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# The dopamine transporter is differentially regulated after dopaminergic lesion

Domingo Afonso-Oramas [a], Ignacio Cruz-Muros [a,d], Pedro Barroso-Chinea [b,d], Diego Álvarez de la Rosa [c], Javier Castro-Hernández [a], Josmar Salas-Hernández [a], Teresa Giráldez [e], Tomás González-Hernández [a,d,*]

[a] Departamento de Anatomía, Facultad de Medicina, Universidad de La Laguna, Tenerife, Spain
[b] Centro de Investigación Médica Aplicada, Universidad de Navarra, Spain
[c] Departamento de Fisiología, Facultad de Medicina, Universidad de La Laguna, Tenerife, Spain
[d] Centro de Investigación Biomédica en Red de Enfermedades Neurodegenerativas, Instituto de Salud Carlos III, Spain
[e] Unidad de Investigación, Hospital Universitario Ntra Sra de Candelaria, Tenerife, Spain

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ABSTRACT

The dopamine transporter (DAT) is a transmembrane glycoprotein responsible for dopamine (DA) uptake, which has been shown to be involved in DA-cell degeneration in Parkinson's disease (PD). At the same time, some studies suggest that DAT may be regulated in response to dopaminergic injury. We have investigated the mechanisms underlying DAT regulation after different degrees of dopaminergic lesion. DAT is persistently down-regulated in surviving midbrain DA-neurons after substantial (62%) loss of striatal DA-terminals, and transiently after slight (11%) loss of DA-terminals in rats. Transient DAT down-regulation consisted of a decrease of glycosylated (mature) DAT in the plasma membrane with accumulation of non-glycosylated (immature) DAT in the endoplasmic reticulum-Golgi (ERG) compartment, and recovery of the normal expression pattern 5 days after lesion. DAT redistribution to the ERG was also observed in HEK cells expressing rat DAT exposed to $MPP^+$, but not after exposure to DAT-unrelated neurotoxins. In contrast to other midbrain DA-cells, those in the ventrolateral region of the substantia nigra do not regulate DAT and degenerate shortly after slight DA-lesion. These data suggest that DAT down-regulation is a post-traslational event induced by DA-analogue toxins, consisting of a stop in its glycosylation and trafficking to the plasma membrane. Its persistence after substantial DA-lesion may act as a compensatory mechanism helping maintain striatal DA levels. The fact that neurons which do not regulate DAT die shortly after lesion suggests a relationship between DAT down-regulation and neuroprotection.



## Introduction

The dopamine transporter (DAT) is a membrane glycoprotein specific to dopaminergic (DA-) cells, whose physiological role is the reuptake of released dopamine (DA) into presynaptic DA-terminals, regulating the time and intensity of DA signalling in extracellular space (Amara and Kuhar, 1993; Gainetdinov et al., 1998b; Giros and Caron, 1993; Uhl, 2003). Given that the cytosolic levels of DA directly depend on DA uptake, and that DA metabolism is the main source of reactive oxygen species in DA-cells (Adams et al., 2001; Luo and Roth, 2000), DAT has also been involved in the degeneration of DA-cells in Parkinson's disease (PD). This idea is supported by evidence of an anatomical correlation between the distribution of DA-neurons expressing high DAT mRNA levels and those showing high vulnerability to degeneration in PD (Cerruti et al., 1993; Hurd et al., 1994; Uhl et al., 1994), and by the fact that DAT can also transport natural and synthetic DA-analogue neurotoxins (Blum et al., 2001; Schober, 2004). Furthermore, its pharmacological blockade or deficient expression makes DA-cells resistant to these neurotoxins (Bezard et al., 1999; Gainetdinov et al., 1997; Kopin, 1992). The finding of an increase in the relative extracellular concentration of DA after a partial lesion of the mesostriatal system has suggested that DAT may at the same time be regulated in response to a DAergic insult (Bezard et al., 2001; Stachowiak et al., 1987), and consequently during the course of PD. This idea is reinforced by *in vitro* studies reporting a DA uptake decrease in striatal synaptosomes and DAT transfected cells after exposure to oxygen radicals or 1-methyl-4-phenylpyridinium ($MPP^+$), the active metabolite of the DA-analogue neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (Berman et al., 1996; Chagkutip et al., 2003; Fleckenstein et al., 1997a; Gulley et al., 2002). However, important aspects concerning this phenomenon are still unknown. The clarification of details such as the lesion degree required for inducing DAT

