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Intranasal Administration of Rotenone to Mice Induces Dopaminergic Neurite Degeneration of Dopaminergic Neurons in the Substantia Nigra.

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Note

Intranasal Administration of Rotenone to Mice Induces Dopaminergic Neurite Degeneration of Dopaminergic Neurons in the Substantia Nigra

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Exposure to environmental neurotoxins is suspected to be a risk factor for sporadic progressive neurodegenerative diseases. Parkinson's disease has been associated with exposure to the pesticide rotenone, a mitochondrial respiration inhibitor. We previously reported that intranasal administration of rotenone in mice induced dopaminergic (DA) neurodegeneration in the olfactory bulb (OB) and reduced olfactory functions. In the present study, we investigated the DA neurons in the brains of mice that were administered rotenone intranasally for an extended period. We found that the olfactory function of mice was attenuated by rotenone administration. Electrophysiological analysis of the mitral cells, which are output neurons in the OB, revealed that the inhibitory input into the mitral cells was retarded. In the immunohistochemical analysis, neurite degeneration of DA neurons in the substantia nigra was observed in rotenone-administered mice, indicating that rotenone progressively initiated the degeneration of cerebral DA neurons *via* the nasal route.

Key words dopaminergic neuron; intranasal administration; olfactory bulb; rotenone; substantia nigra

Environmental chemicals are suspected to be risk factors for neurodegenerative diseases.[1] One case control study indicated an association between Parkinson's disease (PD) and the use of a group of pesticides that inhibit mitochondrial complex I, which included rotenone and paraquat.[2] PD is characterized by degeneration of dopaminergic (DA) neurons in the substantia nigra pars compacta (SNpc).[3] DA neurons are vulnerable to reactive oxygen species (ROS) generated by aberrant mitochondrial respiration.[4] Subcutaneous chronic infusion or direct brain infusion of rotenone induces DA neurodegeneration in the substantia nigra (SN) and features of PD in rats, suggesting that exposure to neurotoxins such as rotenone is a risk factor for PD.[5,6]

In general, exposure of the human brain to environmental chemicals occurs following the intake of such chemicals by ingestion, cutaneous contact, or inhalation. Various chemicals absorbed at the olfactory mucosa reach the brain directly through passive diffusion from the extra-neural space of olfactory nerves to the cerebrospinal fluid and/or by the active axonal transport of olfactory neurons.[7,8] Prediger *et al.* postulated that some forms of PD may be caused by environmental agents delivered to the brain *via* the olfactory mucosa.[9]

PD patients often suffer dysosmia as their first symptom.[10] In the olfactory bulb (OB), a primary center for olfactory information processing, DA neurons function as inhibitory interneurons increasing the dynamic range of information transfer from olfactory receptor neurons to OB neurons[11] and maintaining the odor discriminating ability.[12] Huisman *et al.* reported that DA neurons in the OB increased in postmortem autopsy of PD patients, suggesting an increase in inhibitory inputs of dopamine into olfactory nerve terminals could reduce olfactory functions.[13] We used one-week rotenone administration to the nasal cavity of mice to mimic inhalation exposure to an environmental neurotoxin, and found that the number of DA neurons in the OB was decreased and the olfactory function attenuated by this treatment.[14] Olfactory discrimination deficits in mice lacking the D2 dopaminergic receptor suggest that decreases in DA neurons in the OB also could induce olfactory dysfunctions.[12] However, it has not been ascertained whether the influence of intranasally administered rotenone is limited to the OB.

In the present study, we investigated the progression of the neurotoxic influence of intranasally administered rotenone on the brain for a longer period than in our previous study. To confirm the influence of rotenone in the OB, we performed an olfactory-related behavior test and conducted an electrophysiological analysis of output neurons in the OB after exposure for two weeks. We then investigated the effects of rotenone on DA neurons in other brain regions by performing immunohistochemical staining for tyrosine hydroxylase (TH), a key enzyme in the production of dopamine.

