Behavioral effects of manganese injected in the rat substantia nigra are potentiated by dicumarol, a DT-diaphorase inhibitor

Gabriela Díaz-Véliz*, Sergio Mora, Patricia Gómez, Ma Teresa Dossi, Juan Montiel, Christian Arriagada, Francisco Aboitiz, Juan Segura-Aguilar

Programa de Farmacología Molecular y Clínica, Instituto de Ciencias Biológicas (ICBM), Facultad de Medicina, Universidad de Chile, P.O. Box 16038, Santiago 9, Chile

Abstract

The purpose of this study was to evaluate the contribution of DT-diaphorase inhibition to in vivo neurodegenerative effects of dopamine (DA) oxidation to the corresponding *o*-quinones. The neurotoxicity to nigrostriatal DA neurons was induced by injection of manganese pyrophosphate (Mn^{3+}) complex as a prooxidizing agent alone or together with the DT-diaphorase inhibitor dicumarol into the right rat substantia nigra. The behavioral effects were compared with those induced after selective lesions of dopaminergic neurons with 6-hydroxydopamine (6-OHDA). Intranigral injection of Mn^{3+} and Mn^{3+} plus dicumarol produced significant impairment in motor behavior compared with control animals. However, the effect seen in the Mn^{3+} plus dicumarol injected group was significantly more severe than that observed in the Mn^{3+} alone injected group. In motor activity and rearing behavior, the simultaneous injection of Mn^{3+} plus dicumarol produced a 6-OHDA-like impairment. Similar effects were observed in the acquisition of a conditioned avoidance response (CAR). Dicumarol significantly impaired avoidance conditioning although without affecting the motor behavior. The behavioral effects were correlated to the extent of striatal tyrosine hydroxylase (TH)-positive fiber loss. Rats receiving unilateral intranigral Mn^{3+} and Mn^{3+} plus dicumarol injections exhibited a significant reduction in nigrostriatal TH-positive fiber density in medial forebrain bundle compared with the contralateral noninjected side. In conclusion, this study provides evidence that the neurotoxicity of Mn^{3+} in vivo is potentiated by DT-diaphorase inhibition, suggesting that this enzyme could play a neuroprotective role in the nigrostriatal DA systems.

Keywords: Manganese; DT-diaphorase; Dopamine; Neurodegeneration; Parkinsonism

1. Introduction

Although it is generally accepted that free radicals are involved in the neurodegeneration of the nigrostriatal dopamine (DA) system observed in Parkinson's disease, the exact mechanism of neurodegeneration in vivo is still unknown. Much attention has focused on the fact that oxidation of DA results in the formation of cytotoxic compounds, which can cause massive brain damage if they are allowed to accumulate. However, the rate at which oxygen oxidizes DA at neutral pH is very low (Graham et al., 1978) and probably does not produce any significant amount of reactive oxygen intermediates. Manganese in the Mn^{3+} state is a potent oxidizing agent and can accelerate the oxidation of DA to its *o*-quinone (Barbeau, 1984; Archibald and Tyree, 1987), which instantaneously cyclizes to form aminochrome (Segura-Aguilar and Lind, 1989; Shen and Dryhurst, 1998; Brenneman et al., 1999; Dorman et al., 2000; Lee, 2000). This observation may explain the drastic decrease in the level of DA because this reaction appears to be irreversible (Segura-Aguilar and Lind, 1989). Previously, it was demonstrated that aminochrome resulting from oxidation by DA with Mn^{3+} was toxic in a mouse-derived neuronal cell line (CNh) (Arriagada et al., 2000) and also induces a significant behavioral impairment in vivo (Díaz-Véliz et al., 2002). The one-electron reduction of aminochrome to leukoaminochrome *o*-semiquinone radicals can be one possible source of reactive species (Stokes et al., 1999; Segura-Aguilar et al., 2001; Smythies, 2002). Leukoaminochrome *o*-semiquinone radical is a very reactive metabolite (Segura-Aguilar et al., 1998) that autoxidizes in the presence of oxygen or transition metal ions like manganese, copper or iron (Segura-Aguilar and Lind, 1989;

* Corresponding author. Fax: +56-2-2741628.
E-mail address: gdiaz@med.uchile.cl (G. Díaz-Véliz).