*Abbreviations:* DA, domanine; dSt, dorsal striatum; glyco-DAT, glycosylated DAT form; $MPP^+$, 1-methyl-4-phenylpyridinium; non-glyco-DAT, non-glcyosylated DAT form; SNcv, substantia nigra, caudoventral and lateral region; SNrm, substantia nigra, rostromedial and dorsal region; vSt, ventral striatum; VTA, ventral tegmental area; 6-OHDA, 6-hydroxydopamine.

* Corresponding author. Department of Anatomy, Faculty of Medicine, University of La Laguna, 38207 La Laguna, Tenerife, Spain. Fax: +34 922 660253.
*E-mail address:* tgonhern@ull.es (T. González-Hernández).

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expression changes, if all midbrain DA-cells respond similarly to the neuronal insult, and which cellular mechanisms are involved in this phenomenon, will provide further insight into the role of DAT in the pathogenesis of PD. This study has been focused on these questions by using molecular, morphological and pharmacological techniques in animal and cellular models of PD.

## Material and methods

### *Intracerebroventricular injection of 6-hydroxydopamine in rats*

6-hydroxydopamine (6-OHDA) is a hydroxylated analogue of DA which has been used extensively in animal models of PD. Similarly to DA, 6-OHDA has a high affinity for DAT, which carries the toxin inside the DA-neuron. Once inside the cell, 6-OHDA undergoes auto-oxidation producing oxidative stress because of the formation of free radials (Blum et al., 2001; Schober, 2004). We have developed a rat model of PD based on the intracerebroventricular (i.c.v.) injection of 6-OHDA which causes bilateral and dose-dependent degeneration of mesostriatal DA-neurons, and a motor syndrome composed of hypokinesia, purposeless chewing and catalepsy (Gonzalez-Hernandez et al., 2004; Rodriguez et al., 2001a, 2001b). Furthermore, the topographical pattern of DA-cell degeneration matches that observed in PD (Damier et al., 1999; Hirsch et al., 1988). The loss of DA-cells in the caudo-ventral and lateral region of the substantia nigra (SNcv) is higher than that in its rostro-dorsal and medial region (SNrm), and is higher in this region than in the ventral tegmental area (VTA). In order to study the effects of two different degrees of DA-lesion, animals were injected with either 150 μg or 350 μg of 6-OHDA in this work.

Experimental protocols were approved by the Ethical committee of the University of La Laguna (Reference #091), and are 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. Anaesthesia, pre-surgery treatment and intraventricular injection protocols followed Rodriguez et al. (2001a; 2001b). A total of 45 male Sprague–Dawley rats (350–400 g) supplied by Charles River (L'Arbresle, France) were used. They were injected in the third ventricle with vehicle (0.9% saline solution with 0.3 μg/μl ascorbic acid, $n=10$) or a single dose (150 μg, $n=30$, or 350 μg, $n=5$) of 6-OHDA (6-hydroxydopamine hydrochloride, Sigma, St. Louis, MO; in 7.5 μl of vehicle per injection; 1 μl/min). Bearing in mind that the bilateral degeneration of DA-cells can cause adipsia and aphagia (Ungerstedt, 1971; Zigmond and Stricker, 1973), the intake of food and water was monitored following the 6-OHDA injection. No body weight loss was observed. Rats were killed at 24 h, 48 h, 5 days or 3 weeks after injection.

### *DAT antibodies*

DAT expression was studied by immunohistochemistry and western-blot using antibodies raised against different DAT fragments which recognize the glycosylated and non-glycosylated DAT forms. The characterization of these antibodies is described in supplementary material. See also Cruz-Muros et al. (2009).

### *Morphological study*

Animals were deeply anaesthetised (chloral hydrate, 400 mg/kg, ip) and transcardially perfused with 0.9% saline (150 ml) and 4% paraformaldehyde in 0.1 M phosphate-buffered saline pH 7.4 (PBS, 400 ml). Midbrains and forebrains were immersed in a cryoprotective solution of 30% sucrose in the same buffer overnight at 4 °C, cut into 25 μm coronal sections (50 μm thick for cell count) with a freezing microtome, and collected in 6–8 parallel series. Solutions used for perfusion and cryoprotection were treated with 0.1% diethylpyrocarbonate (DEPC) and autoclaved to inactivate RNases.

