

## $\alpha$ -synuclein and tyrosine hydroxylase expression in acute rotenone toxicity

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**Abstract.** Chronic low-dose (2-3 mg/kg/day) rotenone infusion produces clinical features and biological markers of Parkinson's disease (PD) in some rats. A significant proportion of rats, however, die of acute rotenone toxicity. Most studies have focused on chronic rotenone-infused rats. It has not been established if the animals that die of acute low-dose rotenone toxicity manifest clinical or pathological evidence of PD. In the present study, six rats that received continuous 3 mg/kg/day subcutaneous rotenone infusion, became moribund and were euthanized after five days were compared with ten vehicle infused animals sacrificed 14, 28 or 56 days after placebo infusion. All rotenone-infused rats had significant motor function decline beginning one day after the infusion and progressive worsening in the physical condition until they became severely akinetic, at which point they were euthanized. In the substantia nigra of rotenone-treated rats, four of six had reduced numbers of tyrosine hydroxylase-positive neurons and all six had increased nigral  $\alpha$ -synuclein expression. Our observations show that even a short duration of low-dose subcutaneous rotenone infusion can induce clinical and pathological markers of PD in some rats. The pathophysiology of the enhanced susceptibility to PD in some animals remains to be established.

### Introduction

Parkinson's syndrome (PS) is characterized by a combination of bradykinesia, rigidity and tremor. The most common pathological variant of PS is Parkinson's disease (PD) (1-3) which is characterized by marked loss of substantia nigra (SN) dopaminergic neurons and Lewy body inclusions. The

Lewy bodies (LB) are rich in  $\alpha$ -synuclein (4). Mutation of  $\alpha$ -synuclein is known to produce Parkinsonian features and LB pathology (5,6). The loss of dopaminergic neurons and formation of LB inclusions do not go hand in hand as evident by absence of Lewy bodies in 1-methyl-4-phenyl-1, 2, 3, 6 tetrahydropyridine (MPTP) exposed human subjects that have marked loss of SN neurons (5,7,8). Rotenone is a lipophilic compound found in many plants of the leguminosae family, which are widely distributed in the world. After chronic infusion with rotenone, some rats develop clinical features of PD (9). These animals show reduced TH immunoreactivity in the SN and  $\alpha$ -synuclein-positive inclusions in the SN neurons (9,10). There are, however, wide variations in the level of rotenone toxicity in rats. In one study 36% of the rotenone-infused animals died within the first three days while others survived for a long time (10). Studies of PD markers have so far focused on chronic rotenone-infused animals (9-12). To our knowledge there is no information on pathological markers of PD in the animals that die of low-dose rotenone toxicity during the first week of infusion. We report on PD markers in rats that suffered from acute rotenone toxicity on low-dose subcutaneous infusion.

### Materials and methods

**Animals and treatment.** All procedures involving animals were in strict accordance with guidelines established by the Canadian Council on Animal Care, and approved by the University of Saskatchewan Animal Care Committee.

Male Lewis rats, weighing between 344-396 g at the time of surgery, were randomly divided into those that received vehicle (placebo) or rotenone infusion. Alzet osmotic minipumps (model 2ML4, Durect Corporation, Cupertino, CA) (10) were aseptically filled with rotenone dissolved in equal amounts of dimethyl sulfoxide (DMSO) and polyethylene glycol (PEG) (all from Sigma, St. Louis, MO) or with DMSO/PEG vehicle for implantation. The animals were anesthetized with 5% isoflurane in pure oxygen and maintained on 1-2% isoflurane in oxygen during surgery. The minipump was placed subcutaneously in the back using an aseptic technique. Rotenone-treated animals were continuously infused with 3 mg/kg per day (calculated on weight at the time of surgery) and placebo animals received DMSO/PEG at the same rate of fluid infusion as the rotenone-treated rats. Each animal was housed separately in a cage and maintained on a 12-h light

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and dark cycle with free access to food and water. In rotenone-treated animals that did not die or require euthanizing, and the control animals, the pump was replaced with a new pump containing the same fluid on the 29th day (the day when the first surgery was performed is day 0). Throughout the treatment the animals were monitored daily for weight loss and for motor manifestations. During the first 3 weeks they were weighed daily and subsequently every 3 days. When an animal lost more than 5% of the pre-treatment weight, it was given food supplement Ensure (Ross Laboratories, OH, USA) 5-8 ml and/or lactated Ringer's USP solution 10 ml twice daily. When further deterioration occurred such that the animal could not be fed adequately and was unable to ambulate, the institutional guidelines required discontinuing the dietary supplement and the animal was euthanized (13).