Shen and Dryhurst, 1998; Paris et al., 2001), initiating a redox cycling process (Baez et al., 1995). This aberrant one-electron metabolism of aminochrome can be prevented by a two-electron reduction of aminochrome to leukoaminochrome, catalyzed by DT-diaphorase (Segura-Aguilar and Lind, 1989; Segura-Aguilar et al., 1998, 2001). There is evidence that DT-diaphorase, an enzyme that in rat substantia nigra constitutes the 98% of the total quinone reductase activity (Schultzberg et al., 1988), prevents aminochrome one-electron reduction by reducing aminochrome with two electrons to leukoaminochrome (Segura-Aguilar and Lind, 1989). The selective inhibition of this enzyme leads to an autoxidative cascade due to the ability of leukoaminochrome *o*-semiquinone to induce redox cycling (Baez et al., 1995). Thus, very low concentrations of aminochrome can produce a large amount of reactive oxygen species (Segura-Aguilar et al., 2001). In vitro studies have demonstrated that DT-diaphorase is inhibited by dicumarol (Schultzberg et al., 1988; Segura-Aguilar and Lind, 1989; Paris et al., 2001). Recently, we demonstrated that inhibition of DT-diaphorase is a requirement for Mn^{3+} to produce a 6-hydroxydopamine (6-OHDA)-like rotational behavior in rats (Segura-Aguilar et al., 2002).

To evaluate the contribution of DT-diaphorase inhibition to in vivo neurodegenerative effects of oxidation products of DA, we injected into substantia nigra (1) Mn^{3+} as a general prooxidizing agent to accelerate the oxidation of endogenous DA and (2) dicumarol as a selective inhibitor of DT-diaphorase. We evaluated the degeneration of the nigrostriatal pathway through the expression of spontaneous motor activity and avoidance conditioning, considering the influence of integrity of DA systems on these behaviors.

2. Methods

2.1. Animals

Fifty adult, male Sprague–Dawley rats, weighing 180–220 g, were housed six per cage in a temperature-controlled vivarium under a 12:12 light/dark cycle (lights on from 08:00 to 20:00 h) with free access to food and water. The experimental protocols followed the Guide for Care and Use of Laboratory Animals and were approved by the Faculty of Medicine Committee. The rats were assigned to five experimental groups injected with (1) Tris-HCl vehicle, (2) dicumarol, (3) Mn^{3+}, (4) dicumarol plus Mn^{3+} and (5) 6-OHDA.

2.2. Drugs

The following compounds were purchased from Sigma (St. Louis, MO): dicumarol (3,3′ -methylene-bis-4-hydroxycoumarin) and 6-OHDA hydrobromide. The Mn^{3+} pyrophosphate complex was prepared according to Archibald and Fridovich (1982).

2.3. Intranigral injections

Rats were anesthetized with an intraperitoneal injection of sodium pentobarbital (30 mg/kg) and placed in a David Kopf stereotaxic frame. With the rat skull oriented according to Paxinos and Watson (1986), 2 μl of one of the following solutions were injected into the right substantia nigra (coordinates relatives to bregma were AP = − 4.8, L = − 1.8, V = − 8.2): dicumarol (0.7 μg), Mn^{3+} (6 μg), dicumarol plus Mn^{3+} and 6-OHDA (8 μg). Each drug was dissolved in 0.1 M of Tris-HCl (pH 7.9) and injected at a rate of 1 μl/min during 2 min. Doses of Mn^{3+} and dicumarol were selected based on previous studies (Segura-Aguilar et al., 2002). 6-OHDA, a neurotoxin that may produce selective DA denervation following intranigral administration (Ungerstedt et al., 1974), was dissolved in physiological saline containing 0.1% ascorbic acid. To minimize the possibility of back flow, the needle was kept in place for an additional minute on completion of the injection. Control animals were injected with a similar volume of Tris-HCl vehicle. Ten rats were assigned to each of five experimental groups. Fourteen days after injection, behavioral end points were evaluated.

