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The Parkinson's Disease-Related Protein DJ-1 Protects Dopaminergic Neurons in vivo and Cultured Cells from Alpha-Synuclein and 6-Hydroxydopamine Toxicity

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Key Words
α-Synuclein · DJ-1 protein · 6-Hydroxydopamine · Parkinson's disease · Therapy

Abstract
Background: Dopaminergic degeneration is a major finding in brains of patients with Parkinson's disease (PD), together with Lewy bodies, intraneuronal inclusions mainly composed of the fibrillogenic protein α-synuclein (α-syn). The familial-PD-related protein DJ-1 was reported to reduce dopaminergic degeneration triggered by α-syn or by the dopaminergic-selective neurotoxin 6-hydroxydopamine (6-OHDA). ***Objective:*** The aim was to further investigate the role of DJ-1 in dopaminergic degeneration and to see whether a cell-permeable recombinant form of DJ-1 (TAT-DJ-1) could restore dopamine depletion in vivo, thus representing an innovative therapeutic approach. ***Methods:*** We developed in vitro (PC12/TetOn cells and mouse primary mesencephalic neurons) and in vivo models [including DJ-1 knockout (–/–) mice] to investigate DJ-1 in dopaminergic degeneration. ***Results:*** We found that in PC12/TetOn cells overexpressing α-syn with the familial-PD linked mutation A30P, DJ-1 silencing increased α-syn (A30P) toxicity. Primary mesencephalic neurons from DJ-1 (–/–) mice were more vulnerable to a cell-permeable form of α-syn (TAT-α-syn) and to 6-OHDA. Intrastriatally administered TAT-DJ-1 reduced 6-OHDA toxicity in vivo in C57BL/6 mice. Finally, when we injected TAT-α-syn (A30P) in the striatum of DJ-1 (–/–) animals, dopamine was depleted more than in the control strain. ***Conclusion:*** DJ-1 appears to have a protective role against dopaminergic degeneration triggered by α-syn or 6-OHDA, reinforcing the possible therapeutic importance of this protein in PD.

Introduction

Parkinson's disease (PD) is a common neurodegenerative movement disorder and a major challenge on account of the lack of therapies, which in addition to attenuating symptoms should also prevent neurodegeneration [1, 2]. The neurochemical basis relies on the depletion of dopamine (DA) in the striatum, due to the neurodegeneration of striatum-projecting dopaminergic neurons in the substantia nigra. Almost all PD cases are sporadic, only in a minor

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fraction (around 5%) has a genetic alteration been seen in a group of genes linked to rare familial forms and coding for proteins with no apparent functional interactions [3–5]. Consequently, a major question is the relationship between these proteins, whose changes lead ultimately to a similar pathology. Single-point mutations or multiplications of the gene PARK-1 [alias α-synuclein (α-syn); GenBank: NM_000345; OMIM: No. 168600] and single-point mutations or deletions of the PARK-7 gene (alias DJ-1; GenBank: NM_007262; OMIM: No. 606324) cause an autosomal dominant and an autosomal recessive form of familial PD, respectively [6–9]. α-syn aggregates are present in Lewy bodies, a PD hallmark [10]. An α-syn-dependent etiopathological hypothesis states that in PD α-syn aggregates and gains a toxic function that seems related to its oligomeric state [11, 12]. Transgenic models developed to support this hypothesis resulted in mild to severe phenotypes [13, 14]. Confirming the importance of α-syn dosage in the development of PD, PARK-1 triplication has been associated with familial PD [15, 16]. The physiological function of α-syn is not clear, though some α-syn neuroprotective features that deal with chaperone-like activity, DA homeostasis and correct synaptic vesicular trafficking have been described [17–20].

DJ-1 mutations are rare single-nucleotide substitutions or deletions leading to a nonfunctional protein [21–24]. DJ-1 function in the nervous system deals with antioxidant activity [25, 26], chaperone-like properties [27, 28], mitochondrial physiology [29, 30] and DA homeostasis [31, 32].

The interaction between α-syn and DJ-1 has been explored in cell models of α-syn overexpression, suggesting that DJ-1 is involved in preventing α-syn toxicity by upregulating HSP70 gene transcription (GenBank NM_005345) [33], which prevented α-syn toxicity in vivo and in vitro [34, 35]. As no fully satisfactory genetic model of PD is available, the toxin 6-hydroxydopamine (6-OHDA), both in vitro and in vivo, is widely used to reproduce dopaminergic degeneration for experimental purposes [36]. We set out to further clarify the importance of DJ-1 for the neurodegeneration triggered by α-syn or 6-OHDA in independent cellular and animal models, including *PARK-7* null mice (DJ-1 –/–).

Materials and Methods

PC12/TetOn (α-syn) Cell Lines

PC12/TetOn cell lines were prepared as described [37]. Cells were cultured on disposable plasticware coated with poly-D-lysine at 37°C, 5% CO_2 in RPMI-1640 medium (Invitrogen, Carlsbad, Calif., USA) supplemented with 10% tetracycline-free horse serum, 5% fetal calf serum (Sigma Aldrich, St. Louis, Mo., USA), 2 mM L-glutamine, 100 UI/ml penicillin and 100 mg/ml streptomycin (Invitrogen). To start α-syn expression, freshly prepared doxycycline was added to the culture medium (range: 0.1–1 μg/ml).

Primary Mesencephalic Culture

Brain tissue was removed from fetal mice on embryonic day 13 and the mesencephalic region isolated under a stereotactic microscope. After tissue dissociation, the cells were plated (50,000 cells/cm^2) in 8-well chamber slides (Thermo Fisher Scientific, Waltham, Mass., USA) precoated with poly-D-lysine in Dulbecco's minimal essential medium supplemented with 2% B27, 1% fetal bovine serum, 2 mM L-glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin and 1 μM cytosine arabinoside (Invitrogen). Cultures were kept at 37°C in a humidified 5% CO_2 atmosphere for 7 days before the experiment.

