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Communication

Copper Neurotoxicity in Rat Substantia Nigra and Striatum Is Dependent on DT-Diaphorase Inhibition

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The dependence of copper neurotoxicity on DT-diaphorase inhibition was suggested from results obtained from a cell line derived from substantia nigra. Therefore, the aim of this study was to evaluate whether $CuSO_4$ neurotoxicity in vivo, which was evaluated by determining the contralateral rotation and loss of tyrosine hydroxylase immunostaining, was dependent on DT-diaphorase inhibition by dicoumarol. Animals unilaterally and intranigrally injected with 0.25 nmol of $CuSO_4$ and 2 nmol of dicoumarol presented a significant and characteristic contralateral rotational behavior ($P < 0.01$) when they were systemically stimulated with apomorphine (0.5 mg/kg s.c.), similar to that observed in rats injected unilaterally with 6-hydroxydopamine as a positive control. The behavioral effects correlated with the lost of tyrosine hydroxylase-positive staining, since animals unilaterally and intranigrally injected with 0.25 nmol of $CuSO_4$ together with 2 nmol of dicoumarol exhibited extensive loss of tyrosine hydroxylase-positive fiber density in the striatum ($P < 0.01$) and cell loss in the substantia nigra ($P < 0.01$). Our results support the idea that $CuSO_4$ neurotoxicity is dependent upon DT-diaphorase inhibition.

Introduction

Copper is a trace element that plays an important role in cell physiology as a cofactor of several enzymes, such as cytochrome oxidase, CuZn-superoxide dismutase, dopamine-β-hydroxylase, amine oxidases, Cu monooxygenases, and nitrite reductase/multicopper oxidase tyrosinase (*1, 2*). However, copper overload can be toxic and result in Wilson disease, a disorder in which a genetic mutation in the copper transporter ATPase (ATP7B) results in the accumulation of copper. Interestingly, 69% of patients with Wilson disease present neurological symptoms, of which Parkinsonism is predominant (62%) (*3*). The high frequency of Parkinsonism in patients with Wilson disease suggests the existence of a selective neurotoxic mechanism of copper in the brain, since the Fenton reaction catalyzed by reduced forms of copper in the presence of hydrogen peroxide is so nonspecific that it cannot explain the selective symptoms of copper overload. Young workers who worked in the copper smelting industry also develop Parkinsonism (*4*).

It has been reported that copper can form a complex with dopamine, and it has been found to be neurotoxic in a cell line derived from substantia nigra. This neurotoxic action of the copper−dopamine complex is dependent on (i) the existence of dopamine uptake, (ii) dopamine oxidation to aminochrome, and (iii) inhibition of DT-diaphorase, which catalyzes the two-electron reduction to aminochrome (*5*). These results can explain the specific neurotoxic action of copper in the brain. However, the specific neurotoxic action of copper in a cell line cannot be extrapolated to the whole brain. Therefore, the aim of this study was to determine whether copper sulfate neurotoxicity in vivo is dependent on DT-diaphorase. To evaluate the contribution of DT-diaphorase inhibition to in vivo neurodegenerative effects of copper, we injected $CuSO_4$, a neurotoxic agent, into the right substantia nigra, alone or together with dicumarol, a selective inhibitor of DT-diaphorase. We evaluated the degeneration of the nigrostriatal pathway through the expression of motor responses (rotational model and spontaneous motor activity) and avoidance conditioning, considering the influence of the integrity of dopaminergic systems on these behaviors and immunochemistry.

Experimental Procedures

Chemicals. The following compounds were purchased from Sigma Chemical Co. (St. Louis, MO): cupric sulfate ($CuSO_4$), dicumarol (3,3′-methylene-bis-4-hydroxycoumarin), and 6-hydroxydopamine hydrobromide.

Animals. Adult male Sprague−Dawley rats, weighing 180−220 g at the time of intranigral stereotaxical injection and 220−250 g at the time of behavioral experiments, were housed

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in groups of six in a well-ventilated, temperature (22 ± 1 °C)-controlled environment under a 12:12 light−dark cycle (lights on from 08.00 to 20.00 h) with free access to food and water. 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 experimental protocols were approved by the local Animal Care and Use Committee. The minimum number of animals and duration of observation required to obtain consistent data were employed.