2.4. Spontaneous motor activity

Spontaneous motor activity was evaluated as described previously (Mora and Díaz-Véliz, 1993). Each rat was individually placed into a Plexiglas cage (30 × 30 × 30 cm). The floor of the cage was an activity platform (Lafayette Instrument, Lafayette, IN) connected to an electromechanical counter. To avoid the influence of disturbing noises, the platform was placed into a soundproof chamber and the observations were made through a closed TV circuit. Spontaneous motor activity was recorded every 5 min during a 15-min period. Simultaneously, the number of times each rat reared, the number of headshakes and the time (s) spent in grooming behavior were also recorded.

2.5. Active avoidance conditioning

Active avoidance conditioning was as described previously (Mora and Díaz-Véliz, 1993). Immediately after the spontaneous motor activity test, each rat was individually placed in a two-way shuttle box (Lafayette Instrument) composed of two stainless steel modular testing units. Each unit was equipped with an 18-bar insulated shock grid floor, two 28-V DC lights and a tone generator (Mallory Sonalert 2800 Hz; Lafayette Instrument). Electric shocks were provided to the grid floor by a Master shock supply (Lafayette Instrument). The rats were trained over 50 trials after a 5-min period of habituation. The trial consisted of the presentation of a tone that after 5 s was overlapped with a 0.20-mA footshock until the animal escaped to the opposite chamber, with maximum shock duration of 10 s. A conditioned

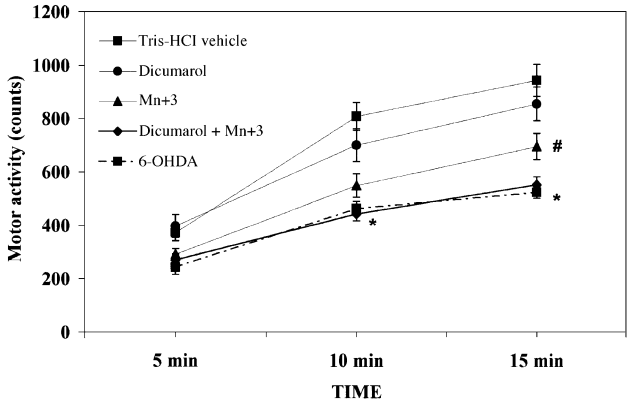

Fig. 1. Spontaneous motor activity in rats treated with Tris-HCl vehicle, dicumarol, Mn^{3+}, dicumarol plus Mn^{3+} and 6-OHDA. This behavior was measured 2 weeks after intranigral administration (see Section 2). Each point of the curve represents the mean ± S.E.M. ($n=10$) of spontaneous motor activity during a 15-min observation period. For statistical comparisons, one-way ANOVA was used followed by post hoc Newman–Keuls' test. * $P<.05$, compared with Tris-HCl vehicle and dicumarol injected groups. # $P<.05$, Mn^{3+}-injected rats compared with all other experimental groups.

avoidance response (CAR) was defined as a crossing to the opposite chamber within the first 5 s (tone alone).

2.6. 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 (Winkler et al., 2002). 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 Microsystems, Buffalo, NY). Coronal sections at forebrain medial bundle level were processed for immunohistochemical demonstration of tyrosine hydroxylase (TH; monoclonal anti-TH antibody clone TH-2; dilution 1:1000; Sigma) to evaluate the consequences of intranigral injections of dicumarol, Mn^{3+}, dicumarol plus Mn^{3+} and 6-OHDA. Immunohistochemistry was performed with the ExtrAvidin Peroxidase Staining kits (Sigma). The degree of DA nigrostriatal lesion was expressed as the percentage of reduction in TH-positive fiber density in the injected side as compared with the contralateral noninjected side. The quantification of TH-positive fiber density was made by counting the total fibers in a transversal section of the medial bundle using a computer image analysis system consisting of an Olympus BH-2 microscope equipped with a Degus video camera coupled to a Power Mac G4 computer with the NIH Image analysis software.