DJ-1 Cloning and TAT-Fused Protein Generation

Human DJ-1 full-length cDNA was cloned starting from a human brain cDNA library (Clontech, Palo Alto, Calif., USA) by rapid amplification of cDNA end PCR. The amplified cDNAs were then cloned into pRSET expression vectors (Invitrogen) and fully sequenced. To generate the fusion proteins TAT-DJ-1, the sequence coding for a peptide containing the translocation domain of the HIV-1 protein TAT (underlined) and a 6XHis-tag (MRGSHHHHHHGMARGYGRKKRRPASPGAS) was inserted in frame before the N terminus of the corresponding cDNA. The fusion protein was then expressed and purified using standard recombinant techniques [38]. The TAT-fused form of α-syn (A30P) and the TAT-fused form of green fluorescent protein (TAT-GFP) were purified as described [39, 40].

6-OHDA Challenge and TAT-Fused Protein Treatment

To challenge cells with 6-OHDA, 60×10^3 PC12/TetOn cells were seeded in a 96-well plate and incubated overnight. The next day, the medium was changed, a freshly prepared 6-OHDA dilution was added (final concentration 50 μM), and the cells were incubated for 24 h. Viable cells were counted by the methylthiazolyl tetrazolium (MTT) colorimetric assay (Promega, Madison, Wisc., USA).

As for primary mesencephalic neurons, starting from the 8-well chamber slide culture described above, we exposed cells to increasing doses of 6-OHDA (10–100 μM) for 24 h, then cells were fixed and the number of neuronal cells/well was assessed by neuronal nuclear antigen (NeuN) immunocytochemistry. The number of tyrosine hydroxylase (TH)-positive neurons was counted in the same well and the TH-positive/NeuN-positive percentage was calculated.

When mesencephalic neurons were challenged with TAT-α-syn (wild-type, WT, or A30P), cells were seeded as previously described and exposed for 72 h to increasing amounts of the recombinant proteins (10, 50, 100 μM) [41]. Viability was then measured by TH-positive/NeuN-positive cell percentage as just described.

RNA Interference

RNA interference was tested as follows: 80×10^3 PC12/TetOn cells were seeded in a 24-well plate and grown overnight. The next day, the medium was replaced with 250 μl of fresh complete medium, and 2 predesigned double-stranded small-interfering RNAs (siRNAs) against the DJ-1 mRNA sequence (siRNA-A sense: 5′-GGGACCAUAUGAUGUGGUGtt-3′; antisense: 5′-CACCACAUCAUAUGGUCCCtc-3′; siRNA-B sense: 5′-GGAGCAGGAAAACCGGAAGtt-3′; antisense: 5′-CUUCCGGUUUUCCUGCUCCtt-3′;

Ambion, Austin, Tex., USA) were added independently in 50 µl serum-free Optimem (Invitrogen) at 100 nM final concentration using as vehicle SilentFect liposome formulation (BioRad Laboratories, Hercules, Calif., USA) for 24–96 h, without any other medium change, to downregulate DJ-1. To prove specific DJ-1 silencing, a negative control siRNA was used (CT–), comprising a 19-bp scrambled sequence with 3′-dT overhangs that has no significant homology to any known human gene sequence (Ambion). Cell viability was then assessed by the MTT assay, or cells were lysed in the presence of a wide-range protease inhibitor to obtain a total protein extract.

Western Blotting and Slot Blot

Proteins were denatured by heating for 5 min at 95°C in Laemmli sample buffer twice, briefly spun and about 25 µg of total protein extract was run on sodium dodecyl sulfate polyacrylamide gel electrophoresis (12%) and transferred to a nitrocellulose membrane (BioRad). The membrane was incubated overnight with a primary antibody, for 1 h with a horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology, Santa Cruz, Calif., USA) and the detection was done with Immobilon Western Chemiluminescent HRP substrate (luminal; Millipore Corporation, Billerica, Mass., USA). Signals were quantified by densitometry, using National Institutes of Health software (ImageJ). Slot blot for α-syn aggregate detection was performed as described [37].

Immunocytochemistry

Primary mesencephalic cells were cultured on 8-well plastic chamber slides (Thermo Fisher Scientific) for 7 days before the assay. Cells were then fixed with 4% paraformaldehyde (Merck, Germany) and permeabilized using 0.5% Triton X-100, 0.2% fetal calf serum in phosphate-buffered saline (PBS). Then, they were incubated overnight at 4°C with a primary antibody in PBS + 1% horse serum, followed by a Cy3- or Cy5-conjugated secondary antibody (Jackson Immuno Laboratories, West Grove, Pa., USA) diluted 1:200 in PBS + 1% horse serum. Cells were then analyzed with a fluorescence microscope coupled to a digital camera (Olympus Corporation, Tokyo, Japan).

Animal Surgery and Ethics

Nine-week-old male C57BL/6 mice (Charles River, Italy) or matched PARK7 null mice (DJ-1 –/–; http://jaxmice.jax.org/strain/006577.html, The Jackson Laboratory, Bar Harbor, Me., USA) were used. They were housed in groups of 5 in a sound-proof room until surgery, after which they were housed singly until the end of the experiment. Mice were maintained on a 12-hour light/dark cycle (lights on from 7.00 a.m. to 7.00 p.m.), at a constant temperature of 21 ± 1°C and 60% relative humidity with free access to standard diet and tap water.

Mice were anesthetized using avertin 2.5% (400 mg/kg, i.p.) and placed into a stereotactic frame adapted for mice (David Kopf Instruments, Tujunga, Calif., USA). 6-OHDA was dissolved at a concentration of 2 µg/µl in 0.2% ascorbic acid in saline, and 2 µl was injected at a rate of 0.5 µl/min in the left striatum. Control animals received the same volume of saline with 0.2% ascorbic acid. The needle was left in place for 5 min after the injection before retraction. The injection was done with a Hamilton syringe at the following coordinates: AP +1 mm, L +2 mm, V −2.8 mm. 6-OHDA-lesioned mice were sacrificed 10 days after treatment.