MATERIALS AND METHODS

Mice All animal experiments were conducted in accordance with the Asahikawa Medical University Guidelines for the Use of Laboratory Animals and were approved by the committee of Asahikawa Medical University for Animal Care and Use (approval ID: 14090). The mice (20–25-week-old female BALB/c mice) were housed at room temperature (24–26°C) under a 12-h light/dark cycle. Rotenone (Sigma-Aldrich, St. Louis, MO, U.S.A.) was dissolved in dimethyl sulfoxide (DMSO) to make a 0.1 M stock solution. To prevent hydrolytic degradation, the rotenone stock solution was diluted with water just before administration to mice. As described previously,[14] a rotenone dilution (rotenone: 0.35 mg/kg; DMSO: 9.86 mg/kg) or vehicle (DMSO: 9.86 mg/kg) was injected into the right-side nasal cavity of mice. These single administrations were given once a day for 2 weeks.

Avoidance of Butyric Acid The odor preferences of the mice were tested in a Plexiglas Y-maze apparatus (long-arm length: 45 cm; short-arm length: 40 cm; arm width: 10 cm) as previously described.[14] A 20-μL aliquot of butyric acid (undiluted or with 25% diluent) or water absorbed in a piece of

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filter paper in a petri dish was placed at the end of each short arm of the maze. Clean air was supplied from the end of each short arm to the end of the long arm. A mouse was initially placed at the end of the long arm, and the duration that the animal spent in each short arm during a 4-min test period was recorded. The "preference ratio" was defined as the ratio of time spent in the butyric acid arm to time spent in both short arms.

Electrophysiological Analysis of Mitral Cells The OBs were dissected out from mice under deep anesthetization with pentobarbital sodium (150 mg/kg). The OBs were cut into parasagittal slices at a thickness of 200 μm with a microslicer. The electrophysiological properties of the mitral (MT) cells were compared in OB slices of the controls and the rotenone-administered mice. Whole-cell patch-clamp recordings were performed as previously described.[14] Action potentials and voltage-gated currents of the MT cells were measured in Ringer's solution (125 mM NaCl, 2.5 mM KCl, 26 mM $NaHCO_3$, 1.25 mM NaH_2PO_4, 2 mM $MgCl_2$, 1 mM $CaCl_2$, 11 mM glucose, pH 7.4) saturated with 95% O_2 and 5% CO_2 mixed gas by using glass electrodes filled with K-gluconate intracellular solution (140 mM K-gluconate, 2 mM $MgCl_2$, 2 mM ATP, 0.5 mM ethylene-glycol bis(2-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), 10 mM *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid (HEPES), pH 7.2/KOH). Stepwise current injection was performed on 9 cells from each of the control and rotenone-administered mice. For the measurement of spontaneous inhibitory postsynaptic currents (sIPSCs), antagonists of glutamate receptors (20 μM D(−)-2-amino-5-phosphonovaleric acid and 20 μM 6-cyano-7-nitroquinoxaline-2,3-dione) and voltage-gated sodium channels (1 μM tetrodotoxin) were added to Ringer's solution to block the excitatory activity of MT cells. Glass electrodes were filled with CsCl intracellular solution (115 mM CsCl, 15 mM NaCl, 2 mM $MgCl_2$, 2 mM ATP, 0.5 mM EGTA, 10 mM HEPES, pH 7.2/CsOH) including 10 mM tetraethylammonium chloride, a voltage-gated potassium channel blocker. Spontaneous events of the control (n=748) and rotenone-administered mice (n=484) were pooled from 7 and 13 cells, respectively.

Immunohistochemistry Under deep anesthesia with ketamine and xylazine, mice were processed by cardiac perfusion with phosphate buffered saline (PBS) and 4% paraformaldehyde in PBS. The brain was dissected out and cut into slices at a thickness of 50 μm with a microslicer. For the fluorescence detection of TH-positive dopaminergic neurons, parasagittal sections were stained with anti-TH monoclonal antibody (mAb) (1 : 2000; Sigma-Aldrich) and Alexa Fluor 488 Goat anti-mouse immunoglobulin G (IgG) antibody (1 : 1000; Life Technologies, Carlsbad, CA, U.S.A.) as described previously.[14] The sections were mounted onto slide glass using ProLong Diamond Antifade Mountant with 4′-6-diamidino-2-phenylindole (DAPI) (Life Technologies). Fluorescent images were obtained using a BX51 fluorescence microscope and a DP72 camera (Olympus, Tokyo, Japan).