Intranigral Stereotaxical Injection. The animals were anesthetized with an intraperitoneal injection of sodium pentobarbital (30 mg/kg) and placed in a David Kopf stereotaxical frame with the skull oriented according to the Paxinos and Watson atlas (*6*). A total of 2 μL of the following solutions was injected into the right substantia nigra using a Hamilton microsyringe (coordinates relatives to bregma were AP = −4.8, L = −1.8, and V = −8.2): $CuSO_4$ (0.25 nmol) and dicumarol (2 nmol), both dissolved in 0.1 M Tris-HCl (pH 7.9), and 6-hydroxydopamine (32 nmol), a neurotoxin that may produce selective dopaminergic denervation following intranigral administration (*7*), dissolved in physiological saline containing 0.1% ascorbic acid. All solutions were injected at a rate of 1 μL/min during 2 min. To minimize the possibility of back flow, the needle was kept in place for an additional minute upon completion of the injection. Control animals were injected with a similar volume of Tris-HCl vehicle. Eight rats were assigned to each of five experimental groups. After surgery, rats were allowed to recover for 7 days prior to conducting rotational responses to apomorphine (0.5 mg/kg sc). Fourteen days after injection, the rats were submitted to other behavioral experiments using a fixed design of spontaneous motor activity followed by conditioned avoidance training.

Rotational Behavior. The rotational model is based on a unilateral lesion of the nigrostriatal DA system with the neurotoxin 6-OHDA (*8*). Rats with this lesion show a postural deviation that can be expressed as a contralateral rotation behavior when they are stimulated with apomorphine (0.5 mg/kg, sc). One week after intranigral drug injection, each rat was placed in a LE 902 Rotometer (Panlab, Barcelona, Spain) connected to a LE 3806 multicounter (Panlab, Barcelona, Spain) to evaluate the presence of motor asymmetry. Rotational behavior testing was carried out and allowed for free movement of the rat for 90 min. The sensor measured and provided a separate output for continuous movement in the clockwise or counter-clockwise directions. Results were expressed as the mean total number of complete 360° turns in either direction during the entire period of observation.

Immunohistochemistry. When the behavioral study was complete, rats were sacrificed with an overdose of sodium pentobarbital and perfused intracardially with 0.9% saline followed by 4% buffered formaldehyde solution (*9*). The brains were removed, postfixed in a 4% buffered formaldehyde solution for 24 h, and cryopreserved in 30% sucrose, and 10 μm frozen sections were cut in a cryostat (Leica CM1800, Leica Microsystem Inc. NY). Frozen sections were cut in a cryostat (Leica CM1800, Leica Microsystem Inc. NY). For TH-immunohistochemistry in the striatum and substantia nigra, the sections were precoated with 5% normal goat serum (NGS)/0.15% Triton X-100 in 0.1 M phosphate buffer (PB, pH 7.4) and incubated overnight at room temperature with a 1:500 dilution of rabbit anti-TH antibody (Pel Freez) in 2.5% NGS/0.15% Triton X-100 in PB. This was followed by incubation with a 1:250 dilution of biotinylated goat antirabbit antibody in 0.15% Triton X-100 in PB and then avidin−biotin−peroxidase complex (ABC, Vector). The degree of dopaminergic nigrostriatal lesion in the striatum was expressed as the percentage of the reduction in TH-positive fiber density in the injected side as compared to the contralateral noninjected side. To estimate the specific TH-staining density, the optical density readings were corrected for nonspecific background density, as measured from the completely denervated parts of the striatum in the animals. The degree of dopaminergic nigrostriatal lesion in the substantia nigra was expressed as the reduction in the number TH-positive cells.