To evaluate the protective role of TAT-DJ-1 against 6-OHDA, C57BL/6 mice were first implanted with a cannula in the striatum, injected with 4 µg of TAT-DJ-1 or recombinant DJ-1 (rDJ-1) on days –1, +1 and +4 (considering 6-OHDA administration as day 0) and sacrificed 10 days after the 6-OHDA injection (6 mice/group).

For the treatment with TAT-α-syn (A30P), 2 µl of the recombinant protein (or of TAT-GFP as negative control) corresponding to increasing amounts (0.05–2.5 µg) were injected in the left striatum as described above, and the mice were euthanized 10 days after the injection.

Procedures involving animals and their care were conducted in conformity with the institutional guidelines at the IRCCS Institute for Pharmacological Research Mario Negri in compliance with national (Decreto Legge No. 116/92, Gazzetta Ufficiale, supplement 40, February 18, 1992; Circolare No. 8, Gazzetta Ufficiale, July 14, 1994) and international laws and policies (EEC Council Directive 86/609, OJL 358, 1, December 12, 1987; Guide for the Care and Use of Laboratory Animals, US National Research Council, eighth edition, 2011).

Striatal Dopamine Content

The mice were killed by cervical dislocation; the brains were rapidly removed and immediately placed in ice-cold saline. The right and left striata were then dissected on an ice-cold plastic dish. Tissue samples were weighed, transferred into 10 volumes of 0.1 M perchloric acid and homogenized by sonication (Branson Ultrasonic Corporation, Danbury, Conn., USA). Homogenates were left at 4°C for 30 min to complete deproteinization, and, after centrifugation at 4,800 *g* for 10 min at 4°C, the supernatants were collected for chromatographic assay. DA and serotonin (5-HT) tissue levels were measured by high-performance liquid chromatography (HPLC) coupled to electrochemical detection, as detailed elsewhere [42]. Data were recorded and analyzed with a computer-assisted data system (Azur, Datalys, France).

Immunohistochemistry

After perfusion, the mouse whole brain was removed and transferred into 4% paraformaldehyde (Merck) at 4°C. After 3–4 h, the brains were removed and left at 4°C in 30% sucrose and PBS solution and were cut into 30-µm coronal sections on a cryostat (Leica, Germany). Free-floating sections were stained for TH (Chemicon International, Temecula, Calif., USA, 1:1,000) and detected by diaminobenzidine reaction (Sigma Aldrich).

The quantitative assessment of TH-positive neurons was performed essentially as described [43]. Shortly, TH immunoreactive cell bodies were counted in 3 consecutive coronal sections by an operator blind to the treatment using a ×40 objective in the ipsilateral and contralateral substantia nigra pars compacta.

Antibodies

The following primary antibodies were used to run immunoblotting or immunochemistry: anti-DJ-1 polyclonal antibody (dilution 1:1,000; Santa Cruz Biotechnology); anti-α-tubulin monoclonal antibody (dilution 1:5,000; Abcam, Cambridge, UK), anti-oligomer (A11) polyclonal antibody (dilution 1:250; Invitrogen), anti-6XHis-tag mouse monoclonal antibody (1:1,000; Abcam), anti-TH monoclonal antibody (dilution 1:500; Chemicon International), anti-NeuN polyclonal antibody (dilution 1:500; Abcam).

Statistical Analysis

Statistics were analyzed with StatView version 5.0. One-way ANOVA (or 2-way ANOVA) was used, followed by Dunnett's or Tukey's post hoc test. Significance was set at $p = 0.05$.