2.7. Statistical analysis

Results were expressed as mean ± S.E.M. Data of spontaneous motor activity comparing treatment and time were analyzed using a two-factor repeated-measures analysis of variance (ANOVA). Data of rearing behavior and avoidance conditioning comparing the treatment effects were analyzed using one-factor repeated-measures ANOVA. Each ANOVA was followed by a post hoc Newman–Keuls' Multiple Comparison Test. Student's t test was used to compare reduction in TH-positive fiber density in the injected side versus the contralateral noninjected side. In all cases, α was set at .05.

3. Results

3.1. Spontaneous motor activity

Fig. 1 shows the time course of intranigral injection on spontaneous motor activity. Two-way ANOVA revealed a significant effect of Treatment [$F(4,135)=30.15$, $P<.0001$] and Time [$F(2,135)=117.78$, $P<.0001$]. The interaction between Treatment and Time was also significant [$F(8,135)=2.47$, $P<.05$], suggesting that the effect of treatment on motor activity was dependent on the time of observation. Subsequent multiple comparison tests indicated that motor activity was not affected as a result of injection of dicumarol at any time. Mn^{3+} plus dicumarol injection significantly depressed motor activity in the same way that 6-OHDA injection compared with control rats ($P<.01$). At 15 min, rats injected with Mn^{3+} showed significantly less motor activity compared with Tris-HCl vehicle and dicumarol-injected rats; moreover, this effect was potentiated with the simultaneous injection of Mn^{3+} plus dicumarol ($P<.01$ compared with rats injected with Mn^{3+} alone). These rats exhibited a motor activity similar to 6-OHDA-injected rats.

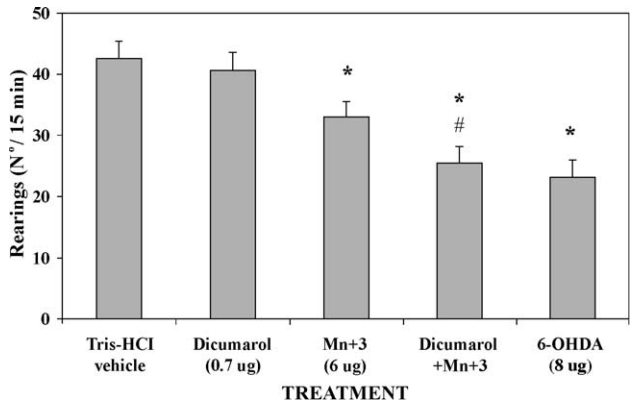

Fig. 2. Rearing activity in rats treated with Tris-HCl vehicle, dicumarol, Mn^{3+}, dicumarol plus Mn^{3+} and 6-OHDA. This behavior was measured 2 weeks after intranigral administration (see Section 2). Each bar represents the mean ± S.E.M. ($n=10$) of rearing behavior in a 15-min observation period. For statistical comparisons, one-way ANOVA was used followed by post hoc Newman–Keuls' test. * $P<.05$, compared with Tris-HCl vehicle and dicumarol injected groups. # $P<.05$, Mn^{3+}-injected rats compared with all other experimental groups.