For colorimetric immunostaining against TH, the coronal sections were stained with anti-TH antibody (1 : 10000), biotinylated goat anti-mouse IgG antibody (1 : 1000; Vector Laboratories, Burlingame, CA, U.S.A.), and ABC (Elite ABC kit; Vector Laboratories) as described previously.[14] The colorimetric chemical reaction was developed with 3,3′-diamino-benzidine (DAB)/H_2O_2 (0.05% DAB and 0.003% H_2O_2 in 0.05 M Tris–HCl buffer, pH 7.6) for 2 min. The images of TH-immunoreactive neurons were obtained using a BX51 microscope and a DP72 camera. The integrated value of TH-immunoreactive signal intensity in the rectangular grid (50×500 μm) from the SNpc to the ventral end of the substantia nigra pars reticulata (SNr) was measured using Image-J software (National Institutes of Health, U.S.A.). After subtracting the values from the ventral end of the SNr as background, the signal values from the SNr were normalized by the values from the SNpc. The average value was calculated from 3 sections from an individual mouse. The average values from 3 of the control and 3 of the rotenone-administered mice were subjected to the statistical analysis.

Statistical Analysis The data on odor preference ratio and relative signal intensity against anti-TH antibody were compared by ANOVA with Tukey *post-hoc* testing. For the cumulative probabilities, the statistical comparison of the distribution was performed by Kruskal–Wallis test followed by Scheffe's multiple comparison.

RESULTS

Rotenone Administration Attenuated TH-Immunoreactivity in the Brain DA neurons are more vulnerable than other neurons to mitochondrial respiration inhibitors such as rotenone.[5,15] To investigate the effects of rotenone on DA neurons in the brain, parasagittal sections were immunostained with anti-TH antibody two weeks after intranasal administration. In the section from the control mouse, DA neurons were clearly stained in the OB and SNpc (Fig. 1A). In addition, the striatum and the SNr were also stained with anti-TH antibody depending on the axonal projections from the DA neurons in the SNpc.[16] Similar to the previous results obtained in mice one week after intranasal rotenone administration,[14] TH-positive signals in the OB were diminished by rotenone administration (Fig. 1B). In addition, TH-positive signals in the striatum and

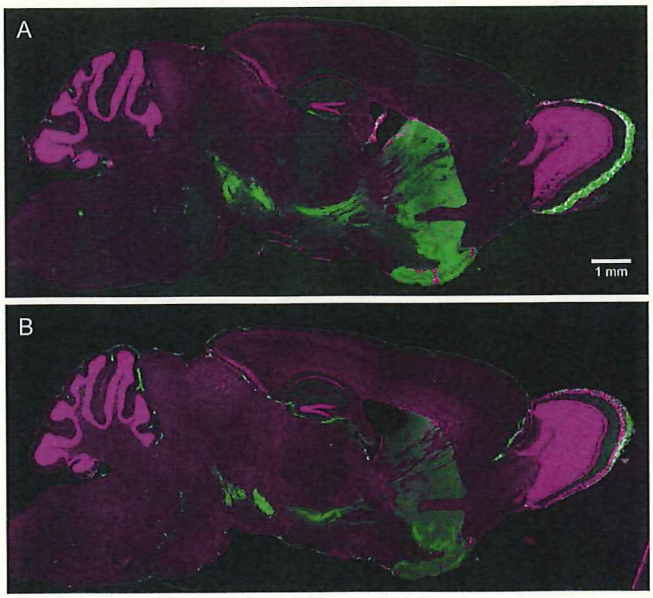

Fig. 1. Immunostaining of DA Neurons in the Brain

Sagittal sections of the brains from control (A) and rotenone-administered mouse (B) are shown. Green and magenta indicate TH-positive signals and DAPI, respectively. Similar results were obtained in five independent experiments.