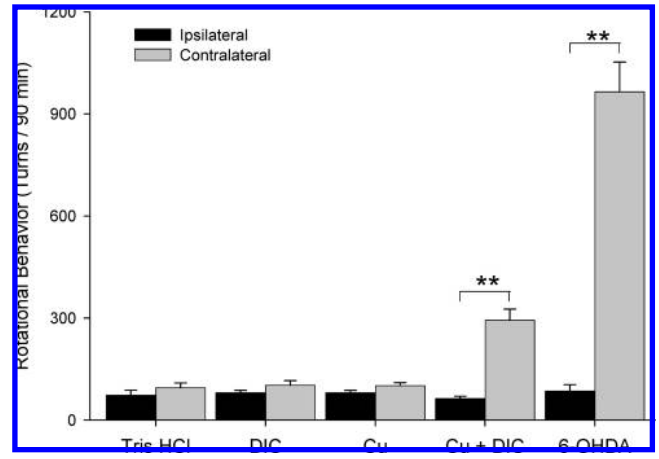

Figure 1. Rotational behavior induced by $CuSO_4$ and dicoumarol. Contralateral rotation was induced by injection of apomorphine (0.5 mg/kg sc) in rats with intracerebral injections of Tris-HCl vehicle, 2 nmol of dicoumarol (DIC), 0.25 nmol of $CuSO_4$ (Cu), and 6-hydroxydopamine (6-OH-DA) into the right substantia nigra. This behavior was measured 7 days after intranigral administration. Values are the means ± SEM of eight rats in each group. Bars represent the total ipsilateral and contralateral rotation in a 90 min observation period. For statistical comparisons, one-way ANOVA was used followed by post hoc Newman−Keuls test to compare contralateral with ipsilateral rotations for each treatment (**P < 0.01).

Data Expression and Statistical Analysis. Behavioral data are expressed by the means ± SEM for each parameter in the different treatment groups. The statistical analysis of these results consisted of a one-way (treatment) ANOVA with the iner-STAT-a v1.3 program and a Newman−Keuls test for multiple comparisons when appropriate. In all cases, α was set at 0.05.

Results

Rotational Behavior. Rotational behavior evaluated after apomorphine (0.5 mg/kg, sc) treatment is shown in Figure 1. The one-way ANOVA shows a significant effect of the treatment, $F(9.70) = 75.97$, $p < 0.0001$. ANOVA shows a significant effect for contralateral rotation, $F(4.35) = 73.25$, $p < 0.0001$, but not for ipsilateral rotation, $F(4.35) = 0.92$, $p = 0.517$. $CuSO_4$ at 0.25 nmol and 2 nmol of dicoumarol (*10*) individually did not induce contralateral rotation (Figure 1). However, the simultaneous injection of 0.25 nmol of $CuSO_4$ with 2 nmol of dicoumarol induced a significant and clear contralateral rotation after injection of apomorphine ($p < 0.01$), which was similar to the rotation of 6-hydroxydopamine-injected rats used as a positive control (Figure 1).

Immunohistochemistry. We studied what happens in the striatum when you inject $CuSO_4$ into the substantia nigra, since the nigrostriatal system has cell bodies in the substantia nigra and the axons have synapses in the striatum. A significant reduction in the tyrosine hydroxylase-positive fiber density (55