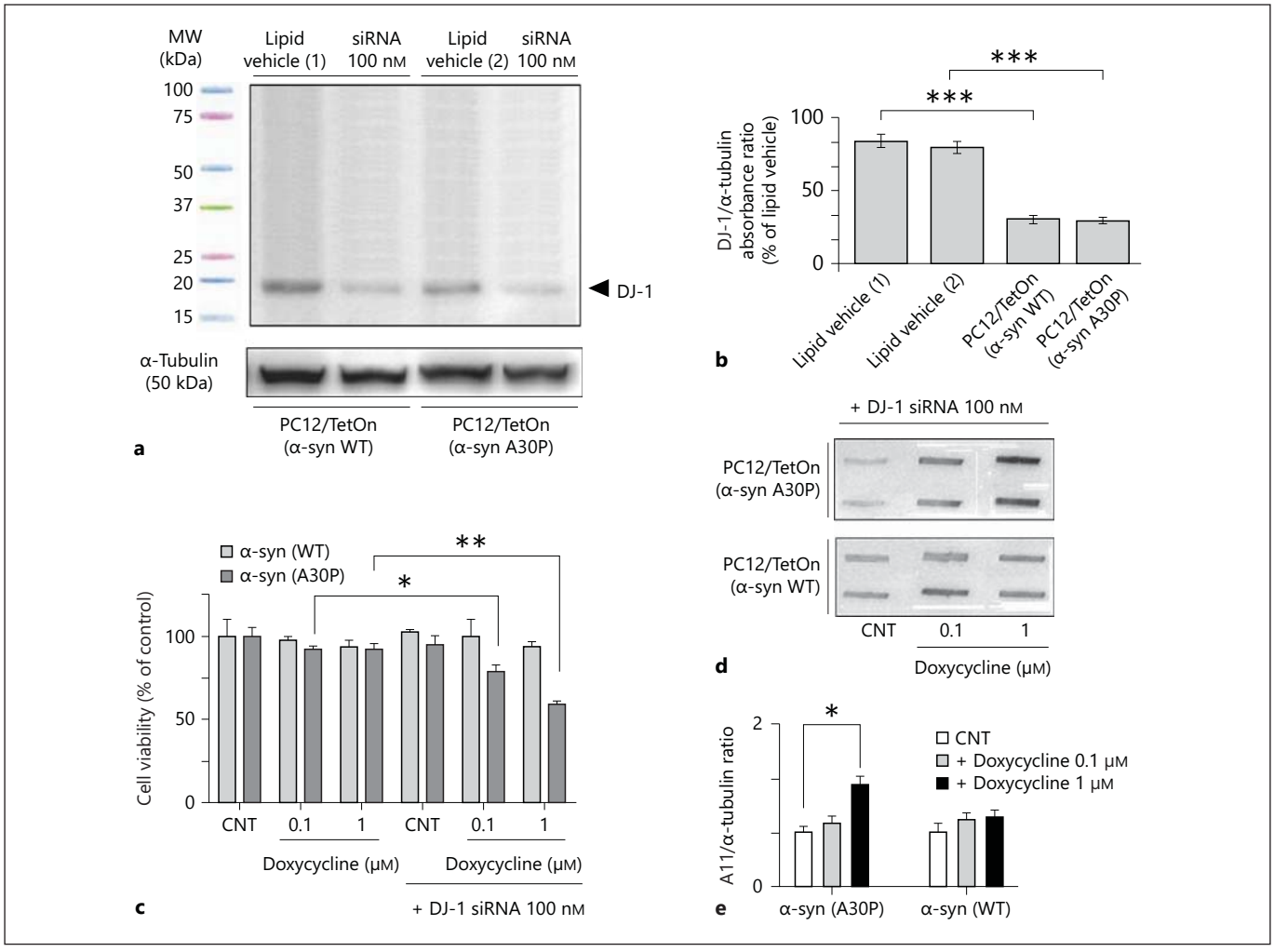

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Fig. 1. DJ-1 downregulation in PC12/TetOn discloses α-syn toxicity. **a** Western blotting showing DJ-1 downregulation by siRNA methodology in PC12/TetOn cells. The best condition was 100 nM siRNA for 48 h. Lipid vehicle is the control condition where cells were exposed to the transfecting agent only. **b** Densitometric quantification of DJ-1 silencing. Values are the means ± standard deviation of 4 independent replicates; *** $p < 0.001$, 1-way ANOVA and Dunnett's test. **c** MTT assay assessing the effect of DJ-1 silencing in doxycycline-induced PC12/TetOn. Cells were exposed for 48 h to DJ-1 siRNA and then induced with increasing doses of doxycycline for a further 48 h. * $p < 0.05$, ** $p < 0.01$, 2-way ANOVA and Tukey's post hoc test. CNT = Control (without doxycycline). **d** Representative slot blot showing the effect of DJ-1 silencing on α-syn aggregation. PC12/TetOn cells were treated as just described, and α-syn oligomeric species formation was assessed by A11 reactivity. **e** Densitometric quantification of 3 independent replicates, performed by normalizing the A11 signal to α-tubulin immunoreactivity in the same blots (data not shown); * $p < 0.05$, 1-way ANOVA and Tukey's post hoc test. CNT = Control (without doxycycline).

Results

DJ-1 Silencing Affects α-syn (A30P) Toxicity in PC12/TetOn Cells

To assess a relationship between DJ-1 and α-syn toxicity, we used siRNA technology to downregulate the DJ-1 level in PC12/TetOn cells, which overexpressed human α-syn (WT or mutated A30P) in a doxycycline-dependent inducible way. First, we followed DJ-1 downregulation over time, starting from 24 to 96 h after siRNA treatment. Both PC12/TetOn lines showed significant downregulation starting from 48 h, and the reduction was still evident at 96 h (data not shown). The best condition was siRNA 100 nM for 48 h (fig. 1a, b). After that, we investigated the effect of DJ-1 silencing in doxycycline-induced PC12/TetOn. Cells were first exposed for 48 h to DJ-1 siRNA, then induced with the antibiotic (0.1 or 1 μM) for a further 48 h. Cell viability was assessed by the MTT assay. For the DJ-1 silenced clone ex-