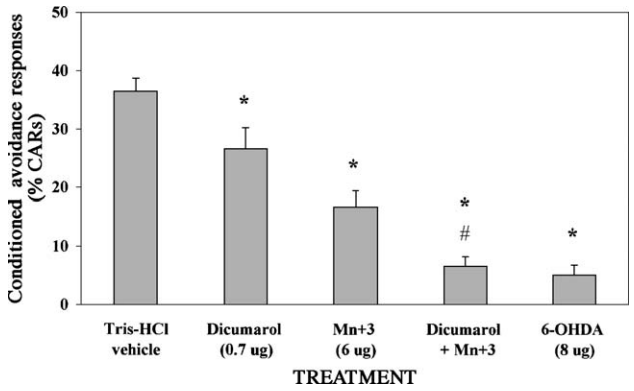

Fig. 3. CAR in rats treated with Tris-HCl vehicle, dicumarol, Mn^{3+}, dicumarol plus Mn^{3+} and 6-OHDA. This behavior was measured 2 week after intranigral administration (see Section 2). Each bar represents the mean ± S.E.M. ($n=10$) of the percentages of CAR for 50 trials. For statistical comparisons, one-way ANOVA was used followed by post hoc Newman–Keuls' test. $^{*}P<.05$, compared with Tris-HCl vehicle and dicumarol injected groups, and $^{\#}P<.05$, Mn^{3+}-injected rats compared with all other experimental groups.

Fig. 2 illustrates the effects of intranigral injection on total rearing behavior at 15 min of observation. One-way ANOVA showed a significant effect of Treatment [$F(4,45)=13.13$, $P<.0001$] on the number of rears. Multiple comparisons indicated that Mn^{3+} injections significantly depressed this behavior with respect to Tris-HCl vehicle and dicumarol-injected groups. This effect was potentiated with the simultaneous injection of Mn^{3+} plus dicumarol ($P<.01$ compared with rats injected with Mn^{3+} alone), and these rats exhibited a behavior similar to 6-OHDA-injected rats. All treatments failed to produce significant changes on other motor behaviors, such as head shaking and grooming behavior (data not shown).

3.2. Active avoidance conditioning

The results of the active avoidance conditioning are showed in Fig. 3. One-way ANOVA revealed a significant effect of the Treatment [$F(4,45)=31.95$, $P<.0001$] on the acquisition of CAR. Post hoc comparisons indicated that all treatments significantly impaired this behavior. A separate injection of dicumarol or Mn^{3+} significantly impaired this behavior ($P<.05$ and .001, respectively). However, the simultaneous injection of dicumarol and Mn^{3+} potentiated the impairment in the acquisition of CAR ($P<.0001$). These rats showed a similar response that was observed in those injected with 6-OHDA and a significant lower performance than in rats injected with dicumarol alone ($P<.05$) and Mn^{3+} alone ($P<.05$). No significant differences were observed concerning the footshock thresholds among the different groups (0.25 ± 0.05 mA).

3.3. Immunohistochemistry

Unilateral injection of Mn^{3+} and Mn^{3+} plus dicumarol results in a significant loss of TH immunoreactivity within the nigrostriatal pathway (Fig. 4). Rats receiving unilateral