Before sacrifice the animal was deeply anesthetized with sodium pentobarbital (Abbott Laboratories, Montreal, Canada), 40 mg/kg, i.p. and was sacrificed by decapitation. The brain was removed immediately and cut into two equal halves along the longitudinal fissure. The left half was stored at  $-70^{\circ}\text{C}$  and the right half was fixed in 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS, pH 7.4) for 2 days and then cryoprotected in 30% sucrose for 2-3 days at  $4^{\circ}\text{C}$ . Finally, the right half of the brain was frozen in 2-methylbutane prechilled with dry ice and then stored at  $-70^{\circ}\text{C}$  until further analysis. The brains from the rotenone-infused rats and those from the placebo-infused rats were processed identically.

**Immunohistochemistry.** Coronal sections of the striatum (40  $\mu\text{m}$ ) and the substantia nigra (30  $\mu\text{m}$ ) were cut on a freezing microtome (Leica SM 2000R) and collected in 24-well plates containing 0.01 M PBS. The detection of TH and  $\alpha$ -synuclein immunoreactivity was performed using conventional avidin-biotin-immunoperoxide technique as described previously with minor modifications (9). Free-floating sections were rinsed three times in 0.01 M PBS and then pretreated with 0.3% hydrogen peroxide in PBS for 30 min to eliminate endogenous peroxidase activity. After rinsing three times in 0.01 M PBS, the sections were pre-incubated at room temperature for 1 h with a blocking solution containing 5% normal horse or goat serum and 0.3% Triton X-100 in 0.01 M PBS. They were then incubated at  $4^{\circ}\text{C}$  with monoclonal mouse anti-TH (1:1000, Chemicon, Temecula, CA, USA) or polyclonal rabbit anti- $\alpha$ -synuclein (1:1000, Sigma) for 48 h and then at room temperature for an additional 2 h. The primary antibodies were localized with a Vectastain ABC kit (Vector Laboratories, Burlingame, CA, USA). The reaction product was visualized with the development solution containing 0.03% DAB and 0.03% hydrogen peroxide in 0.01 M PBS (pH 7.4) for 5-10 min. The sections were mounted onto polylysine-coated slides, air dried, dehydrated, cleared, and coverslipped. For the purpose of immunohistochemical control we performed the procedures as above except we omitted the primary antibodies.

**Quantification of dopaminergic neurons in the substantia nigra.** Every 6th section (from bregma -4.8 to -6.04 mm) of the substantia nigra was immunostained for TH (e.g. section 1, 7, 13) and for  $\alpha$ -synuclein (e.g. section 2, 8, 14). The quantification of TH-positive neurons in the substantia nigra

compacta (SNc) was performed with stereological protocol (14). TH-positive neurons in every 6th section of SNc were counted. The number of TH-positive neurons counted in all sections was multiplied by 6 to obtain the total number of TH positive neurons in SNc. The counting was performed with a  $\times 40$  objective.

**Densitometric analysis.** Every 6th section (from bregma 2.2 to -0.26 mm) of the striatum was immunostained for TH or for  $\alpha$ -synuclein. Digital images were obtained using an Olympus BH2-RFCA microscope fitted with a Spot-RT digital camera (Diagnostic Instruments, Sterling Heights, MI) and analyzed using Image-Pro Plus software (Media Cybernetics, Silver Spring, MD). The measured areas of the striatum (target areas) were determined as follows. The outer border of the striatum was defined by the lateral ventricle medially and by the corpus callosum dorsally and laterally. The areas of the corpus callosum were measured as the areas of background. The measured areas of the substantia nigra (target areas) were throughout the substantia nigra (SN). Peduncle areas were measured as the areas of background. With Image-Pro Plus software, the data were obtained as a gray-scale value of the measured area; i.e. the bigger the number the darker the staining, the smaller the number the lighter the staining. The following formula was used to calculate the percentage difference score (DS) in optical density (15):

$$\frac{\text{Target gray scale} - \text{Background gray scale}}{(\text{Target gray scale} + \text{Background gray scale})/2}$$

**Clinical features.** All rats were videotaped in a round area of 40-cm diameter for 2 min on the day before implanting the pump. After minipump implantation, video tapes were made on the first day, every third day subsequently and on the day of sacrifice or euthanasia. The clinical features were analyzed based on those videotapes.

**Statistics.** Rotenone-treated and placebo rats were compared using Student's t-test. All analyses were carried out using SPSS software (13.0 for Windows, 2004). The level of significance was set at  $P < 0.05$ .

