Stable Expression of Short Interfering RNA for DT-Diaphorase Induces Neurotoxicity

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DT-Diaphorase has been proposed to play a neuroprotective role in dopaminergic neurons by preventing aminochrome neurotoxicity. There are several studies supporting this idea, but in all studies, we used dicoumarol, an inhibitor of DT-diaphorase. We have designed and developed two siRNA to silence the expression of DT-diaphorase to study its role in aminochrome metabolism. We transduced RCSN-3 cells with retroviral particles containing a pRetroSuper plasmid coding a siRNA for DT-diaphorase. The cells selected in the presence of puromycin generated a stable cell line RCSN-3Nq6 and RCSN-3Nq7 with low expression of DT-diaphorase (27% and 33% of wild type, respectively). A significant cell death was observed in RCSN-3 cells expressing siRNA Nq6 and Nq7 for DT-diaphorase when were incubated with 100 μM aminochrome during 48 (4- and 3.5-fold, respectively; $P < 0.01$). These results support the protective role of DT-diaphorase against aminochrome neurotoxicity in dopaminergic neurons containing neuromelanin and show that Nq6 and Nq7 siRNA are very useful tools to study the role of DT-diaphorase in aminochrome metabolism.

Introduction

Parkinson's disease is characterized pathologically by the selective loss of neuromelanin-containing neurons in the substantia nigra pars compacta and locus coeruleus. Oxygen catalyzes dopamine oxidation to dopamine *o*-quinone, which automatically cyclizes in several steps to form aminochrome at physiological pH (for a review, see ref *1*). Aminochrome, the precursor of neuromelanin that accumulates with age in human substantia nigra (*2*), is able to participate in two neurotoxic reactions such as (i) the formation of adducts with proteins such as α-synuclein that inhibits α-synuclein fibrillization and enhances and stabilizes protofibril formation that seems to be essential for its neurotoxic effects (*3, 4*); and (ii) aminochrome can be reduced by flavoenzymes that transfer one-electron to leukoaminochrome-*o*-semiquinone radical, which is extremely reactive with oxygen and is neurotoxic (*5*−*10*).

DT-Diaphorase, (EC.1.6.99.2) prevents aminochrome participation in neurotoxic pathways such as the one-electron reduction to form the leukoaminochrome *o*-semiquinone radical (*5*−*10*) and the formation of adducts with proteins such as α-synuclein generating neurotoxic protofibrils (*11, 12*). However, the inhibitor of DT-diaphorase dicoumarol was used in these studies to investigate its role in aminochrome metabolism. Therefore, the aim of this study was to test the hypothesis that DT-diaphorase protects the catecholaminergic cell line RCSN-3 by using siRNA for DT-diaphorase.

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Experimetal Procedures

Chemicals. Dopamines, DME/HAM-F12 nutrient mixture, were purchased from Sigma-Aldrich. (St. Louis, MO, USA). Polyclonal antibodies against β-actin, DT-diaphorase, goat anti rabbit IgG-HRP, and luminol reagent were obtained from Santa Cruz Biotechnology Inc. (California, USA). LIVE/DEAD Viability/Cytotoxicity Kit was from Molecular Probes (Eugene, OR, USA). Aminochrome was synthesized in our laboratory as described previously (*10*). Aminochrome synthesis was performed by oxidizing 5 mM dopamine with 10 ng of tyrosinase in buffer and 20 mM MES at pH 6.0 at 1 mL volume total for 15 min at room temperature. Aminochrome was separated from free dopamine and tyrosinase with a CM-sephadex C50−100 column (18 × 0.7 cm) equilibrated with 20 mM MES at pH 6.0.