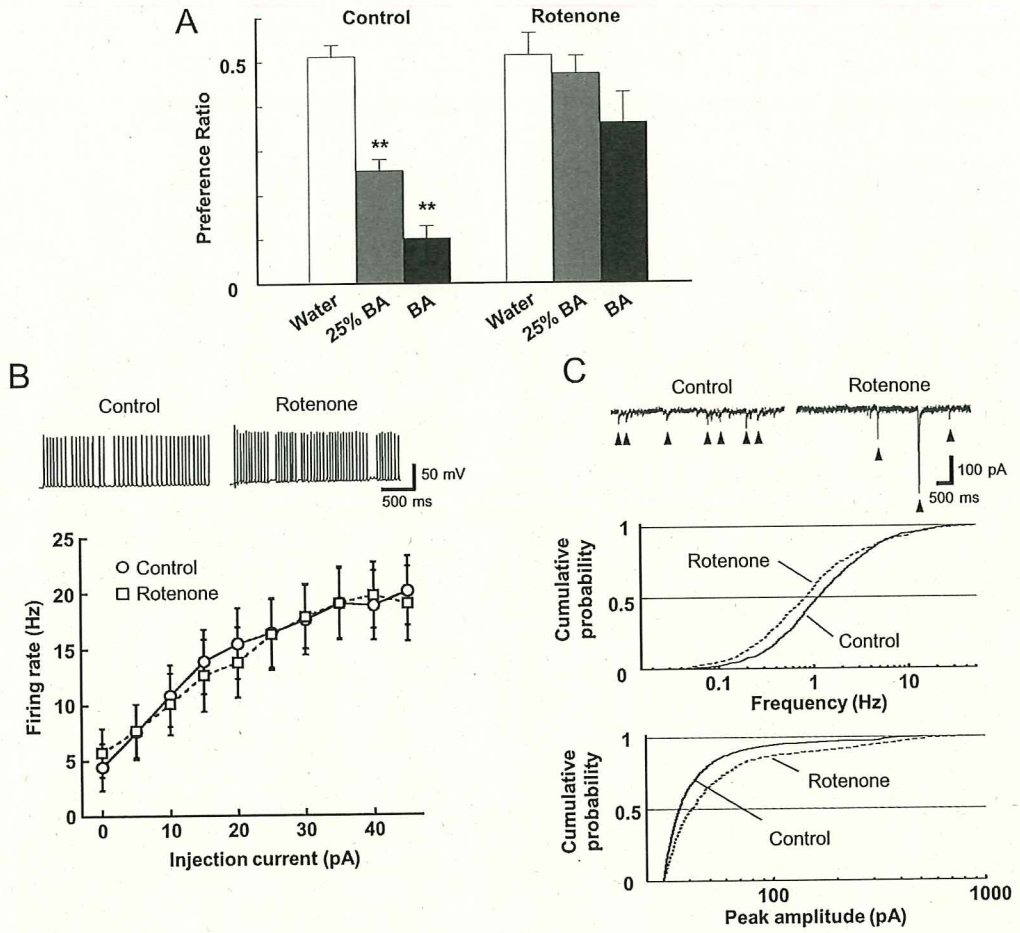

Fig. 2. The Intranasal Administration of Rotenone Attenuated Olfactory Functions

(A) The mice administered rotenone exhibited avoidance of butyric acid. The combinations of odorants in the short arms of the maze were water–water (white column), water/25% dilution of butyric acid (25% BA; gray column), or water–undiluted butyric acid (BA; black column). Both control mice (n=10) and rotenone-administered mice (n=12) were subjected to the odor preference test. Data are the mean±standard error (S.E.). **p<0.01 *vs.* water, Tukey *post-hoc* test. (B) The excitability of the MT cells. Twenty pico ampere current injection elicited the firing of action potentials (top). The firing rate of the MT cells from control (circle) and rotenone-administered mice (square) in response to the indicated injected current are shown (bottom). (C) sIPSCs recorded from the MT cells (top; arrowheads). Cumulative probabilities of sIPSC frequencies calculated in the controls (solid line) and the rotenone-administered mice (broken line; middle). The curve shift to the left reflects the decrease of frequency (p<0.01). Cumulative probabilities of sIPSC amplitudes (bottom). The shift to the right reflects an enhancement of the amplitude (p<0.01). The symbols are the same as in the middle panel.

SN were also attenuated by rotenone administration.