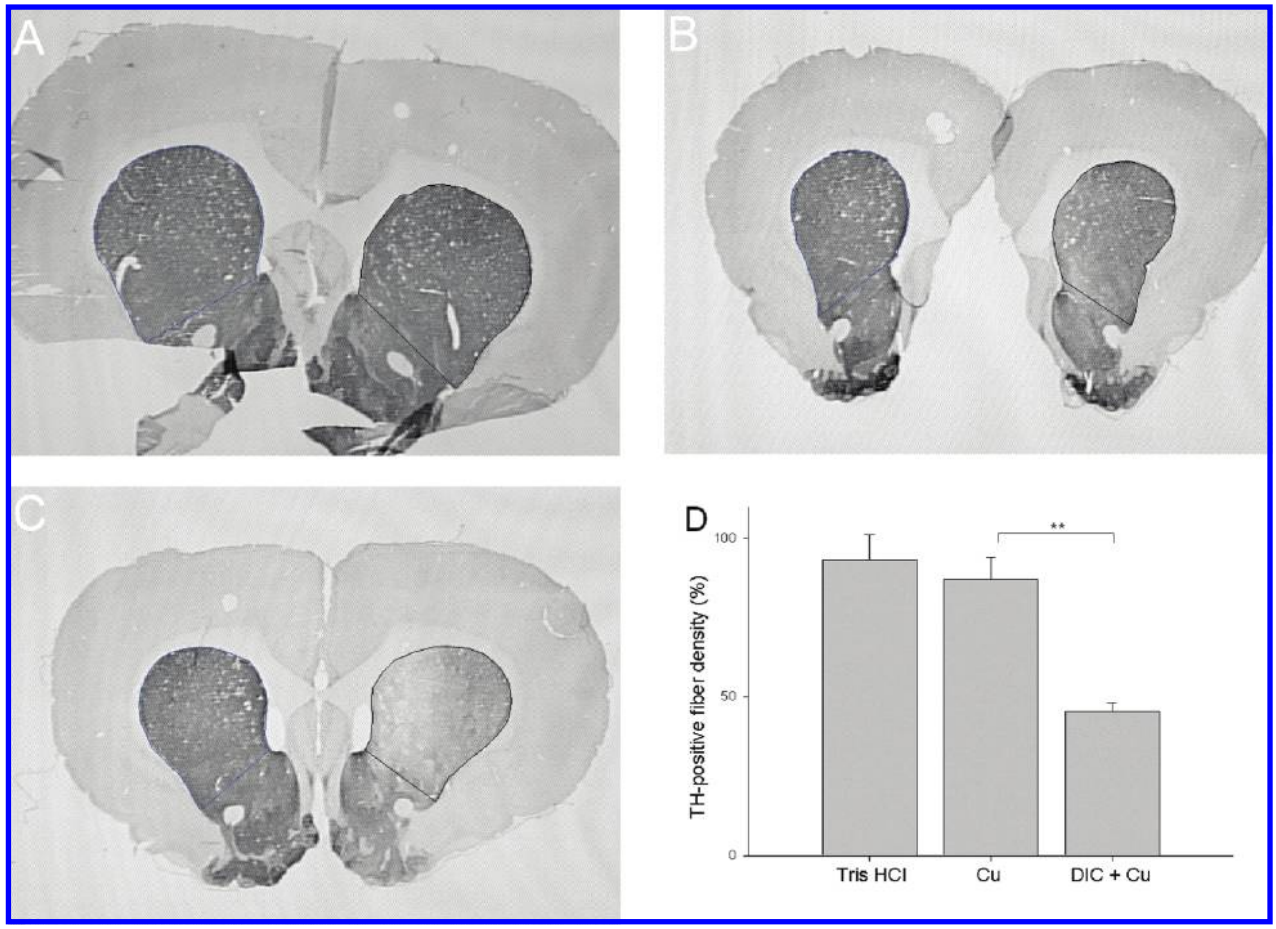

Figure 2. Reduction in tyrosine hydroxylase-positive fiber density in striatum. The effect of intracerebral injection in the right side of the substantia nigra on tyrosine hydroxylase-positive fiber density in striatum was determined in rats injected with Tris-HCl vehicle (A), 0.25 nmol of $CuSO_4$ (B), and 0.25 nmol of $CuSO_4$ together with 2 nmol of dicoumarol (DIC + Cu) (C). The quantification of tyrosine hydroxylase-positive fiber density is shown in panel D. Each bar represents the means ± SEM ($n = 3$) of the percentages of tyrosine hydroxylase (TH)-positive fiber loss in the striatum as compared with the contralateral control intact side for the Tris-HCl vehicle, 0.25 nmol of $CuSO_4$ (Cu), and 0.25 nmol of $CuSO_4$ together with 2 nmol of dicoumarol (DIC + Cu) groups. For statistical comparisons, one-way (treatment) ANOVA with the inerSTAT-a version 1.3 program was used to compare the untreated left side with the injected right side (**$P < 0.01$).

± 3%, $P < 0.01$; $n = 3$) was observed in the right side of the striatum when the rats were injected with 0.25 nmol of $CuSO_4$ together with 2 nmol of dicoumarol into the right side of the substantia nigra (Figure 2C,D). No significant lost of tyrosine hydroxylase-positive staining was observed in rats injected with 0.25 nmol of $CuSO_4$ alone (Figure 2 A,D). We also studied what happens in the substantia nigra when the rats were injected with 0.25 nmol of $CuSO_4$ together with 2 nmol of dicoumarol into the right side of the substantia nigra (Figure 3C). However, in the substantia nigra, we measured the number of cell bodies that survived the treatment. A significant reduction of tyrosine hydroxylase-positive cell bodies was observed in the right side of the substantia nigra when rats were injected with $CuSO_4$ together with dicoumarol into the right side of the substantia nigra (38 ± 3%; $P < 0.01$; $n = 4$; Figure 3D). No significant lost of tyrosine hydroxylase positive staining was observed in rats injected with 0.25 nmol of $CuSO_4$ alone (Figure 3B,D). We studied what happens in the striatum when you inject $CuSO_4$ into the substantia nigra since the nigrostriatal system has cell bodies in the substantia nigra and the axons have synapses in the striatum.