Cell Culture. The RCSN-3 cell line grows in monolayers, with a doubling time of 52 h, a plating efficiency of 21%, and a saturation density of 56,000 cells/cm^2 in normal growth media composed of DME/HAM-F12 (1:1), 10% bovine serum, 2.5% fetal bovine serum, and 40 mg/L gentamicin sulfate (*13*). The cultures were kept in an incubator at 37 °C with 100% humidity, and the cells were grown in atmospheres of both 5% or 10% CO_2 (*13*). The cell morphology was determined by staining the cells with 0.5 μM Calcein AM for 45 min at room temperature in the dark (Molecular Probes). The cell morphology was determined by using a phase contrast microscope equipped with fluorescence using the following the filters: 450−490 nm (excitation) and 515−565 nm (emission) (*13*).

Western Blot. An RCSN-3 cell homogenate (100 μg) was separated on SDS−polyacrylamide-gel electrophoresis (10% w/v) and transferred electrophoretically to a 0.2 μm nitrocellulose membrane that was incubated with antibodies against DT-diaphorase and α/β-actin overnight at room temperature and detected with goat anti rabbit IgG-HRP-linked antibodies using luminol.

Plasmid Clones. For siRNA duplexes and cloning, we chose sequences to target human (NM_000903.2, NM_001025433.1, and NM_001025434.1, respectively), rat (NM_017000.2), and mouse (NM_008706.3). The siRNA duplexes were synthesized with 2 nt deoxythymidine 3′-overhangs (TAG Copenhagen) as described

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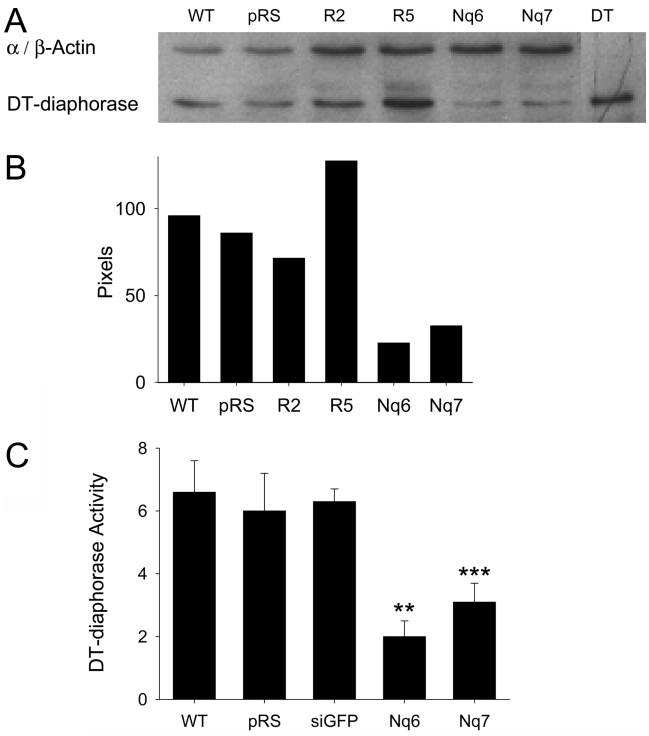

Figure 1. Effect of siRNA on DT-diaphorase expression in RCSN-3 cells. A strong decrease in DT-diaphorase expression was observed in RCSN-3 cells expressing Nq6 siRNA (Nq6) and Nq7 siRNA (Nq7) determined by using the Western blot technique (A). The quantification of this Western blot presented in A was performed by measuring the pixels of DT-diaphorase/(α/β) actin by using ScionImage software (B). DT-Diaphorase expression was also determined in RCSN-3 wild type (WT) cells and transduced with retroviral particles without siRNA (pRS), RCSN-3 cells expressing R2 siRNA (R2), and R5 siRNA (R5), and we used purified DT-diaphorase (DT) as the positive control for antibodies. Determination of DT-diaphorase activity expressed as μmol/min/mg protein was also significantly decreased in RCSN-3 cells expressing Nq6 and Nq7 siRNA (C). The values are the mean ± SD ($n = 3$). The statistical significance was assessed using analysis of variance (ANOVA) for multiple comparisons and Student's t test. (**$P < 0.01$; ***$P < 0.001$).