## Results

**Selection of animals for the study.** Fifty animals received rotenone and 17 received placebo infusion. Four (8%) of the rotenone-treated animals were found dead during the first week and 14 others (28%) became moribund and required euthanizing during the first week. Of the latter 14 rats, six were randomly chosen for this study. Ten placebo-treated rats randomly chosen for sacrifice, three after 14 days, three after 28 days and four after 56 days, were used in this study.

**Clinical symptoms of the rotenone-treated rats.** All placebo-treated rats remained healthy after minipump implantation throughout the course of follow up. All six rotenone-treated rats included in this study manifested motor function impairment including unsteady ambulation ( $n=5$ ), walking backwards ( $n=4$ ), slowed mobility ( $n=4$ ), hunched back posture ( $n=4$ ), and circling movement ( $n=3$ ). Some motor impairment was evident one day after infusion which became worse until the

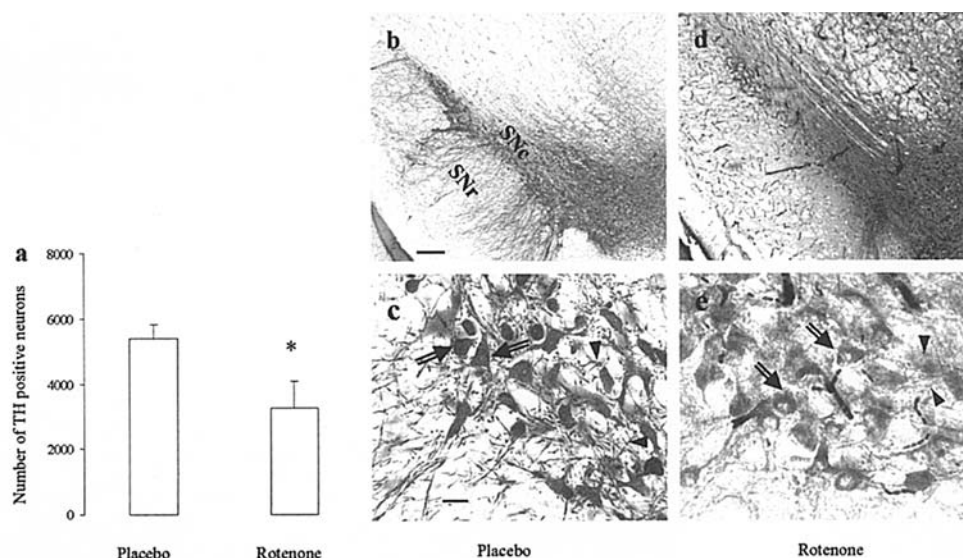


Figure 1. Tyrosine hydroxylase (TH)-positive neurons and fibers in substantia nigra in controls and rotenone-treated animals. Comparison between placebo (n=10) and rotenone (n=6) groups revealed a significantly reduced number of TH-positive neurons in SN pars compacta (SNc) in the rotenone-treated group ( $P<0.05$ ) (a). The data is expressed as mean  $\pm$  SE. Representative photographs are shown in b-e. In placebo rats, TH-positive neurons in SNc and abundant fine TH-positive fibers in substantia nigra pars reticulata (SNr) (b). The outline and processes of TH-positive neurons (indicated by arrows) and neurites (indicated by arrowheads) among neurons were observed (c). (d and e) From rotenone-infused rats. The number of TH-positive neurons in SNc is reduced and TH-positive fibers in SNr are not visible (d). The neurons (indicated by arrows) showed lower intensity of TH immunostaining and indistinct outlines. The debris of neuronal and neurite lysis (indicated by arrowheads) was observed (e). Scale bar=200  $\mu$ m (b) and 20  $\mu$ m (c).

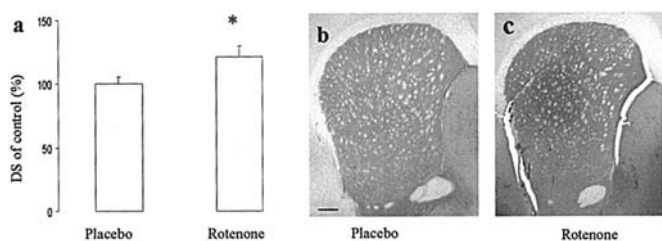


Figure 2. Effect of acute rotenone toxicity on the level of TH in striatum. Comparison between placebo (n=10) and rotenone-treated (n=6) groups revealed significantly increased levels of TH in striatum in the rotenone-treated group ( $P<0.05$ ) (a). The data is expressed as mean  $\pm$  SE. Representative photographs are shown in b and c. The density of TH immunostaining in rotenone-treated rats (c) was higher than that in placebo rats (b) and the round area with the darkest staining was also observed (c). Scale bar, 400  $\mu$ m (b).