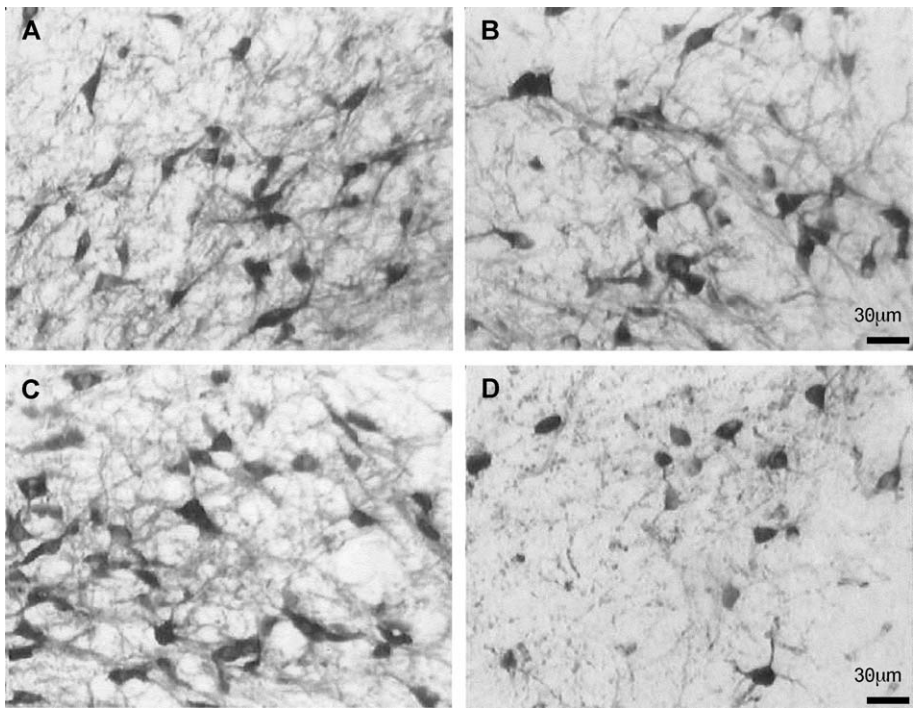

Fig. 4. The effect of Mn^{3+} and dicumarol on dopaminergic neurons in substantia nigra. Photomicrographs showing TH-positive fiber loss in Mn^{3+} (B) and Mn^{3+} plus dicumarol (D) intranigral injected side compared with the contralateral control intact side (A and C; see Section 2). Bar = 30 um.

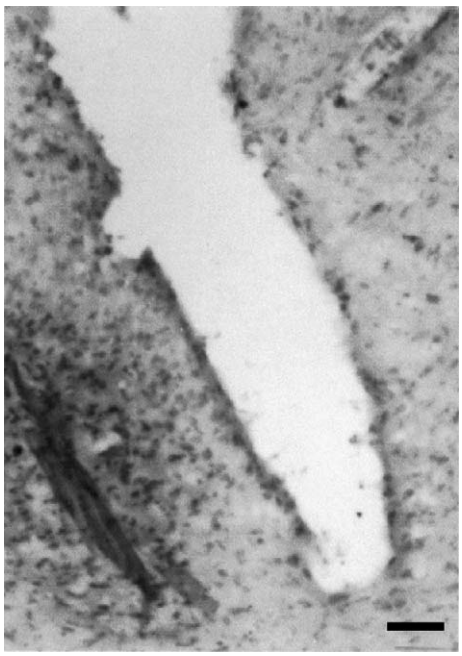

Fig. 5. Photomicrograph of Nissl-stained section showing the dicumarol injection site. Bar = 100 um. Signs of hemorrhage or inflammatory damage around the site of dicumarol injection were not observed by histological analysis using Nissl stain.

intranigral Mn^{3+} and Mn^{3+} plus dicumarol injections exhibited a $61.9 \pm 5.9\%$ ($P < .005$) and $78.2 \pm 5.1\%$ ($P < .005$) reduction in nigrostriatal TH-positive fiber density compared with the contralateral noninjected side. The simultaneous injection of dicumarol and Mn^{3+} potentiated the loss of TH-positive fiber density compared with the injection of Mn^{3+} alone ($P < .05$). The reduction of TH immunoreactivity in rats receiving unilateral injection of dicumarol and 6-OHDA was $22.7 \pm 2.5\%$ ($P < .05$) and $85.1 \pm 4.7\%$ ($P < .005$), respectively. Signs of hemorrhage or inflammatory damage around the site of dicumarol injection were not observed by histological analysis using Nissl stain (Fig. 5).