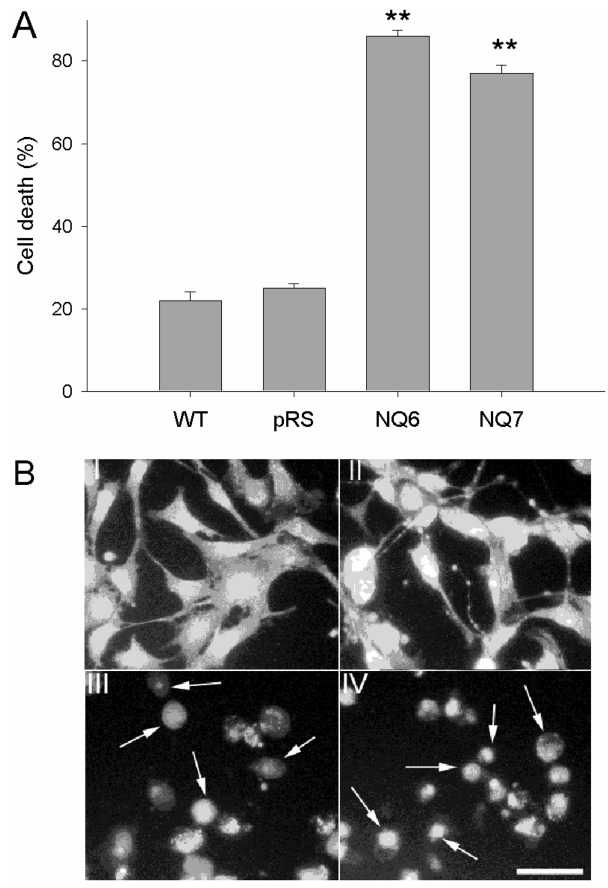

Figure 2. Effect of siRNA for DT-diaphorase on RCSN-3 cells. (A) A significant increase in cell death was observed in RCSN-3 cells expressing Nq6 or Nq7 siRNA when the cells were treated with 100 μM aminochrome during 48. RCSN3 cells wild type (WT) and expressing pRetroSuper plasmid alone (pRS) did not exhibit a significant cell death. The values are the mean ± SD ($n = 3$). The statistical significance was assessed using analysis of variance (ANOVA) for multiple comparisons and Student's t test. (**$P < 0.01$). (B) The elongated cell shape observed in RCSN-3 wild type cells (I) and RCSN-3 cells expressing pRetroSuper plasmid alone (II) after 48 h treatments with 100 μM aminochrome was changed in RCSN-3 expressing Nq6 (III) and Nq7 siRNA (IV) that showed a reduced volume and spherical shape. The cell morphology was determined by using Calcein AM and fluorescence microscopy. The scale bar is 60 μm.

before (*14*). We synthesized the following oligonucletides sense siRNA to target the consensus sequences 5′-TGCCATGAAG GAGGCTGCT-3′ (Nq6) and 5′-GACAACCAGATCAAAGCTA-3′ (Nq7) in rat, human, and mouse genes Nq6 sense 5′-GATC-CCCTGCCATG AAGGAGGCTGCTTTCAAGAGAAGCA GC-CTCCT TCATG GCATTTTTGGAAA-3′ and antisense 5′-AGCTTTTCCAAAAATGC ATGAAGGAGGCTGCTTCTCTT GAAAGCAGCCTCCTTCATGGCAGGG-3′; Nq7 sense 5′-GATC-CCCGACAACC AGATCAAAGCTATTCAAGAGATAGCTTTG-ATCTGGTT GTC TTTTTGGAAA-3′ and antisense 5′-AGCTTTTC-CAAAAAGACAACCAGATCAAA GCTATCTCTTGAATAGCT-TTGATCTGGTTGTCGGG-3′. We synthesized the following oligonucletides sense siRNA to target rat DT-diaphorase R2 sense 5′-GATCCCCGGCCGCCTGAGCCCGGATATTCAAGAGATATC-CGGGCTCAGGCGGCCTTTTTGGAAA and antisense 5′-AGCTT-TTCCAAAAAGGCCGCCTGAGCCCGGATATCTCTTGAATATC-CGGGCTCAGGCGGCCGGG-3′; and R5 sense 5′-GATCCCCCCT-CAACTGGTGT ACAGCATTCAAGAGATGCTGT ACACCAGT-TGAGG TTTTTGGAAA-3′ and antisense 5′-AGCTTTTCCA-AAAACCTCAACTGGTGTACAGCATCTCTTGAATGCTGTA-CACCA GTTGAGGGGG-3′. The plasmid pRetroSuper and the oligonucleotides coding for the siRNA were incubated with the restriction enzymes HindI and BgIII and allowed to ligate under standard conditions using a ratio of 1:10 plasmid and oligonucleotides coding for the siRNA.