Intranasally Administered Rotenone Attenuated Olfactory Function We previously demonstrated that mice administered rotenone *via* the nasal cavity for one week did not avoid an aliquot of 25% butyric acid in the Y-maze arm, whereas they continued to avoid an aliquot of 100% butyric acid.[14] Then, we again employed an odor preference test using butyric acid to explore whether two weeks of rotenone administration would aggravate the olfactory dysfunction. The data from each group were cast into a two-factor ANOVA with administration (control or rotenone) and concentration of butyric acid as factors. This analysis revealed a significant effect of administration (F(1, 20)=16.858, p<0.01). Tukey *post-hoc* testing revealed that the control mice significantly avoided the smell of butyric acid both at the 25 and 100% concentrations (p<0.01; Fig. 2A). Rotenone-administered mice avoided neither the 25% nor the 100% butyric acid (p=0.982, 0.137), indicating that rotenone administration reduced the avoidance of butyric acid.

Rotenone Administration Altered the Inhibitory Input into Mitral Cells in the Olfactory Bulb To investigate the functional alteration of the neurons in the OB of rotenone-administered mice, the electrophysiological properties of the MT cells, which are the main glutamatergic output neurons in the OB, were analyzed. Analysis of the excitability of the MT cells revealed that frequent firing of action potentials was elicited by stepwise current injection in both the control and rotenone-administered mice (Fig. 2B, top). No statistically significant differences in firing frequency were observed between the controls and rotenone-administered mice (Fig. 2B, bottom). Thus the data suggest that intranasal rotenone administration did not affect the excitability of the MT cells even when rotenone was administered for a longer period. Because DA neurons in the glomerular layer of the OB are thought to be inhibitory interneurons for the MT cells,[17,18] we next recorded the sIPSCs of the MT cells under pharmacological suppression of the excitatory postsynaptic current. The sIPSCs recorded from the MT cells of the rotenone-administered mice showed lower frequency and larger amplitudes than those of the control mice (Fig. 2C, top). The distribution of the sIPSCs frequency recorded from rotenone-administered mice was shifted toward a lower frequency than that recorded from the controls (p<0.01; Fig. 2C, middle), implying a reduction in the number of inhibitory synaptic inputs. Statistically significant

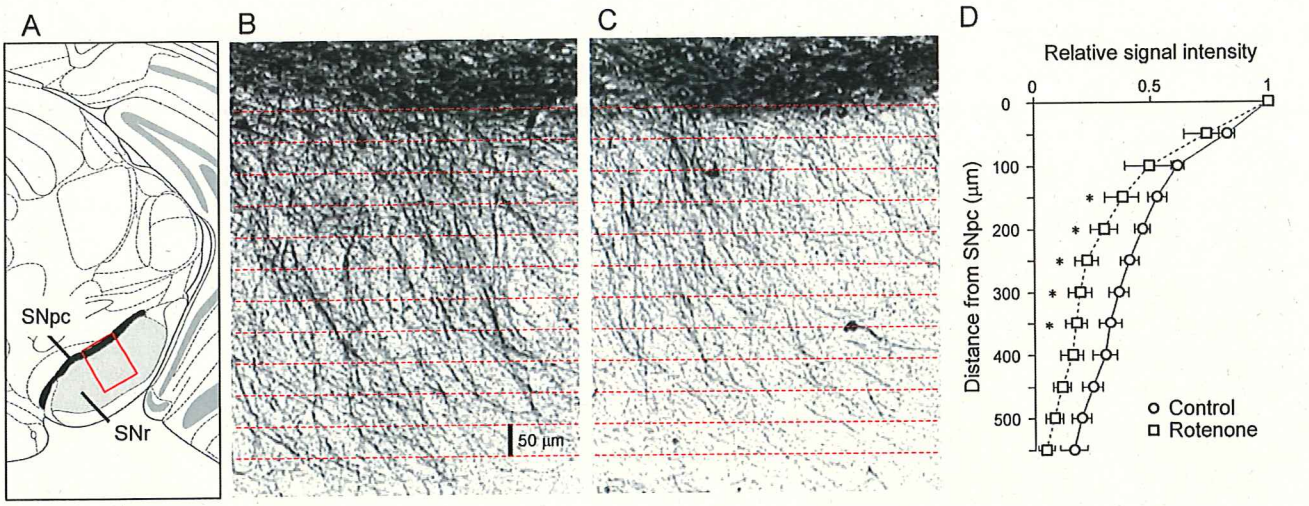

Fig. 3. Immunostaining of DA Neurites in the SN

(A) The red square on the mouse brain atlas[21] (bregma −3.08 mm) indicates the region subjected to colorimetric staining. (B, C) Colorimetric anti-TH immunostaining in the SN of control and rotenone-administered mice, respectively. Red broken lines indicate the center position of rectangular grids that were subjected to signal intensity measurement. (D) Relative TH-immunoreactive signal intensity in the SN of controls (circle) and rotenone-administered mice (square). Data are the mean±S.E., n=3. *p<0.05, Tukey *post-hoc* test.