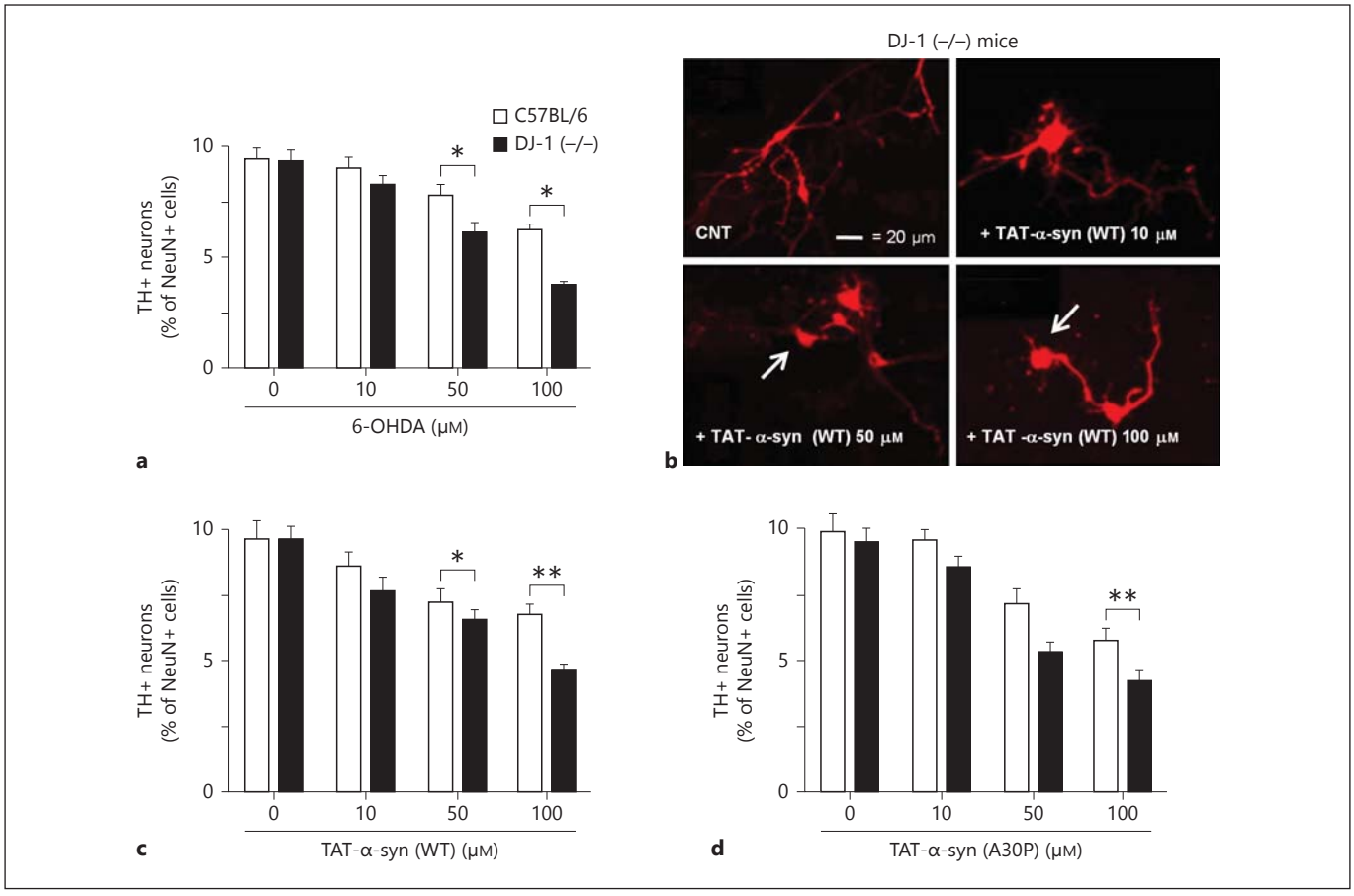

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Fig. 2. DJ-1 (–/–) mesencephalic primary neurons are more vulnerable than C57BL/6 neurons to 6-OHDA or TAT-α-syn. **a** Dose-response curve of 6-OHDA toxicity. Primary neurons were cultured as described in the Methods and challenged with increasing amounts of 6-OHDA. * $p < 0.05$, 2-way ANOVA and Tukey's post hoc test. **b** Immunocytochemistry for TH of mesencephalic primary neurons from DJ-1 (–/–) mice, showing neurodegeneration after TAT-α-syn (WT) treatment. The arrows point to degenerated neurons with reduced harborization. A similar morphological response was evident in mesencephalic neurons from C57BL/6 control mice, or when we used TAT-α-syn (A30P) in both strains (data not shown). CNT = Control. Magnification ×20. **c**, **d** TAT-α-syn (WT) and TAT-α-syn (A30P) toxicity in C57BL/6 and DJ-1 (–/–) mesencephalic primary neurons. TAT-α-syn was added to the medium after 7 days from culture start and, after a further 72 h, the number of TH-positive cells (TH+) was counted and expressed as percentage of NeuN-positive cells (NeuN+) in the same well. * $p < 0.05$, ** $p < 0.01$, 2-way ANOVA and Tukey's post hoc test.

pressing α-syn (A30P), we detected a doxycycline dose-dependent decrease in cellular viability (fig. 1c). To verify if this reduction correlated with α-syn aggregation, we checked for oligomeric form generation with A11 antibody under doxycycline induction and DJ-1 silencing [37]. We noticed an increase in the A11/α-tubulin ratio in the PC12/TetOn line expressing α-syn (A30P; fig. 1d, e).

Mesencephalic Neurons from DJ-1 (–/–) Mice Are More Susceptible to Both 6-OHDA and TAT-α-syn

To evaluate DJ-1's neuroprotective role in a more physiological model, we exposed mesencephalic primary neurons prepared from DJ-1 (–/–) embryos to 6-OHDA for 24 h. As shown in figure 2a, neurons from DJ-1 (–/–) mice were more susceptible to 6-OHDA toxicity than neurons from C57BL/6 control mice. The percentage of TH-positive neurons decreased in C57BL/6 cultures from 10 to 100 μM 6-OHDA but in DJ-1 (–/–) neurons the same 6-OHDA concentrations induced a greater mortality, which became significant in comparison to control mice from 50 μM.

Mesencephalic neurons were then exposed to the fusion protein TAT-α-syn (WT) or TAT-α-syn (A30P). To confirm the TAT-α-syn-induced dopaminergic toxicity, we first did TH immunocytochemistry in treated cells.

After TAT-α-syn treatment, there were morphological signs of neuronal degeneration (fig. 2c). We move on viability assessment by TH-positive cell count. TH-positive neurons from DJ-1 (–/–) mice were more vulnerable to TAT-α-syn toxicity (both WT and mutated A30P) than TH-positive neurons from C57BL/6 mice (fig. 2c, d).

TAT-DJ-1 Counteracts 6-OHDA Toxicity in vivo

To see whether DJ-1 was able to protect against dopaminergic degeneration in vivo, we treated C57BL/6 mice with the recombinant protein TAT-DJ-1 during 6-OHDA challenge. To verify the presence of the fusion protein at the striatal level, a first injection of TAT-DJ-1 was done and repeated after 48 h. Ten days after the second injection, the presence of the protein was detected by immunohistochemistry using an antibody against its 6XHis-tag. We found a positive and diffuse signal in the striatum, more marked than on the mock-injected contralateral side (fig. 3a).

Having confirmed the bioavailability of TAT-DJ-1, we set up a neuroprotection experiment based on 2 distinct injections of TAT-DJ-1 as described above, 1 day before (day –1) and 1 after (day +1) the challenge with 6-OHDA. There was a significant protection of the TAT-DJ-1 protein (fig. 3b), measured as the inhibition of 6-OHDA-induced striatal DA depletion 10 days after neurotoxin challenge; a recombinant form of DJ-1 without the TAT-sequence (rDJ-1) gave no such protection in the same experimental setting (fig. 3b).