Production of Retroviral Particles. HEK-293T cells were transfected with a mix of 6 μg of DNA of pRetroSuper coding for siRNA, 6 μg DNA of packing plasmid pMDG, and FUGENE HD (Roche) in a relationship (8:2 DNA/FUGENE HD).

Transduction of RCSN-3 Cells. RCSN-3 cells were incubated in a T-75 flask to a 40−50% confluence where 2 mL of supernatant containing retroviral particles was added to the cells in the presence of 4 μg/mL Polybrene. The cells that survive the highest puromycin concentration were trypsinized and were subcultured.

Cell Death. Cell death was determined by counting live and dead cells after staining with 0.5 μM Calcein AM and 5 μM ethidium homodimer-1 for 45 min at room temperature in the dark (LIVE/DEAD Viability/Cytotoxicity Kit, Molecular Probes). Calcein AM is a marker for live cells, and ethidium homodimer-1 intercalates into the DNA of dead cells. Cells were counted with a phase contrast microscope equipped with fluorescence using the following filters: Calcein AM 450−490 nm (excitation) and 515−565 nm (emission), and ethidium homodimer-1, 510−560 nm (excitation) and LP-590 nm (emission).

DT-Diaphorase Activity. DT-diaphorase activity was determined in Tris/HCl buffer at pH 7.5 containing 0.08% Triton X-100 by using 500 μM NADH or 500 μM NADPH as the electron donor and 77 μM cytochrome c and 10 μM menadione as the electron acceptor. The reaction was measured spectrophotometrically by following the reduction of cytochrome C, which continuously reoxidized the reduced menadione at 550 nm, employing an extinction coefficient of 18.5 mM^{-1} cm^{-1}. DT-diaphorase activity