Discussion

The high frequency of Parkinsonism symptoms in patients with Wilson disease (*3*) suggests the existence of a specific mechanism of copper neurotoxicity, which affects dopaminergic neurons. Studies in a cell line derived from rat substantia nigra (RCSN-3) showed that copper is selectively neurotoxic in cells that have dopamine uptake since it is not toxic in cells lacking dopamine uptake (*5*). The results presented in this study support this idea, since we observed contralateral rotation when 0.25 nmol and 2 nmol of $CuSO_4$ are injected together with dicoumarol (Figure 1A). The rotational model is based on the unilateral lesion of a specific neurotoxin of dopaminergic neurons. This model was developed using 6-hydroxydopamine (*7*), and it has been considered as a prototype for studying Parkinsonism and drugs with putative antiparkinsonian actions (*11–14*). The fact that $CuSO_4$ induces contralateral behavior suggests an explanation for why copper overload in brain induces Parkinsonism in Wilson disease. The main, but not exclusive, neurotoxic action of copper is on dopaminergic system since the most predominant neurological symptom in Wilson disease is Parkinsonism (62%) (*3*). Interestingly, young workers, who work in the copper smelting industry also develop Parkinsonism (*4*).

Neuromelanin is able to chelate metals (*15*), and its content in rats is lower than in humans. However, neuromelanin is not able to chelate copper and prevent the formation of copper−dopamine complexes, since copper forms a complex with extracellular