To improve neuroprotection, we increased the number of TAT-DJ-1 injections. Mice were treated with TAT-DJ-1 (or rDJ-1) 3 times, on days –1, +1 and +4 (considering the 6-OHDA injection as day 0). HPLC analysis of striatal DA 10 days after 6-OHDA challenge indicated a stronger neuroprotective effect of TAT-DJ-1 against 6-OHDA, while rDJ-1 gave no protection (fig. 3c). Striatal levels of 5-HT did not change, confirming the selective effect of 6-OHDA and TAT-DJ-1 treatment (fig. 3d).

To support the rescue of dopaminergic system failure by TAT-DJ-1, we examined dopaminergic neurons in the substantia nigra by immunohistochemistry and TH-positive cell count. Compared with 6-OHDA-treated mice, TH immunoreactivity was greater in the 6-OHDA + TAT-DJ-1 group, while in mice treated with rDJ-1 in the presence of the neurotoxin this effect was not evident. These results were confirmed by TH-positive cell count (fig. 3e, f).

DJ-1 (–/–) Mice Have Greater DA Depletion after TAT-α-syn (A30P)

We set up an in vivo model of TAT-α-syn (A30P) toxicity in C57BL/6 mice. We injected the fusion protein (0.05–2.5 μg) in the left striatum, using as negative control a TAT-fused form of GFP, considering that a recombinant α-syn might have toxic effects also without the TAT-fused sequence [44]. First, we verified that the TAT-α-syn (A30P) protein was detectable at the striatal level 10 days after injection. We were able to reveal a positive diffuse signal in the hemisphere lesioned with 2.5 μg of TAT-α-syn (A30P) using an antibody against its 6XHis-tag (fig. 4a). Striatal tissues of TAT-α-syn (A30P)-treated mice were then collected for HPLC analysis of DA and 5-HT levels (fig. 4b, c). We found a dose-dependent depletion of DA in the TAT-α-syn (A30P)-treated animals, while 5-HT levels were unchanged. No difference in DA or 5-HT levels was detected in the TAT-GFP group (data not shown).

TAT-α-syn (A30P) toxicity was confirmed by TH immunohistochemistry and TH-positive neuron count in the substantia nigra. The TAT-GFP group did not show any reduction in TH staining or TH-positive neuron count, but TAT-α-syn (A30P)-injected animals did (fig. 4d, e).

Finally, we quantified the toxicity of TAT-α-syn (A30P) in DJ-1 (–/–) and C57BL/6 mice by comparing the DA levels in the two groups. DJ-1 (–/–) mice had about half the DA level of C57BL/6 animals treated with the same amount of TAT-α-syn (A30P; 2.5 μg; fig. 4f).

Fig. 3. TAT-DJ-1 counteracts in vivo dopaminergic degeneration triggered by 6-OHDA. **a** 6XHis-tag immunoreactivity. Picture showing the brain presence of TAT-DJ-1 10 days after its injection at the striatal level. The arrow points to the injection site. **b**, **c** C57BL/6 mice were lesioned with 6-OHDA (4 μg) in order to induce dopaminergic cell loss and DA depletion. To test the protective action of TAT-DJ-1, a group of animals was injected with the fusion protein twice (**b**) or 3 times (**c**), as described in the Methods. A different group received rDJ-1, together with 6-OHDA. * $p < 0.05$, ** $p < 0.01$, 1-way ANOVA and Tukey's post hoc test (6 mice/group). **d** 5-HT levels referring to the experiment in **c**. Results are expressed as micrograms per gram of wet weight of striatum. **e** TH immunoreactivity in the substantia nigra: control group (vehicle alone); mice lesioned with 6-OHDA; mice treated with TAT-DJ-1 together with 6-OHDA; mice treated with rDJ-1 and 6-OHDA; mice injected with the recombinant proteins alone (TAT-DJ-1 or rDJ-1). In each picture, the dashed line separates the two brain hemispheres. The injection was always given into the hemisphere marked by the label, and the contralateral one was used as control. **f** Quantification of TH-positive (TH+) neuron number in the substantia nigra of mice receiving the treatment described in **e**. Results are shown as direct count number. *** $p < 0.001$, 1-way ANOVA and Tukey's post hoc test (3 mice/group).