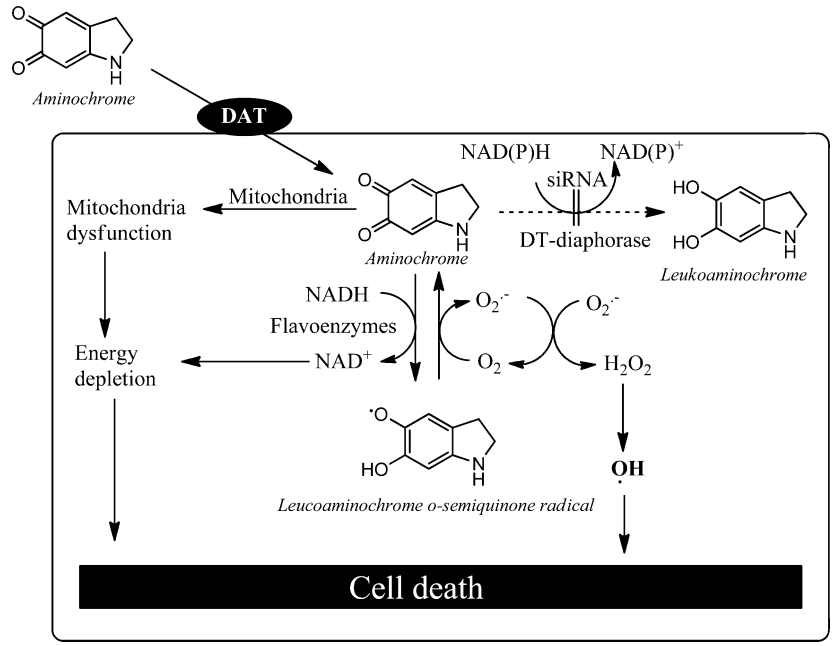

Figure 3. Possible mechanism for siRNA for DT-diaphorase induced cell death. The low expression of DT-diaphorase as a consequence of the expression of siRNA toward the enzyme will allow the formation of aminochrome adducts with mitochondria proteins resulting in mitochondria dysfunction. In addition, aminochrome can be reduced by one-electron to form the leukoaminochrome *o*-semiquinone radical, which is extremely reactive with oxygen. It auto-oxidizes, generating a redox cycle between aminochrome and the leukoaminochrome *o*-semiquinone radical, resulting in the depletion of NADH, which is required for ATP synthesis in the mitochondria and oxygen that is also required for ATP synthesis in the mitochondria. The mytochondria dysfunction and lack of NADH and oxygen will prevent the formation of ATP required for cell function and survival. The redox cycle will also induce the formation of superoxide radicals, which spontaneously or enzymatically generate hydrogen peroxide, which is the precursor of hydroxyl radicals.

was calculated by inhibiting the quinone reductase activity with dicoumarol (*15*).

Results

To study the protective role of DT-diaphorase against the neurotoxic metabolism of aminochrome, we have synthesized two different siRNA duplexes specific for DT-diaphorase mRNA, the sites being conserved on the human, mouse, and the rat gene (siRNA Nq6 and Nq7) and two other siRNA duplexes specific for only rat DT-diaphorase mRNA (siRNA R2 and R5). We transduced the catecholaminergic cell line RCSN-3 cells with a supernatant containing retroviral particles with pRetroSuper coding for siRNA Nq6, Nq7, R2, and R5 collected at 72 h. As a control, we transduced RCSN-3 cells with the pRetroSuper plasmid without siRNA. A dramatic inhibition of 73 and 67% in DT-diaphorase expression in RCSN-3 cells transduced with siRNA Nq6 and Nq7, respectively, was observed (Figure 1A and B). However, the siRNA R2 and R5 did not induce reduction but rather stimulation in DT-diaphorase expression (Figure 1A). The DT-diaphorase activity in RCSN-3 cells transduced with siRNA Nq6 and Nq7 for DT-diaphorase were significantly decreased to 2.0 ± 0.5 μmol/min/mg ($P < 0.01$); 70% inhibition and 3.1 ± 0.6 ($P < 0.001$); 55% inhibition, respectively. No significant changes in DT-diaphorase activity was observed in RCSN-3 cells transduced with pRetroSuper plasmid alone (6.0 ± 1.2 μmol/min/mg) or coding for GFP 6.3 μmol/min/mg with RCSN-3 wild type cells (6.6 ± 1.0 μmol/min/mg) (Figure 1C).

The protective role of DT-diaphorase against the neurotoxic effects aminochrome was determined by incubating the RCSN-3 cells expressing Nq6 or Nq7 siRNA with 100 μM aminochrome during 48 h. At this concentration, aminochrome induced 21% cell death in RCSN-3 wild type cells contrasting with a significant increase in cell death in RCSN-3 cells expressing Nq6 siRNA (87 ± 1.5% cell death; $P < 0.01$;) and Nq7 siRNA (78 ± 2.0% cell death, $P < 0.01$) (Figure 2A). No significant cell death in RCSN-3 cells expressing Nq6 or Nq7 siRNA incubated with 100 μM aminochrome was observed at 12 or 24 h (not shown). The treatment of RCSN-3 cells expressing siRNA for DT-diaphorase with 100 μM aminochrome induced a dramatic morphological change characterized by the loss of their elongated shape and reduction in their volume, adopting a spherical shape (Figure 2B).

