome represents an alternative for a preclinical model of Parkinson's disease (Muñoz et al. 2016; Muñoz and Segura-Aguilar, 2016). It is important to remark that aminochrome induces mitochondrial dysfunction, alpha-synuclein aggregation to neurotoxic oligomers, protein degradation dysfunction, and oxidative

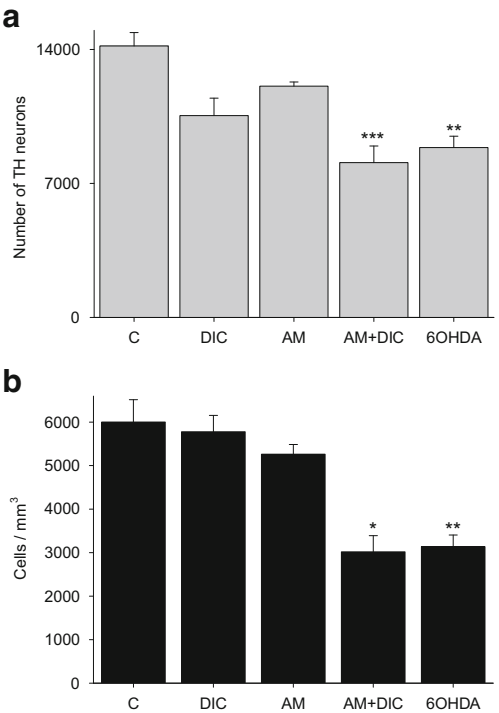

Fig. 3 Estimation of T-OH+ cell number in SNpc. **a** Number of T-OH-immunopositive neurons estimated with stereological analysis. In comparison to the control group (*C*), the number of T-OH (+) neurons is reduced in the group treated with 6-OHDA versus the group, aminochrome 0.8 nmol + dicoumarol. *Bars* represent the total number of T-OH-immunopositive cells estimated in the SNpc within the dorsal portion in the ipsilateral side of control and lesioned animals. **b** Estimation of cell number per volume measured (cell density/mm^3). There is a significant difference in cell density for the aminochrome plus dicoumarol group, also for the 6-OHDA group versus the control group (*C*). Difference between groups for T-OH estimated cell density as cell number were analyzed using one-way ANOVA followed by a Bonferroni multi-comparison post hoc test; $P < 0.05$*, $P < 0.001$**, $P < 0.0001$***. Values are expressed as mean ± SEM ($n \leq 4$)

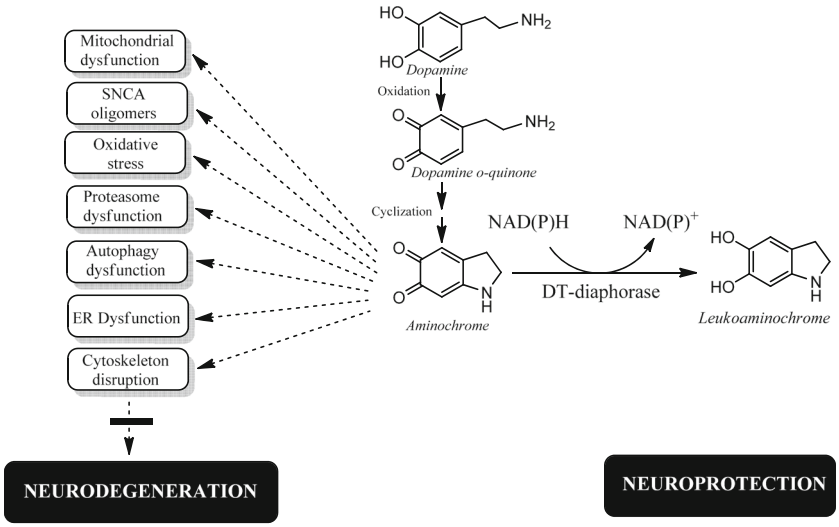

Fig. 4 DT-diaphorase neuroprotective action. Dopamine oxidation to neuromelanin involves the formation of *o*-quinones that can be neurotoxic. Aminochrome, the most stable *o*-quinone formed during dopamine oxidation, induces mitochondria dysfunction, the formation of alpha-synuclein (*SNCA*) neurotoxic oligomers, oxidative stress, proteasome dysfunction, autophagy dysfunction, and endoplasmic reticulum stress and cytoskeleton disruption. Interestingly, DT-diaphorase prevents aminochrome neurotoxicity by reducing aminochrome with two electrons to leukoaminochrome. In this study, DT-diaphorase prevented the loss of dopaminergic neurons and apomorphine-induced contralateral rotational behavior, indicative of the protective role of DT-diaphorase

and endoplasmic reticulum stress (Aguirre et al. 2012; Arriagada et al. 2004; Paris et al. 2011; Fuentes et al. 2007; Muñoz et al. 2012a, 2012b, 2015; Huenchuguala et al. 2014; Zafar et al. 2006, Xiong et al. 2014)—thereby reproducing cellular mechanisms associated with the onset and progression of Parkinson's disease.

In conclusion, aminochrome chronic neurotoxicity in the nigrostriatal system depends on DT-diaphorase inhibition or low expression of this enzyme. The evidence presented in this work is the first step to corroborate in vivo all the evidence presented previously in vitro. These evidences suggest that DT-diaphorase may play a very important role to maintaining dopaminergic neuron viability.

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