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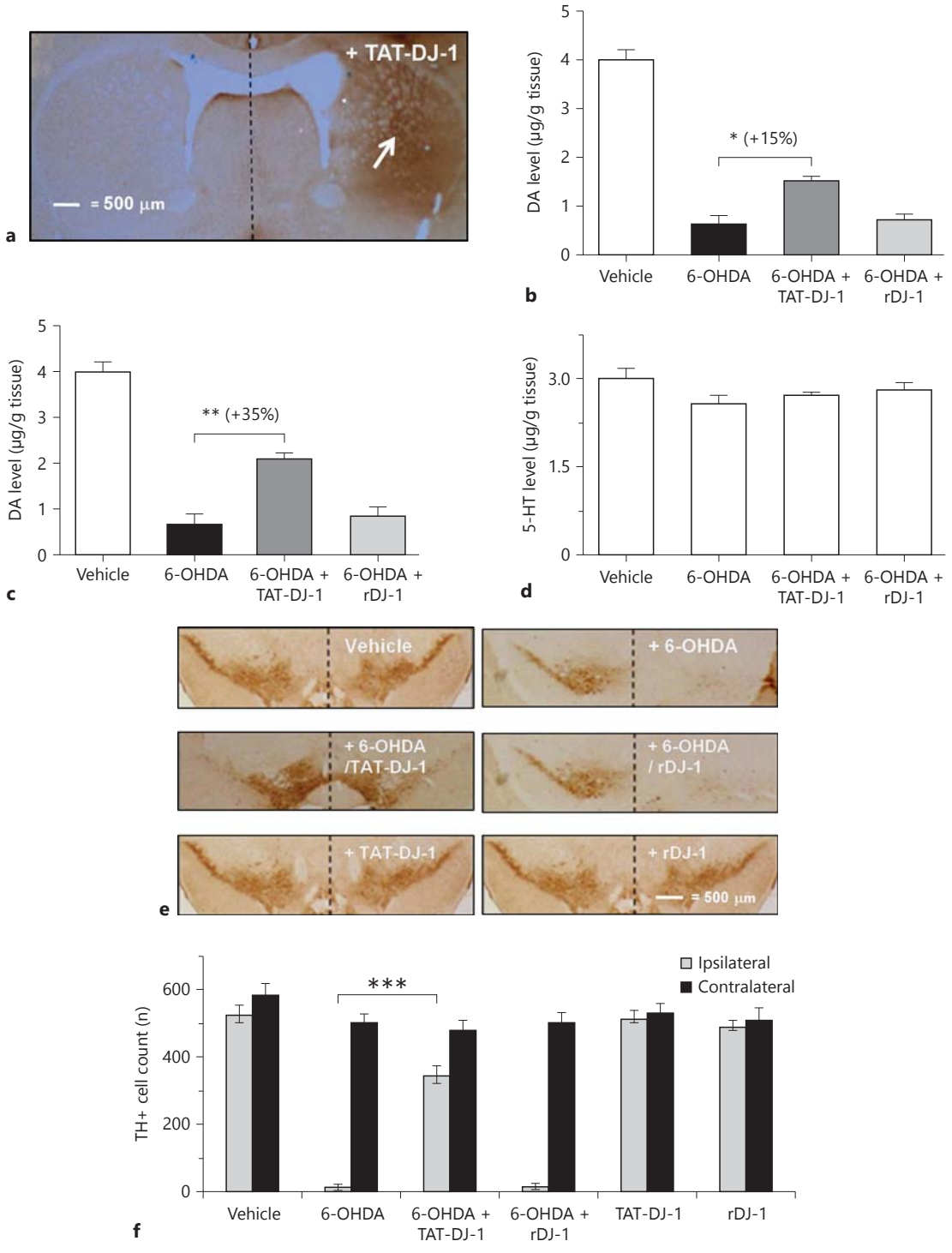

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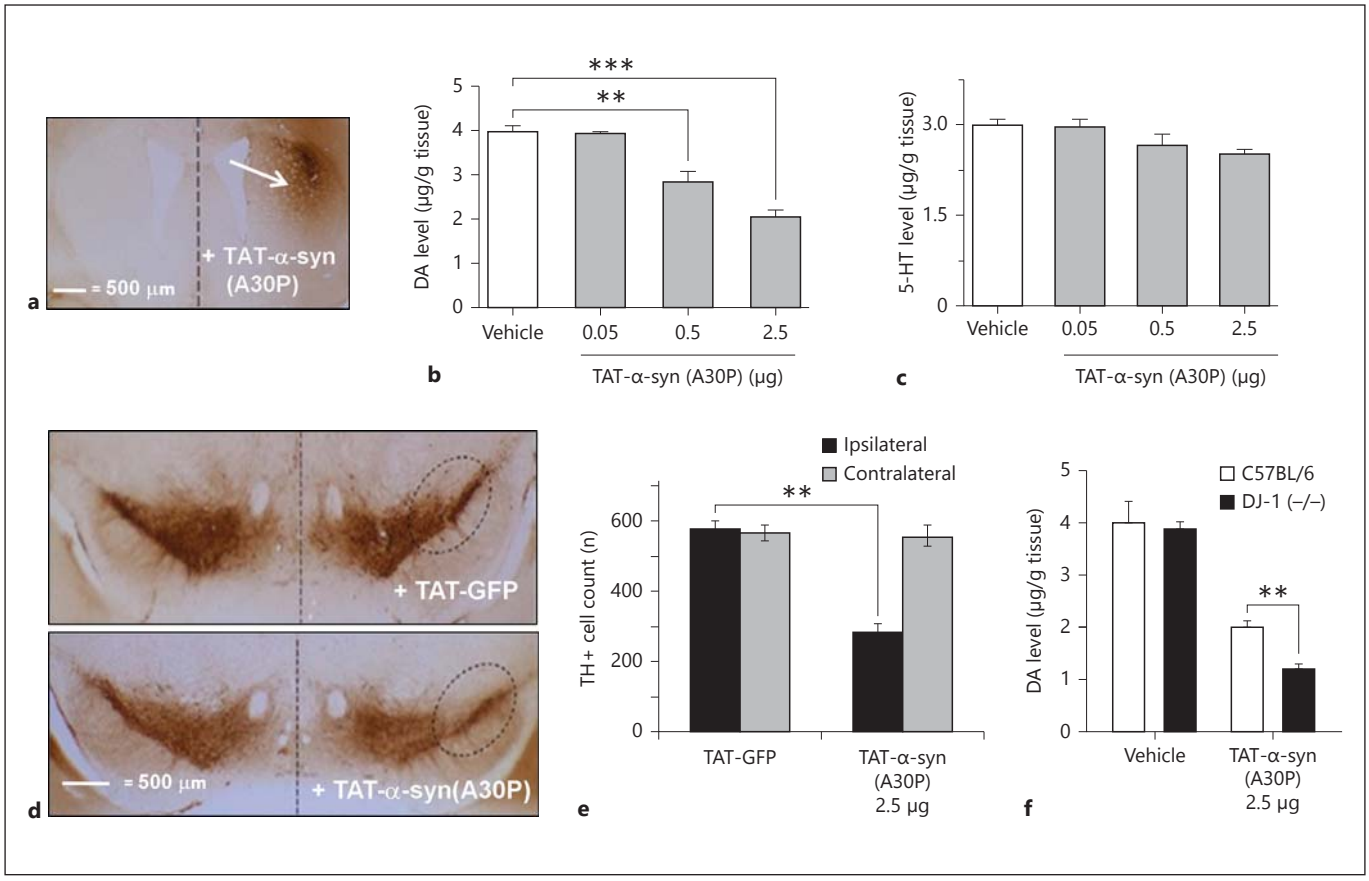

