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Research report

The degeneration of dopaminergic synapses in Parkinson's disease: A selective animal model

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HIGHLIGHTS

- This is an animal model of the initial stages of Parkinson's disease.
- This model induces a selective dopamine denervation/reinervation of the striatum.
- This model is suitable to study the early role of astrocytes on Parkinson's disease.
- This model is suitable to study the dopamine action on the subventricular zone.

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ABSTRACT

Available evidence increasingly suggests that the degeneration of dopamine neurons in Parkinson's disease starts in the striatal axons and synaptic terminals. A selective procedure is described here to study the mechanisms involved in the striatal denervation of dopaminergic terminals. This procedure can also be used to analyze mechanisms involved in the dopaminergic re-innervation of the striatum, and the role of astrocytes and microglia in both processes. Adult Sprague-Dawley rats were injected in the lateral ventricles with increasing doses of 6-hydroxydopamine (12–50 μg), which generated a dose-dependent loss of dopaminergic synapses and axons in the striatum, followed by an axonal sprouting (weeks later) and by a progressive recovery of striatal dopaminergic synapses (months later). Both the degeneration and regeneration of the dopaminergic terminals were accompanied by astrogliosis. Because the experimental manipulations did not induce unspecific damage in the striatal tissue, this method could be particularly suitable to study the basic mechanisms involved in the distal degeneration and regeneration of dopaminergic nigrostriatal neurons, and the possible role of astrocytes and microglia in the dynamics of both processes.

1. Introduction

There is increasing evidence suggesting that the degeneration of dopamine neurons (DAn) in Parkinson's disease (PD) starts in the axon and synaptic terminals and progresses retrogradely to the cell somata in the substantia nigra (SN) (dying-back degeneration). At the time of onset of motor disturbances, more than 70% of dopamine (DA) [1–3], and more than 50% of the tyrosine hydroxylase (TH), DA transporter (DAT) and vesicular monoamine transporter (VMAT2) proteins have been lost in the striatum [4–7]. However, less than 30% of nigral DA cells (compared to aged-matched controls) is lost at this time [1,8–10]. Similar findings have been reported in patients with a medium (3–5 years after the onset of motor disturbances) [11] or long [3,7,12] PD evolution, which also show a more profound DAergic alteration in the striatum than in the SN. Mechanisms involved in the early deterioration of synapses and axons of DAergic neurons in PD are still little known [10,13–15]. Available data suggest that the retrograde degeneration of DAergic cells involves a decline of the axonal trafficking of proteins and mitochondrias [16,17], followed by the aggregation of proteins (e.g. α-synuclein), the accumulation of organelle cargos [18] and the formation of axonal spheroids [19,20]. These axonal disorders are probably modulated by surrounding astrocytes [21,22] and microglia [23], but the actual influence of these cells on the degeneration and regeneration of striatal DAergic synapses is still poorly understood [24–27].

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Available animal models of PD have limitations when it comes to studying the mechanisms involved in the dying-back degeneration of dopaminergic (DAergic) neurons [28–30]. These mechanisms are usually studied by the striatal injection of 6-hydroxydopamine (6OHDA), which after being taken-up by the DAT of the DAn blocks the complex 1 of the synaptic and axonal mitochondrias of this cell and induces its retrograde degeneration [31–35] and the activation of surrounding astrocytes and microglia [36–40]. We began using this method, but two methodological difficulties led us to look for another procedure. The injection of 6OHDA in the striatal tissue causes an unspecific degeneration of non-DAergic striatal cells, which is associated with the penetration of the needle, the hydrostatic pressure generated by the toxic injection and the high 6OHDA-concentration around the needle tip. Because astrocytes and microglia are very sensitive to unspecific local damage [41,42], the selective effect of 6OHDA on the DAn may be masked by the unspecific activation of microglial and astrocytic cells generated by the injection procedure. Previous studies injecting 6OHDA in the cerebrospinal fluids [29,30,43–47] suggested the possibility of introducing the toxic across the brain ventricle, thereby preventing the unspecific effect induced by the injection in the striatal tissue. The present work shows that this method is a selective procedure to study the mechanisms involved in the dying-back degeneration of DAn axons, also providing the possibility of analyzing the mechanisms involved in the dopaminergic re-innervation of the striatum, and the involvement of astrocytes and microglia in both processes.

2. Materials and methods

2.1. Animals and lesions

Experiments were carried out on male Sprague-Dawley rats weighing 300–350 g. Animals were housed at 22 °C, two per cage, under normal laboratory conditions on a standard light-dark schedule with free access to food and water. Experiments were conducted in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC) regarding the care and use of animals for experimental procedures and adequate measures were taken to minimise pain and discomfort.