Discussion

Our results support the proposed neuroprotective role of DT-diaphorase against aminochrome neurotoxicity on the basis of experiments done with dicoumarol, a specific inhibitor of DT-diaphorase (*5*−*10*). Although, DT-diaphorase is defined as the quinone reductase activity inhibited by dicoumarol, we cannot discard that dicoumarol may exert some unspecific effects since dicoumarol has been reported to impair mitochondrial electron transport (*16*). However, the use of specific siRNA for DT-diaphorase drastically decreases the enzyme expression and activity, inducing a significant 4-fold increase in cell death, supporting the protective role against aminochrome neurotoxicity on the basis of experiments with dicoumarol (*5*−*10*). The strong decrease in DT-diaphorase expression induced by siRNA NQ6 and NQ7 prevent the two-electron reduction of aminochrome, allowing aminochrome to participate in neurotoxic reactions such as forming adducts with α-synuclein and inducing and stabilizing neurotoxic protofibrils (*3, 4*) or being reduced by flavoenzymes using NADH or NADPH, which catalyze the transfer of one electron to a leukoaminochrome-*o*-semiquinone radical (*17*). This species is extremely reactive with oxygen, auto-oxidizing with the generation of a redox, and cycling between aminochrome and the leukoaminochrome *o*-semiquinone radical. This redox cycling results in the depletion of NADH, NADPH, and oxygen (Figure 3).

The expression of siRNA NQ6 and NQ7 in RCSN-3 cells prevented DT-diaphorase expression, but the siRNA R2 and R5 did not induce reduction. The siRNA R5 induces stimulation in DT-diaphorase expression. Most probably, this effect is due to three main reasons: (a) R2 and R5 siRNA is not functional against the target mRNA of DT-diaphorase, (b) perhaps the designed siRNA is unspecific to the DT-diaphorase expression, and (c) therefore they might have off-target affects, which will cause stimulations. These effects are common for siRNA since we still lack theoretical predictions for designing the perfect siRNA, even though we do follow recommendations from manufacturers and leading research laboratories (*14*).

It is of interest to mention that aminochrome formed by dopamine oxidation with Mn^{3+} induced acute neurotoxicity in only 2 h when aminochrome is reduced by one-electron to leukoaminochrome *o*-semiquinone radical in the presence of the DT-diaphorase inhibitor dicoumarol. Interestingly, aminochrome did not induce morphological changes under these conditions (*5*) contrasting with the cell death induced by purified aminochrome in 48 h, which resulted in dramatic morphological changes such as reduction of their volume and changes in shape from elongated to spherical. Aminochrome formed by the oxidizing agent Mn^{3+} is the same molecule as the one we used in this study, but the difference is that aminochrome formed by oxidizing dopamine with Mn^{3+} in the cell culture medium contains Mn^{2+} that can be take up into the cell by a divalent metal transporter (DMT-1) expressed in RCSN-3 cells, inducing a different cell death. These morphological changes induced by purified aminochrome is the consequence of the disruption of actins and α-tubulin and β-tubulin cytoskeleton filament networks that became condensed around the cell membrane (*10*). Actin is an important component of the cellular cytoskeleton, which is essential for sculpting and maintaining cell shape (*18*). The cyanobacterial toxin Microcystin-LR induces shrinkage with the shortening and loss of actin filaments, associated with a concentration dependent depolymerization of microtubules (*19*).

Mitochondrial dysfunction is probably one of the mechanisms involved in aminochrome-induced cell death when siRNA significantly decreases DT-diaphorase expression (Figure 3). It was reported that dopamine *o*-quinone can lead to changes in mitochondrial respiration since after exposure to dopamine *o*-quinone, the respiration in the mitochondria proceeded without being coupled to ATP synthesis, an unproductive use of cellular energy (*20*). Dopamine *o*-quinone is the precursor of aminochrome and is a transient compound at physiological pH levels since its amino chain undergoes cyclization to form aminochrome. Stable dopamine *o*-quinone can only be found when the pH is lower than 2.0 (*15*). Proteins involved in various mitochondrial functions were identified as being covalently modified by ^{14}C-dopamine *o*-quinone, including proteins from the tricarboxylic acid cycle and subunits of complex I and III (*21*). Mitochondrial dysfunction induced by the formation of adducts between the aminochrome and mitochondrial proteins leads to a collapse of energy production that is worsened by the lack of NADH and oxygen due to the generation of redox cycling between aminochrome and the leukoaminochrome *o*-semiquinone radical when aminochrome is reduced by flavoenzymes that use NADH as the electron donor (Figure 3) (*17, 22*). Aminochrome induces the disruption of mitochondrial membrane potential and mitochondria damage determined by electron microscopy (*5, 6*).