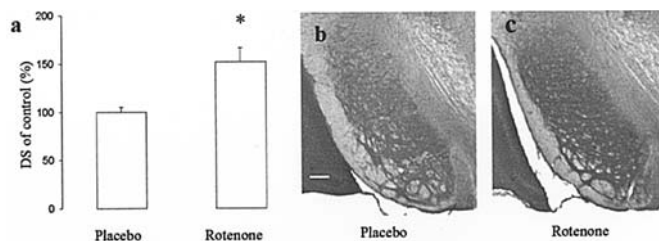


Figure 3. Effect of acute rotenone toxicity on the level of  $\alpha$ -synuclein in SNr. Comparison between placebo (n=10) and rotenone-treated (n=6) groups revealed significantly increased levels of  $\alpha$ -synuclein in SNr in the rotenone-treated group ( $P<0.05$ ) (a). The data is expressed as mean  $\pm$  SE. Representative photographs are shown in b and c. The density of  $\alpha$ -synuclein immunostaining in rotenone-treated rats (c) was higher than that in placebo rats (b). Scale bar, 250  $\mu$ m (b).

rats were severely akinetic and had to be euthanized five days after rotenone infusion. In four of the six rats between the second and fifth day, there was bleeding from the nose and/or hemorrhages in the eyelids.

**Rotenone reduced TH-IR neurons in the SNc.** In rotenone-treated rats, the number of TH-positive neurons in the SNc was reduced to approximately 60% that of controls ( $P<0.05$ ) (Fig. 1a). The representative photographs of SN sections of rats are shown in Fig. 1b-e. In the placebo-treated rats, TH-positive neurons were concentrated in the SNc and dense, fine TH-IR fibers were observed in the substantia nigra pars reticulata (SNr) (Fig. 1b). The outline of the SNc neurons was clear and TH-IR neurites were observed interspersed between them (Fig. 1c). In rotenone-treated rats, the TH-positive neurons were significantly reduced in SNc and the fine TH-IR positive fibers were not visible in the SNr (Fig. 1d). Additionally, the

outline of the TH-positive neurons was indistinct, there was loss of TH-IR neurites and TH staining was reduced in the SNc neurons (Fig. 1e).

**Effect of rotenone on TH immunostaining density in the striatum.** The rotenone-treated rats as a group had increased striatal TH immunostaining density (Fig. 2a). Representative photographs are shown in Fig. 2b and c. In the rotenone-treated animals, the TH staining density in the striatum was higher than that in the placebo group (Fig. 2b and c).

**Rotenone increased  $\alpha$ -synuclein immunostaining density in the substantia nigra.**  $\alpha$ -Synuclein immunostaining density in the SN was significantly increased in the rotenone-infused rats (Fig. 3a). Fig. 3b shows the  $\alpha$ -synuclein immunoreactivity in the placebo-treated rats. Fig. 3c shows  $\alpha$ -synuclein staining in the rotenone-treated rats.



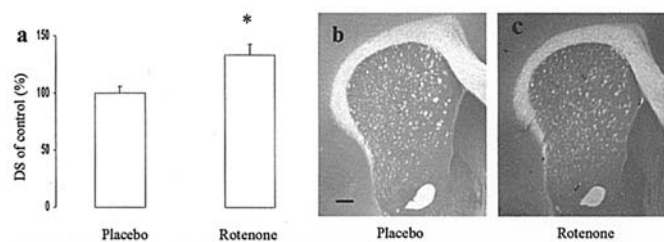


Figure 4. Effect of acute toxicity on the level of  $\alpha$ -synuclein in striatum. Comparison between placebo ( $n=10$ ) and rotenone-treated ( $n=6$ ) groups revealed significantly increased levels of  $\alpha$ -synuclein in striatum in the rotenone-treated group ( $P<0.05$ ) (a). The data is expressed as mean  $\pm$  SE. Representative photographs are shown in b and c. The density of  $\alpha$ -synuclein immunostaining in rotenone-treated rats (c) was higher than that in placebo rats (b). Scale bar, 0.8 mm (b).

**Rotenone increased  $\alpha$ -synuclein staining density in the striatum.** Rotenone-infused rats had significantly increased  $\alpha$ -synuclein immunostaining density in the striatum (Fig. 4a). Representative photographs are shown in Fig. 4b and c. The immunostaining density was higher in rotenone-treated rats (Fig. 4c) than the control rats (Fig. 4b). Despite increased immunostaining there was no aggregation of  $\alpha$ -synuclein into Lewy bodies.