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Fig. 4. TAT-α-syn (A30P) induces a decrease in the DA level that is greater in DJ-1 (–/–) mice. **a** Immunohistochemistry to detect TAT-α-syn (A30P) in the mouse brain. The presence of the recombinant protein (2.5 μg) was assessed by 6XHis-tag reactivity 10 days after injection. The arrow points to the injection site (striatum). **b**, **c** C57BL/6 mice were lesioned with TAT-α-syn (A30P; 0.05, 0.5 or 2.5 μg) to induce dopaminergic cell loss and DA depletion. To verify the toxic effect, the striatal DA level (**b**) and 5-HT level (**c**) were measured by HPLC 10 days after the lesion. ** $p < 0.01$, *** $p < 0.001$, 1-way ANOVA and Tukey's post hoc test (6 mice/group). Results are expressed as micrograms per gram of wet weight of striatum. **d** Immunohistochemistry to assess TH reactivity in C57BL/6 mice in the substantia nigra after the injection of TAT-GFP or TAT-α-syn (A30P). The dotted oval highlights the substantia nigra portion with dopaminergic degeneration after TAT-α-syn (A30P). The vertical dashed lines separate the two hemispheres. The treated hemisphere is the one with the label, and the contralateral one was used as control. **e** Quantification of TH-positive neuron (TH+) number in the substantia nigra of the mice receiving the treatment described in **d**. Results are shown as direct count number. ** $p < 0.01$, 1-way ANOVA and Tukey's post hoc test (3 mice/group). **f** DJ-1 (–/–) mouse striatal DA levels after TAT-α-syn (A30P). Mice were injected with TAT-α-syn (A30P; 2.5 μg) and euthanized after 10 days. The vehicle group received the same volume of hypotonic PBS. ** $p < 0.01$ versus C57BL/6 treated with TAT-α-syn (A30P), 2-way ANOVA and Tukey's post hoc test (6 mice/group). Results are expressed as micrograms of DA per gram of wet weight of striatum.

Discussion

PD is characterized by the degeneration of dopaminergic neurons and DA depletion in the striatum, the main biochemical feature of the disorder. The molecular mechanism of this pathogenetic process is still debated, even though the presynaptic and aggregation-prone protein α-syn might play a major role [6, 7, 11].

We have added evidence of the protective role of DJ-1 against dopaminergic degeneration, also caused by α-syn, starting from in vitro models. The importance of DJ-1 for PD is first of all supported by genetic studies linking DJ-1 loss of function to the early-onset form of the disease. In agreement with this, we found that DJ-1 downregulation seems involved in the toxicity caused by α-syn. We had already drawn a similar conclusion in other models [45], but the present study makes this evidence firmer thanks to the observation of the increased vulnerability to TAT-α-syn toxicity of DJ-1 (–/–) mesencephalic neurons. Recently, others have highlighted the in-

volvement of DJ-1 in neuroprotection in neuron/astrocyte cocultures [46].

An independent confirmation of a relation between DJ-1 and α-syn comes from our experiments with DJ-1 silencing in PC12/TetOn cells, where DJ-1 downregulation triggered the toxicity due to α-syn overproduction and aggregation. The fact that the mutated form A30P only becomes deleterious with DJ-1 silencing probably depends on the fact that the mutation enhances the α-syn aggregation rate, so in this case the reduction of DJ-1 by siRNA is sufficient to unmask toxicity. Instead, in the case of α-syn (WT), DJ-1 reduction alone was probably not enough to trigger toxicity, also considering that PC12/TetOn cells have efficient alternative mechanisms for coping with α-syn aggregation (for instance, macroautophagy and the proteasome) [37].

The in vivo results suggest some discordant data on the role of DJ-1 in counteracting dopaminergic degeneration. In fact, while a protective effect of DJ-1 against MPTP, 6-OHDA or MG132 was reported [47, 48], Ramsey et al. [49] developed a mouse strain overexpressing a mutated form of the α-syn gene (A53T pathogenetic mutation) on a DJ-1 null background whose phenotype was not worsened by the lack of DJ-1. It is possible that our acute model of TAT-α-syn toxicity may have been more aggressive, thus able to highlight DJ-1's neuroprotective role, while the earlier mouse model might have developed compensatory mechanisms for controlling α-syn toxicity in the absence of DJ-1. It should be noticed that our in vivo model of acute TAT-α-syn toxicity offers an easy experimental system, leading to selective dopaminergic degeneration, confirmed by the lack of 5-HT depletion in the TAT-α-syn (A30P)-treated mice. This dopaminergic loss was also observed in a TAT-α-syn (A30P) rat model of PD, where it paralleled a time-dependent impairment in motor function [50]. In agreement with our model, the possibility of acute induction of α-syn toxicity by intrastriatal injection leading to a PD-like phenotype was also reported, even in the absence of a translocation domain in the recombinant protein [51].

The exact molecular mechanisms by which DJ-1 achieves neuroprotection needs to be further investigated, and might be both direct and indirect. For instance, we had found that DJ-1 was able to positively upregulate the chaperone protein Hsp70 [45], which was reported to support neuroprotection against α-syn toxicity and several other PD-linked stressors [52, 53].

A second possibility involves the evidence that TAT-DJ-1 treatment led to an increase in TH-positive neurons. This agrees with the literature suggesting a direct effect of DJ-1 on TH transcription [54]. However, further experiments are required to clarify the neuroprotective pathways sparked off by DJ-1 and whether its protective action towards the dopaminergic system is in the substantia nigra or striatum.

In summary, our results reinforce DJ-1 as a positive regulator of the dopaminergic system, contributing to our understanding of the mechanism leading to PD in the presence of DJ-1 failure that makes dopaminergic neurons more susceptible to oxidative stress or α-syn toxicity.

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Disclosure Statement

The authors have no conflict of interest to disclose.

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