Rats were anaesthetised with ketamine (25–40 mg/kg i.p.; Rhône Mérieux; Lyon, France) and xylazine (3–6 mg/kg i.p.; Bayer, Leverkusen, Germany), and injected in the lateral ventricle (Kopf Instruments, Tujunga, California; coordinates: 1.4 mm lateral to the midline, 0.8 mm posterior to bregma and 4 mm below the dura) with vehicle (0.9% saline solution with 0.3 μg/μl ascorbic acid) or a single dose of 6OHDA (6-hydroxydopamine hydrochloride, Sigma, St. Louis, MO, 12–50 μg in 10 μl of vehicle per injection; 1 μl/min). Some rats ($n = 4$) were injected with 6OHDA in the striatum (8 μg in 4 μl; 1 μl/min). In order to prevent the degeneration of noradrenergic cells, the noradrenaline uptake was inhibited with nortriptyline (30 mg/kg injected i.p. 20 min before 6-OHDA administration; nortriptyline hydrochloride, Sigma, St. Louis, MO).

2.2. Tissue processing

Rats were anaesthetized with chloral hydrate (400 mg/kg i.p.) and transcardially perfused with 200 ml of 0.9% saline solution followed by 400 ml of 4% paraformaldehyde in 0.1 M phosphate buffer pH 7.4 (PBS) 4 h, 1 day, 5 days, 3 weeks and 6 months after 6-OHDA administration, [48]. Brains were removed and stored in the same fixative at 4 °C for 4 h, immersed in a cryoprotective solution of 30% sucrose in the same buffer for 48 h and then cut at 30 μm with a sliding microtome (HM 450, MICROM International GmbH; Walldorf). Brains were cut following axial planes parallel to the surface of the brain cortex and perpendicular to the probe trajectory. Sections were collected in 7 parallel series and processed for immunohistochemistry [49].

2.3. Immunofluorescent labelling

For immunofluorescent labelling, floating sections were first incubated for 1 h at room temperature (RT) in 4% normal goat serum (NGS, Sigma-Aldrich, Madrid) in PBS, and 0.05% Triton X-100 (TX-100, Sigma-Aldrich, Madrid), and overnight in the same solution containing the following primary antibodies: rabbit anti-TH policlonal antibody (1:1000; Sigma, Madrid, Spain) + mouse anti-macrosialin (anti-CD68) monoclonal antibody (1:1000; ABD Serotec, Bionova, Madrid) and chicken anti-GFAP polyclonal antibody (1:2000; Sigma, Madrid, Spain) or mouse anti-TH monoclonal antibody (1:12,000; Sigma, Madrid, Spain). Finally, triple immunofluorescent labeling sections were incubated for 2 h with RhodamineRedX-conjugated goat anti-rabbit IgG (1:3000; Jackson ImmunoResearch), Alexa Fluor 647-conjugated goat antichicken (Invitrogen, Madrid) 1:600 in PBS containing and biotine-conjugated goat antimouse 1:1200 (Jackson ImmunoResearch) in PBS containing 1:200 NGS followed by CY2-conjugated streptavidine (1:200; Jackson ImmunoResearch) in PBS containing 1:200 NGS and single immunofluorescent labeling sections were incubated for 2 h with FITC-goat anti-mouse (1:1200; Jackson ImmunoResearch) in PBS containing 1:200 NGS.

After several rinses, sections were mounted on gelatinized slides, air dried, coverslipped with Vectashield (Vector), and examined under confocal microscopy (Olympus Fluoview FV1000) using appropriate filters. Control experiments were performed for each immunohistochemistry and immunofluorescence study by removing the primary antibody, resulting in negative staining.

2.4. Quantitative analysis

The DAergic denervation after the 6-OHDA administration was evaluated by a densitometric analysis of the DAergic terminals in the striatum. The TH-immunoreactivity of 25 μm × 25 μm × 30 μm voxels located at different distances from the lateral ventricle was computed with the ImageY program (IJ1.46r). Densitometric data were normalized as a percentage of the mean value found in the voxels located 500 μm from the lateral ventricle contralateral to the injection side. In order to prevent differences due to variations in protocol conditions during tissue processing and densitometric analysis, all sections were processed simultaneously using the same protocols and chemical reagents, and all microscopic and computer parameters were kept constant throughout the densitometric study.

Astrocytic reaction to 6-OHDA administration was evaluated by comparing the GFAP-immunoreactivity (immunofluorescence intensity) in the striatum of the perfused brain side with that of the contralateral brain side. A number of variables produce inter-trial fluctuations of immunostaining, a variability which was reduced by normalizing data with those obtained in the same striatum of the contralateral brain side (data were computed as percentages of the mean value computed in the astrocytic processes of the contralateral striatum). At least 2000 astrocytic processes were computed in each experimental condition (computations performed in images of 10 μm × 10 μm × 2 μm digitized with a spatial resolution of 1025 × 1025 pixels). In each brain slice, the same number of astrocytic processes was computed in the striatum of both brain sides. The value used to represent the immunoreactivity of each astrocytic process was the average of its 3000 most immunoreactive voxels. Measurements were performed with the FV10-ASW (version 01.07.01.00) software (Olympus Corporation).