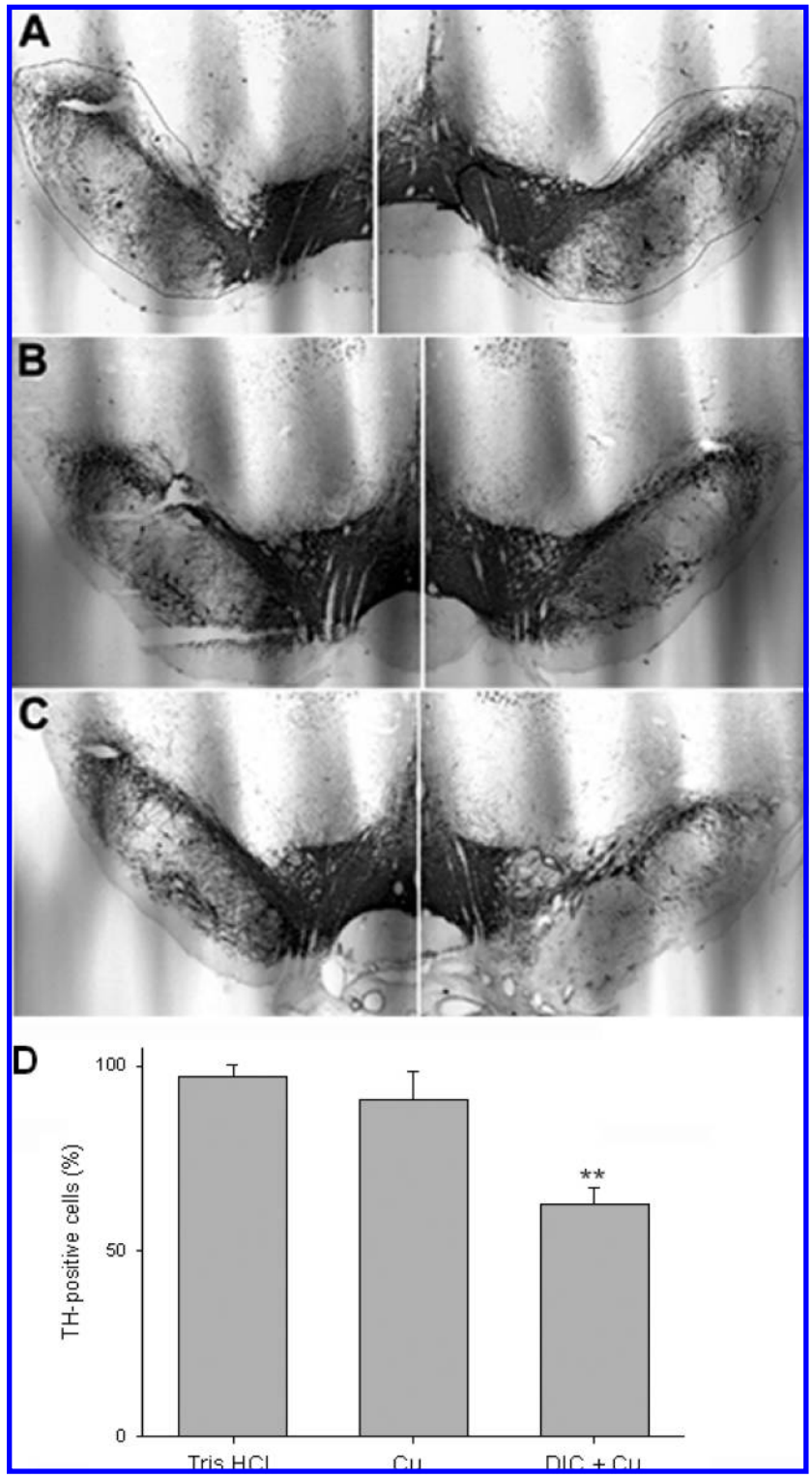

Figure 3. Reduction in tyrosine hydroxylase-positive cells in the substantia nigra. The effect of intracerebral injection in the right side of the substantia nigra on tyrosine hydroxylase-positive cells in the substantia nigra was determined in rats injected with Tris-HCl vehicle (A), 0.25 nmol of $CuSO_4$ (B), and 0.25 nmol of $CuSO_4$ together with 2 nmol of dicoumarol (DIC + Cu) (C). The quantification of tyrosine hydroxylase-positive cells is shown in panel D for the Tris-HCl vehicle, 0.25 nmol of $CuSO_4$ (Cu), and 0.25 nmol of $CuSO_4$ together with 2 nmol of dicoumarol (Cu + DIC) groups. Each bar represents the mean ± SEM ($n = 4$) of the reduction of tyrosine hydroxylase (TH)-positive cell bodies in the treated side of the substantia nigra as compared with the control intact side. For statistical comparisons, one-way (treatment) ANOVA with the inerSTAT-a version 1.3 program was used to compare the untreated left side with the injected right side (**$P < 0.01$).

dopamine released during neurotransmission and neuromelanin is localized inside of dopaminergic neurons.

No contralateral rotation was observed when 0.25 nmol of $CuSO_4$ was injected alone into the substantia nigra. However, a significant contralateral rotation (Figure 1) and loss of tyrosine hydroxylase-positive staining in the striatum (Figure 3C,D) was observed when the same concentration of $CuSO_4$ was injected in the presence of dicoumarol, an inhibitor of DT-diaphorase. DT-diaphorase (EC.1.6.99.2) is a unique flavoenzyme that catalyzes the reduction of two electrons of quinones to hydroquinones and the two-electron reduction of aminochrome (*16*). DT-diaphorase immunoreactivity has been found in neurons of