In conclusion, this study shows that DT-diaphorase is a protective enzyme against aminochrome neurotoxicity and that Nq6 and Nq7 siRNA against DT-diaphorase are very useful tools to study the role of this enzyme in aminochrome metabolism in a more specific way.

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References

(1) Paris, I., Lozano, J., Perez-Pastene, C., Muñoz, P., and Segura-Aguilar, J. (2009) Molecular and neurochemical mechanisms in PD pathogenesis. *Neurotox. Res. 16*, 271–279.

(2) Zecca, L., Fariello, R., Riederer, P., Sulzer, D., Gatti, A., and Tampellini, D. (2002) The absolute concentration of nigral neuromelanin, assayed by a new sensitive method, increases throughout the life and is dramatically decreased in Parkinson's disease. *FEBS Lett. 510*, 216–220.

(3) Conway, K. A., Rochet, J. C., Bieganski, R. M., and Lansbury, P. T., Jr. (2001) Kinetic stabilization of the a -synuclein protofibril by a dopamine-α-synuclein adduct. *Science 294*, 1346–1349.

(4) Norris, E. H., Giasson, B. I., Hodara, R., Xu, S., Trojanowski, J. Q., Ischiropoulos, H., and Lee, V. M. (2005) Reversible inhibition of α-synuclein fibrillization by dopaminochrome-mediated conformational alterations. *J. Biol. Chem. 280*, 21212–21219.

(5) Arriagada, A., Paris, I., Sanchez de las Matas, M. J., Martinez-Alvarado, P., Cardenas, S., Castañeda, P., Graumann, R., Perez-Pastene, C., Olea-Azar, C., Couve, E., Herrero, M. T., Caviedes, P., and Segura-Aguilar, J. (2004) On the neurotoxicity of leukoaminochrome o-semiquinone radical derived of dopamine oxidation: mitochondria damage, necrosis and hydroxyl radical formation. *Neurobiol. Dis. 16*, 468–477.

(6) Fuentes, P., Paris, I., Nassif, M., Caviedes, P., and Segura-Aguilar, J. (2007) Inhibition of VMAT-2 and DT-diaphorase induce cell death in a substantia nigra-derived cell line--an experimental cell model for dopamine toxicity studies. *Chem. Res. Toxicol. 20*, 776–783.

(7) Paris, I., Dagnino-Subiabre, A., Marcelain, K., Bennett, L. B., Caviedes, P., Caviedes, R., Azar, C. O., and Segura-Aguilar, J. (2001) Copper neurotoxicity is dependent on dopamine-mediated copper uptake and one-electron reduction of aminochrome in a rat substantia nigra neuronal cell line. *J. Neurochem. 77*, 519–529.

(8) Paris, I., Martinez-Alvarado, P., Perez-Pastene, C., Vieira, M. N., Olea-Azar, C., Raisman-Vozari, R., Cardenas, S., Graumann, R., Caviedes, P., and Segura-Aguilar, J. (2005) Monoamine transporter inhibitors and norepinephrine reduce dopamine-dependent iron toxicity in cells derived from the substantia nigra. *J. Neurochem. 92*, 1021–1032.

(9) Paris, I., Perez-Pastene, C., Couve, E., Caviedes, P., Ledoux, S., and Segura-Aguilar, J. (2009) Copper dopamine complex induces mitochondrial autophagy preceding caspase-independent apoptotic cell death. *J. Biol. Chem. 284*, 13306–13315.