## Discussion

All ten rats infused with vehicle placebo remained healthy throughout the study. By contrast, all six rotenone-infused rats included in the study had impaired motor function resembling PD features that manifested one day after infusion followed by progressive motor decline requiring euthanizing during the first week. Thus the motor disability was attributable to rotenone and not to the insertion of the pump or the vehicle used to dissolve rotenone.

High-dose rotenone (10–18 mg/kg/day) infusion for one week has been reported to produce neuronal loss in the striatum and globus pallidus, but the SN neurons were spared (16). Individual variations in the response to rotenone infusion have been well-studied in rats (8–10,12), but the reasons for them remain unknown. In chronic low dose (2–3 mg/kg/day) rotenone infusion TH-IR neuronal loss in the SN has been reported in several studies (9–11,17,18). While some reports indicate selective dopaminergic degeneration and  $\alpha$ -synuclein aggregation (10), others found more widespread pathological changes comparable to multiple system degeneration (18).

To our knowledge, there is no literature on PD markers in animals that died early on low-dose (2–3 mg/kg/day) rotenone infusion. We restricted the study to only those rats in which we could identify the time of death (euthanizing) precisely and excluded the rats that died naturally at an unknown time. The two major findings in our rats were: i) the damage to the TH-IR neurons; and ii) the increased  $\alpha$ -synuclein expression in the substantia nigra. The substantia nigra TH-positive neurons manifested some degree of pathology in every rotenone-infused animal in our study. The majority (four of six) had marked loss of TH-positive SNc neurons and reduced number of TH-positive fibers (Fig. 1d and e). The two rotenone-treated animals that had normal TH-positive neuronal count also demonstrated pathological evidence characterized by swollen

neurons and reduced number of TH-positive fibers (photograph not shown). The loss of TH-positive neurons in the SNc paralleled the loss of TH-positive neuropil in the SNr. Whether the dysfunction of the neurons resulted in anterograde degeneration of the neuropil or the damage to neurofibers resulted in retrograde degeneration of neurons as suggested by some studies (9,12) cannot be resolved by the present study.

Chronic rotenone toxicity may show no reduction in TH-IR immunostaining, patchy focal loss or diffuse loss of TH-IR fibers in the striatum (10,12,19,20). We noted similar variations in striatal TH staining. The four rotenone-treated rats that had lost neurons in the SNc showed no elevation in striatal TH staining compared with placebo animals, though the TH staining was increased in rotenone-treated animals as a group. We observed circumscribed areas with higher TH staining in the striatum of an animal that had normal TH-positive neuronal count in SNc (Fig. 2c). The reasons for these variations remain to be elucidated.

Partial damage to the nigrostriatal dopamine (DA) system can cause acute loss of DA innervation and induce compensations for that loss in the striatum (21). Compensatory responses include the increase of TH synthesis, increase of DA release and turnover, and decrease of DA uptake (21). In the present study, however, there was no change downstream in the striatal TH immunostaining when there was loss of nigral neurons.

Lewy body (LB) inclusions are considered as the hallmark of Parkinson's disease pathology (5,22–24).  $\alpha$ -synuclein is the major component of Lewy bodies and Lewy neurites (4,25). Chronic low-dose rotenone infusion can produce  $\alpha$ -synuclein aggregation in SN neurons (9–11) and *in vitro* studies show increased  $\alpha$ -synuclein expression in human dopaminergic (SH-SY5Y) cells after 24-h rotenone exposure (26). We evaluated  $\alpha$ -synuclein expression with immunohistochemical quantification, which revealed increased immunostaining for  $\alpha$ -synuclein in both the substantia nigra (SNr) and striatum in all rotenone-treated animals that became moribund and were sacrificed within one week of infusion. However,  $\alpha$ -synuclein aggregation, i.e. Lewy body formation, was not found in nigral neurons in our study. The pathophysiological significance of  $\alpha$ -synuclein aggregation remains controversial (27,28). Three possibilities have been postulated regarding  $\alpha$ -synuclein aggregation: i) it is directly toxic to the cell; ii) it is an inert marker of an underlying metabolic defect; or iii) it is a protective reaction (28). Increased  $\alpha$ -synuclein staining without aggregation may be a precursor for Lewy body formation. The true significance of increased  $\alpha$ -synuclein staining with or without aggregation remains to be determined.

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