increases in the peak amplitude of sIPSCs from rotenone-administered mice were confirmed by the shift of the probability distribution (p<0.01; Fig. 2C, bottom). It is possible that rotenone altered the inhibitory modulation for the MT cells by decreasing number of DA neurons.

The Degeneration of Dopaminergic Neuronal Neurites in the Substantia Nigra Axons and dendrites often degenerate prior to the loss of the cell bodies in neurodegeneration.[19] To quantify the degree of rotenone-induced neurite degeneration of DA neurons in the SNr, ABC-enhanced colorimetric staining using anti-TH antibody was performed on coronal sections of the SN. In the SNr, TH-positive shaggy dendrites from SNpc were observed in the control mice (Fig. 3B). In contrast, they were attenuated in rotenone-administered mice (Fig. 3C). The relative signal intensity decreased with an increase in distance from the edge of the SNpc both in the control and rotenone-administered mice, and the signal intensity was larger in control mice than rotenone-administrated mice (Fig. 3D). The data from the rectangular grid on the SN were cast into a two-factor ANOVA with administration (control or rotenone) and position (rectangle grid from the SNpc to the ventral SNr) as factors. This analysis revealed a significant main effect of administration (F(1, 48)=37.862, p<0.0001). Tukey *post-hoc* testing revealed that rotenone administration reduced the TH-positive signal intensity in the SNr (p<0.05). Thus these data suggest that intranasal rotenone administration induced DA neurite degeneration in the SNr.

DISCUSSION

In this study, we investigated the effects of longer-term (two weeks *versus* one week in our previous study) intranasal administration of rotenone on the olfactory functions and TH-positive neurons in mice. We found that rotenone impaired olfactory function as assessed by the avoidance of butyric acid in a Y-maze apparatus. The electrophysiological analysis revealed that rotenone influenced the inhibitory input for the MT cells, suggesting that rotenone caused a disturbance of the olfactory information processing in the OB. The TH-immunoreactivity in the OB was diminished by rotenone administration. Moreover, rotenone reduced TH-immunoreactivity in the SNr, indicating neurite degeneration of DA neurons in the SNpc.

The effects of rotenone on the DA neurons in rodents are controversial. Infusion of rotenone to the brain has been shown to decrease the number of TH-positive neurons in the SN of mice,[6] while intranasally administered rotenone did not reduce the numbers of TH-positive neurons in mice and rats.[20] DA neurons located in the SNpc innervate not only the striatum, but also the SNr.[16] Degeneration in neurites is an early pathological feature of many neurodegenerative disorders.[19] Our present observation of neurite degeneration at the SNr induced by intranasally administered rotenone suggests that rotenone initiates the degeneration of DA neurons at the SNpc. Our results using an intranasal rotenone administration model thus suggest that dysosmia arises through a progression of the degeneration of DA neurons induced by inhaled-neurotoxins.

The pathological effects on the OB induced by intranasally administered rotenone in mice are not the same as those seen in PD patients. In the OB of postmortem PD patients, the total number of TH-positive neurons is higher than in controls.[13] However, intranasally administered rotenone mainly reduced the density of TH-positive neurons in the mouse OB and olfactory functions as well as TH-positive neurites in the SNr. A previous HPLC analysis revealed that, 10 min after intranasal administration, rotenone was present in the OB and also at a very small level in the ventral midbrain of mice.[20] Therefore, it is possible that rotenone administered intranasally induces a severe degeneration of dopaminergic neurons in the OB. Further case study will be needed to explore the number of TH-immunoreactive neurons in the OB of PD patients with a history of exposure to rotenone.

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Conflict of Interest The authors declare no conflict of interest.

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