4. Discussion

The results of the current study support the in vivo neurotoxicity of Mn^{3+} and the contribution of DT-diaphorase inhibition by dicumarol to the behavioral consequences of the nigrostriatal pathway degeneration. In fact, Mn^{3+} injected into the rat substantia nigra decreased spontaneous motor activity, rearing behavior and acquisition of an avoidance response. These effects were potentiated by the concomitant administration of dicumarol; in this condition, they were not significantly different to that induced by the injection of 6-OHDA. The rationality for using Mn^{3+} as a prooxidant agent was to accelerate the autoxidation cascade involving oxidation of endogenous DA to aminochrome and leukoaminochrome *o*-semiquinone radical. One-electron reduction of aminochrome to leukoaminochrome *o*-semiquinone radical is responsible for the generation of reactive species involved in the neurodegenerative process (Segura-Aguilar and Lind, 1989; Segura-Aguilar et al., 1998, 2001; Kostrzewa and Segura-Aguilar, 2002). The brain is an important target of attack for transition metal ions, such as Mn^{3+}, due to its great catecholamine concentration and the high speed of oxidative metabolism catalyzed by these metals (Stokes et al., 1999). The present findings are consistent with a recent study that provided evidence that oxidation of DA to aminochrome appears to be an important mediator of the behavioral consequences of oxidative damage (Díaz-Véliz et al., 2002). Our results confirm the suggestion that the neurodegenerative events in dopaminergic systems depend on overproduction of *o*-quinones in vivo. The autoxidation of DA to aminochrome (Segura-Aguilar et al., 1998, 2001) has been postulated to be a normal process because DT-diaphorase prevent one-electron reduction of aminochrome to leukoaminochrome *o*-semiquinone radical (Baez et al., 1995; Segura-Aguilar et al., 1998; Paris et al., 2001). DT-diaphorase is a flavoprotein found in central DA neurons that prevents the formation of leukoaminochrome *o*-semiquinone radicals by reducing aminochrome with two electrons to leukoaminochrome (Segura-Aguilar and Lind, 1989). Previous in vitro studies have demonstrated that in the rat brain DT-diaphorase is inhibited by dicumarol (Schultzberg et al., 1988; Segura-Aguilar and Lind, 1989; Paris et al., 2001). The dense network of DT-diaphorase-immunoreactive fibers in the striatum disappeared along with the dopaminergic innervation after 6-OHDA lesion (Schultzberg et al., 1988). Leukoaminochrome *o*-semiquinone radical has been reported to be responsible for neurotoxic effects of aminochrome in dopaminergic RCSN-3 cells derived from rat substantia nigra when DT-diaphorase was inhibited by dicumarol (Paris et al., 2001). Recently, we informed that Mn^{3+} administration together with dicumarol into the left medial forebrain bundle produced a behavioral pattern characterized by contralateral rotations when the rats were stimulated with apomorphine. This effect was not observed when Mn^{3+} was administered alone (Segura-Aguilar et al., 2002). The rotational model developed by Ungerstedt et al. (1974) is the most established animal assay to study the unilateral nigrostriatal degeneration. In addition, in the present study, we observed that the concomitant intranigral administration of the DT-diaphorase inhibitor dicumarol potentiates the neurobehavioral effects of Mn^{3+}, suggesting that dicumarol enhanced the Mn^{3+} denervation of the nigrostriatal DA system. These behavioral observations were in concordance with the significant loss of TH immunoreactivity within the nigrostriatal pathway after Mn^{3+} and Mn^{3+} plus dicumarol injection.

Both motor activity and associative learning are dependent on the integrity of the DA nigrostriatal system. Then, degeneration of the nigrostriatal dopaminergic system could led to severe disruption of motor and associative behavior (Dubois and Pillon, 1997; Schneider and Pope-Coleman,