### *Immunohistochemistry*

Midbrain and forebrain sections were processed for DAT and TH immunohistochemistry according to the procedure previously described (Gonzalez-Hernandez et al., 2004), and using the following primary antibodies: rabbit anti-DAT polyclonal antibody (sc-14002, 1:400, for non-glycosylated DAT labelling), rat anti-DAT monoclonal antibody (MAB369, 1:800; for glycosylated DAT labelling), and mouse anti-TH monoclonal antibody (Sigma, 1:12,000). Immunoreactions were visible after incubation for 1 h at RT in ExtrAvidin-peroxidase (1:5000, Sigma) in PBS, and after 10 min in 0.005% 3'-3'-diamiobenzidine tetrahydrochloride (DAB, Sigma) and 0.001% $H_2O_2$ in cacodylate buffer 0.05 N pH 7.6.

### *Fluoro-Jade histofluorescence*

Fluoro-Jade staining is a sensitive histofluorescent technique for detecting degenerated neurons in brain tissue (Schmued et al., 1997; Schmued and Hopkins, 2000). This tracer was used to reveal neuronal degeneration 48 h after 6-OHDA injection. Briefly, midbrain sections were mounted with distilled water onto 3-aminopropyltriethoxysilane (APS) coated slides and air-dried at RT for 40 min. The slides were immersed for 3 min in 100% ethyl alcohol, and in 70% alcohol and distilled water for 1 min. They were then transferred to a solution of 0.06% potassium permanganate for 15 min, rinsed for 1 min in distilled water and incubated for 30 min in 0.001% Fluoro-Jade (Histo-Chem, Jefferson, AR) in 0.09% acetic acid. After staining, the sections were rinsed several times in distilled water, dehydrated, immersed in xylene and coverslipped with PDX (BDH Chemicals).

### *Quantitative analysis of immunostained sections*

Bearing in mind the functional division of the mesostriatal system (Fallon and Moore, 1978; Joel and Weiner, 2000) and the degeneration pattern in PD (Bernheimer et al., 1973; Damier et al., 1999; Hirsch et al., 1988) and in our rat model of PD, the midbrain DA-formation was divided into three regions: (1) the SNcv, which includes A9 DA-cells lying in the ventrolateral region of the SN pars compacta and the SN pars reticulata; (2) the SNrm, and (3) VTA (see Fig. 1A). The striatum was divided into the dorsal striatum (dSt), whose DA-afferents come mainly from the SNcv, and the ventral striatum (vSt), whose DA-afferents come mainly from the SNrm and VTA (see Fig. 1D).

### *Cell count*

The analysed parameters were: (1) number and localization of TH-cells three weeks after 6-OHDA injection, and (2) number and localization of TH-cells showing morphological features of neuronal degeneration (neuronal body swelling and FJ histofluorescence) 48 h after 6-OHDA injection. All studies were performed in 5 rats per group. We used the optical dissector method (West, 1999; Williams and Rakic, 1988) and a computer-assisted image analysis system attached to an Olympus microscope (BX51) equipped with a 40×, 0.75 N.A. objective, motorized stage with serial optical sections 2 μm apart in the z axis, and an Olympus camera (DP70). An electronic microcaptor (MT12) was connected to both the microscope and the parallel port of the computer. Cell counting was performed with the computer assisted stereological toolbox software (C.A.S.T.-grid) from Olympus, Denmark. Six 50-μm thick sections 150 μm apart from each other in the rostrocaudal axis were randomly selected in each rat. DA-regions were divided into fields of 150 × 125 μm at a magnification of 400×. Cell counts were performed in four randomly selected boxes of 75 μm ($x$-axis) × 62 μm ($y$-axis) × 20 μm ($z$-axis) per region and section.

*Densitometric analysis* of labelling intensity was performed in DAT-immunostained midbrain somata and in TH-immunostained striatal terminals. The study was performed on 5 rats per group, and six 25-μm thick sections 150 μm apart from each other per rat. In order to prevent