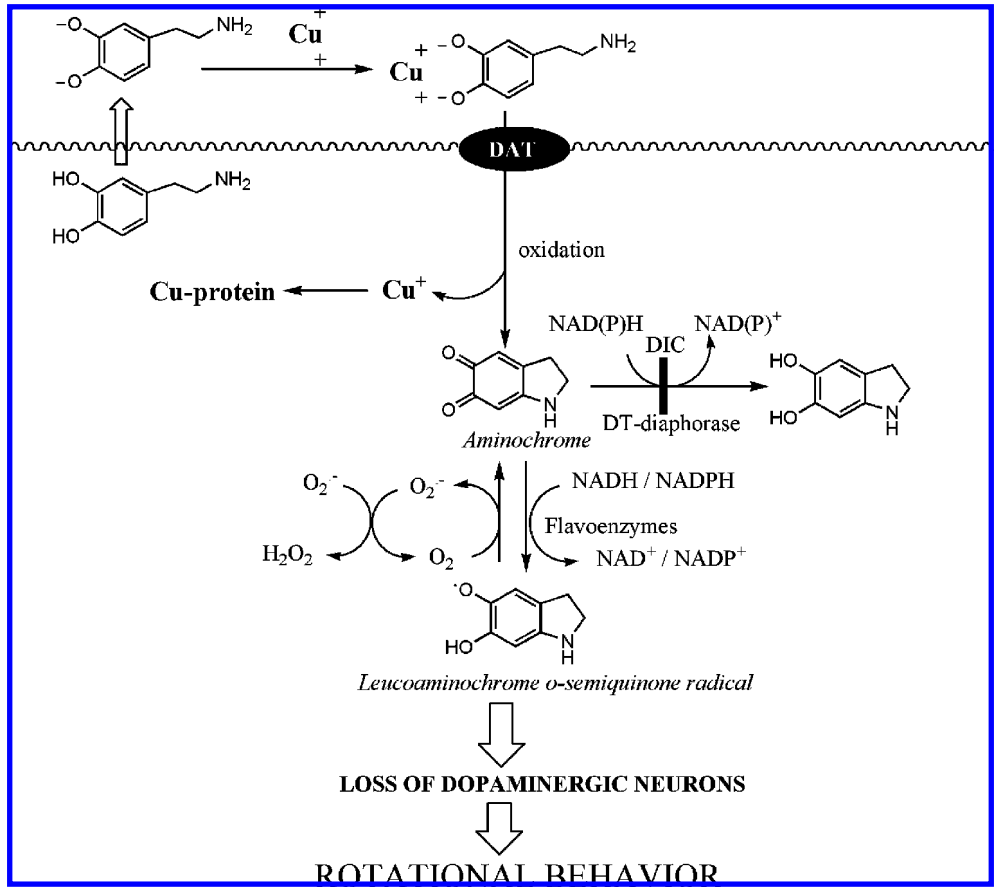

Figure 4. Possible mechanism for $CuSO_4$-induced contralateral rotation and loss of tyrosine hydroxylase-positive fibers. $CuSO_4$ forms a complex with dopamine released during neurotransmission, and the Cu−DA complex is taken up via dopamine transporters into dopaminergic neurons, where dopamine is oxidized to aminochrome. Flavoenzymes, using NADH or NADPH, catalyze the one-electron reduction of aminochrome to leukoaminochrome *o*-semiquinone radical, which autoxidizes to generate a redox cycle when dicoumarol inhibits DT-diaphorase. This redox cycle results in (i) the depletion of NADH, which is required to produce ATP synthesis in the mitochondria; (ii) depletion of NADPH, which is required to maintain GSH in the reduced state necessary to exert its antioxidant action; (iii) the depletion of oxygen, which is required for ATP synthesis in the mitochondria; and (iv) formation of superoxide radicals, which spontaneously or enzymatically generate hydrogen peroxide, the precursor of hydroxyl radicals.

both the substantia nigra and ventral tegmental area, and it colocalizes with tyrosine hydroxylase-like immunoreactivity (*17*). DT-diaphorase has been proposed to be a neuroprotective enzyme of dopaminergic neurons, and its inhibition by dicoumarol induces in cells derived from rat substantia nigra (RCSN-3): Cu toxicity where Cu−dopamine complex is taken up via the dopamine transporter and dopamine is oxidized to aminochrome (*5*); aminochrome toxicity in RCSN-3 cells (*18*); iron toxicity, which is dependent on dopamine uptake in RCSN-3 cells (*19, 20*); and reserpine induced neurotoxicity by increasing dopamine oxidation to aminochrome in RCSN-3 cells (*21*). In addition, in rats, DT-diaphorase inhibition by dicoumarol induces a 6-OH-dopamine-like contralateral rotation and an extensive loss of tyrosine hydroxylase staining in rats injected intracerebrally with the oxidizing agent manganese^{3+} pyrophosphate into the medial forebrain bundle or substantia nigra, respectively (*10, 22*). DT-diaphorase prevents aminochrome neurotoxic actions, such as the one-electron reduction of aminochrome (*5, 18–21*) and formation of α-synuclein protofibrils (*23*). The results presented in this work support the idea that DT-diaphorase plays a neuroprotective role in dopaminergic neurons (*5, 24–26*), as suggested by the contralateral rotation and extensive loss of tyrosine hydroxylase-positive staining observed when $CuSO_4$ was injected together with the DT-diaphorase inhibitor dicoumarol (Figure 4).

In conclusion, this study demonstrates the in vivo behavioral consequences of neurotoxicity of $CuSO_4$ in the nigrostriatal pathway. These effects are dependent on simultaneous injection of $CuSO_4$ and dicoumarol into the substantia nigra, suggesting a neuroprotective role for DT-diaphorase.

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