(10) Paris, I., Perez-Pastene, C., Cardenas, S., Iturra, P., Muñoz, P., Couve, E., Caviedes, P., and Segura-Aguilar, J. (2010) Aminochrome induces disruption of actin, α-, and β-tubulin cytoskeleton networks in substantia-nigra-derived cell line. *Neurotox. Res. 18*, 82–92.

(11) Cardenas, S. P., Perez-Pastene, C., Couve, E., and Segura-Aguilar, J. (2008) The DT-diaphorase prevents the aggregation of a-synuclein induced by aminochrome. *Neurotoxicity Res. 13*, 136.

(12) Segura-Aguilar, J., Cardenas, S., Riveros, A., Fuentes-Bravo, P., Lozano, J., Graumann, R., Paris, I., Nassif, M., and Caviedes, P. (2006) DT-diaphorase prevents the formation of α-synuclein adducts with aminochrome. *Soc. Neurosci. Abstr. 824*, 17.

(13) Paris, I., Lozano, J., Cardenas, S., Perez-Pastene, C., Saud, K., Fuentes, P., Caviedes, P., Dagnino-Subiabre, A., Raisman-Vozari, R., Shimahara, T., Kostrzewa, J. P., Chi, D., Kostrzewa, R. M., Caviedes, R., and Segura-Aguilar, J. (2008) The catecholaminergic RCSN-3 cell line: a model to study dopamine metabolism. *Neurotox Res. 13*, 221–230.

(14) Elbashir, S. M., Harborth, J., Lendeckel, W., Yalcin, A., Weber, K., and Tuschl, T. (2001) Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. *Nature 411*, 494–498.

(15) Segura-Aguilar, J., and Lind, C. (1989) On the mechanism of the Mn^{+3}-induced neurotoxicity of dopamine prevention of quinone-derived oxygen toxicity by DT-diaphorase and superoxide dismutase. *Chem.-Biol. Interact. 72*, 309–324.

(16) González-Aragón, D., Ariza, J., and Villalba, J. M. (2007) Dicoumarol impairs mitochondrial electron transport and pyrimidine biosynthesis in human myeloid leukemia HL-60 cells. *Biochem. Pharmacol. 73*, 427–439.

(17) Segura-Aguilar, J., Metodiewa, D., and Welch, C. (1998) Metabolic activation of dopamine *o*-quinones to *o*-semiquinones by NADPH cytochrome P450 reductase may play an important role in oxidative stress and apoptotic effects. *Biochim. Biophys. Acta 1381*, 1–6.

(18) Cingolani, L. A., and Goda, Y. (2008) Actin in action: the interplay between the actin cytoskeleton and synaptic efficacy. *Nat. Rev. Neurosci. 9*, 344–356.
(19) Gácsi, M., Antal, O., Vasas, G., Máthé, C., Borbély, G., Saker, M. L., Gyori, J., Farkas, A., Vehovszky, A., and Bánfalvi, G. (2009) Comparative study of cyanotoxins affecting cytoskeletal and chromatin structures in CHO-K1 cells. *Toxicol. In Vitro 23*, 710–718.
(20) LaVoie, M. J., and Hastings, T. G. (1999) Dopamine quinone formation and protein modification associated with the striatal neurotoxicity of methamphetamine: evidence against a role for extracellular dopamine. *J. Neurosci. 19*, 1484–1491.
(21) Van Laar, V. S., Dukes, A. A., Cascio, M., and Hastings, T. G. (2008) Proteomic analysis of rat brain mitochondria following exposure to dopamine quinone: implications for Parkinson disease. *Neurobiol. Dis. 29*, 477–489.
(22) Baez, S., Linderson, Y., and Segura-Aguilar, J. (1995) Superoxide dismutase and catalase enhance autoxidation during one-electron reduction of aminochrome by NADPH-cytochrome P-450 reductase. *Biochem. Mol. Med. 54*, 12–18.

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