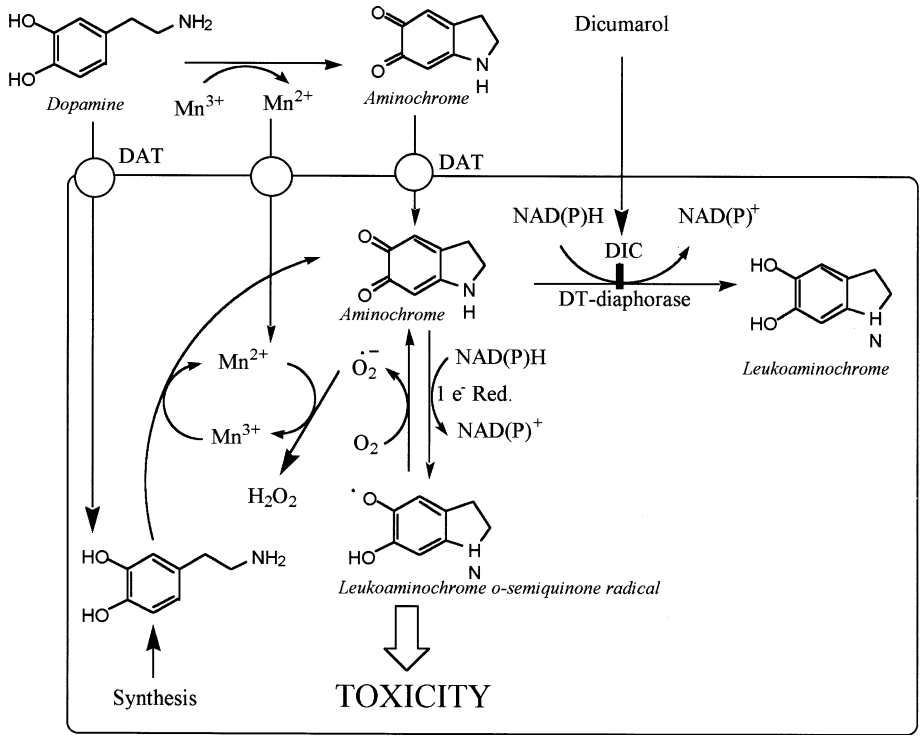

Fig. 6. Possible mechanisms for toxic effects of intracerebral injection of Mn^{3+} together with dicumarol into substantia nigra.

1995; Kulisevsky, 2000). Two-way avoidance is a type of conditioning that results in associative learning. The rat learns to avoid a signaled noxious stimulus (electrical shock) by initiating a locomotor response for moving to another compartment. Then, acquisition of avoidance responses could be altered by changes in locomotor activity. However, the present behavioral data suggest that the influence of intranigral injection on avoidance response was not necessarily a consequence of equivalent changes in spontaneous motor activity. In 6-OHDA intranigral-injected rats, depressed motor activity was clearly accompanied by a decrease in avoidance conditioning. Similar effects were shown by Mn^{3+} plus dicumarol injection and with a lesser effect in Mn^{3+} injection. However, in dicumarol-injected rats, both behaviors were dissociated. In fact, the significant impairment in avoidance behavior was not accompanied by any change in motor activity. In the present study, immunohistochemical assays demonstrate a correlation between percent reduction in nigrostriatal TH-positive fiber density and behavioral consequences. Intranigral injection of dicumarol alone, which induced 22.7% reduction in TH-positive fiber density, led to a significant inhibition of the avoidance acquisition that was not accompanied with any change in spontaneous motor activity. Increased damage of nigrostriatal DA fibers, as observed in Mn^{3+} and Mn^{3+} plus dicumarol intranigral-injected rats, depressed motor activity and decreased avoidance conditioning in 6-OHDA-lesioned rats. These findings led us to suggest that cognitive functions could be more sensitive than motor performance to disruptions in DA nigrostriatal neurotransmission. These data agree with several experimental and clinical investigations of Parkinson's disease, which have shown that cognitive deficit precedes motor impairment (Schneider and Pope-Coleman, 1995; Dubois and Pillon, 1997).

In conclusion, DT-diaphorase inhibition after dicumarol injection is able to induce moderate behavioral changes in the rat and also potentiates the neurotoxic effect of Mn^{3+} when they are injected simultaneously into substantia nigra. These results support the view of neurodegeneration as a consequence of the oxidation of endogenous DA into reactive cytotoxic leukoaminochrome *o*-semiquinone, which is accelerated by metal transition ions like Mn^{3+} (Fig. 6). DA is oxidized to aminochrome by reducing Mn^{3+} to Mn^{2+} (Segura-Aguilar and Lind, 1989), which may react with superoxide radicals to generate hydrogen peroxide and Mn^{3+} (Archibald and Fridovich, 1982). The requirement of the simultaneous injection of Mn^{3+} and inhibition of DT-diaphorase by dicumarol for producing 6-OHDA-like behaviors supports the proposed role of DT-diaphorase as an antioxidant and neuroprotective enzyme of the dopaminergic systems. Thus, the degeneration of DA neurons in parkinsonism may be caused by an imbalance between the factors promoting the oxidation of DA and the availability of neuroprotective defenses.

Acknowledgements

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