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► **To cite this version:**

Marc Deffains, Marie-hélène Canron, Margaux Teil, Qin Li, Benjamin Dehay, et al.. L-DOPA regulates  $\alpha$ -synuclein accumulation in experimental parkinsonism. *Neuropathology and Applied Neurobiology*, 2020, 47 (4), pp.532-543. 10.1111/nan.12678 . hal-03838653

**HAL Id: hal-03838653**

**<https://hal.science/hal-03838653v1>**

Submitted on 9 Nov 2022

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Article type : Original Article

## L-DOPA regulates $\alpha$ -synuclein accumulation in experimental parkinsonism

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**Keywords (up to 8):** Parkinson's disease, levodopa,  $\alpha$ -synuclein, protein aggregation, tau, MPTP, macaque

**Short running title:** L-DOPA ameliorates  $\alpha$ -synuclein pathology in PD

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the [Version of Record](#). Please cite this article as [doi: 10.1111/NAN.12678](https://doi.org/10.1111/NAN.12678)

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## Total number of

- Words (up to 5000): 4798
- Figures : 6

## Abstract

**Aims:** Widespread accumulation of misfolded  $\alpha$ -synuclein aggregates is a key feature of Parkinson's disease (PD). Although the pattern and extent of  $\alpha$ -synuclein accumulation through PD brains is known, the impact of chronic dopamine-replacement therapy (the gold-standard pharmacological treatment of PD) on the fate of  $\alpha$ -synuclein is still unknown. Here, we investigated the distribution and accumulation of  $\alpha$ -synuclein in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) non-human primate of PD and determined the effect of chronic L-DOPA treatment on MPTP-induced  $\alpha$ -synuclein pathology.

**Methods:** We measured the density of  $\alpha$ -synuclein and tau immuno-positive neurons in the substantia nigra, putamen, hippocampal CA1 region, temporal cortex and dentate nucleus of control, MPTP and MPTP+L-DOPA-treated monkeys. Moreover, we also extracted and quantified Triton-X (TX) soluble and insoluble  $\alpha$ -synuclein in putamen and hippocampus samples from a separate cohort of control, MPTP and MPTP+L-DOPA-treated monkeys.

**Results:** MPTP-induced  $\alpha$ -synuclein accumulation in NHP model of PD was not limited to the substantia nigra but also occurred in the putamen, hippocampal CA1 region and temporal cortex. Tau was increased only in the temporal cortex. Moreover, increased intraneuronal TX insoluble  $\alpha$ -synuclein was truncated, but not in the structural form of Lewy bodies. The MPTP-induced increase in  $\alpha$ -synuclein levels was abolished in animals having received L-DOPA in all the brain regions, except in the substantia nigra.

**Conclusions:** Dopamine replacement therapy can dramatically ameliorate  $\alpha$ -synuclein pathology in the MPTP NHP model of PD. Therefore, patient's dopaminergic medication should be systematically considered when assessing  $\alpha$ -synuclein as a biomarker for diagnosis, monitoring disease progression and response to disease-modifying treatments.



## List of abbreviations

AAALAC: Association for the Assessment and Accreditation of Laboratory Animal Care

DRT: dopamine replacement therapy

LBS: Lewy bodies

L-DOPA: 3,4-dihydroxyphenylalanine

MPTP: 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine

NHP: non-human primates

PD: Parkinson's disease

SNpc: substantia nigra pars compacta

TX: Triton-X

## Introduction

Widespread accumulation of aggregation-prone proteins is a key feature of neurodegenerative disorders. In Parkinson's disease (PD) and related-disorders designated as synucleinopathies, misfolded  $\alpha$ -synuclein accumulates either in Lewy bodies (LBs) in neurons in PD and dementia with Lewy bodies or in oligodendroglial cytoplasmic inclusions in multiple system atrophy. In addition to  $\alpha$ -synuclein, these cytoplasmic inclusions contain numerous components, including other aggregation prone proteins such as tau [1, 2]. The spatial and temporal evolution of  $\alpha$ -synuclein accumulation over the course of PD has been described, suggesting an ascending progression from the brainstem, later invading subcortical and cortical territories in the most advanced stages of the disease [3]. Although this scheme has been confirmed in subsequent studies, deviation from this proposed pattern has also been reported (for review, see [4]). Whether this relates to the intrinsic heterogeneity of the disease process, clinical subtypes associated with unconventional patterns of  $\alpha$ -synuclein accumulation, concurrent aging-related formation of LBs or external factors remains a matter of debate.

Given its pivotal role in the pathogenesis of PD,  $\alpha$ -synuclein is considered as a promising biomarker [5]. As such, it is critical to identify if symptomatic treatments used in PD may affect the pattern and extent of  $\alpha$ -synuclein accumulation. This remains an open question due to inherent limitations. Indeed, pathological studies on PD brains are unlikely to be able to reveal any effect of a given symptomatic treatment on protein aggregation due to clinical and pathological heterogeneity, combined with a large variety of drugs used over the course of the disease and incomplete or limited clinical records of drug intake.

Administration of the parkinsonian neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) is the gold-standard for modelling PD in non-human primates (NHPs), recapitulating many of the motor and non-motor clinical features of the disease [6, 7]. With regards to protein misfolding and accumulation, exposure to MPTP leads to a secondary accumulation of  $\alpha$ -synuclein in the substantia nigra [8-10], that can still be detected over 10 years after administration of the toxin, suggesting that this neurotoxin leads to long lasting alterations of  $\alpha$ -synuclein homeostasis [11]. These seminal studies have however focused on the substantia nigra pars compacta (SNpc) and did not investigate other regions of the central nervous system relevant to the pathophysiology of

PD. Furthermore, these studies did not consider other aggregation prone proteins such as tau and did not investigate the potential effect of dopamine replacement therapy (DRT).

The aim of this study was to investigate in the NHP MPTP model of PD the possible occurrence of a widespread synucleinopathy as occurring in the human disease and to determine if the gold-standard pharmacological treatment (i.e., L-DOPA) may influence some neuropathological features such as neuronal loss in the substantia nigra, astrogliosis, and the fate of aggregation prone proteins such as  $\alpha$ -synuclein and Tau.

## **Materials and Methods**

### **Animals**

Brain tissue samples were obtained from the non-human primate brain bank at the Institut des Maladies Neurodegeneratives (Bordeaux, France). Experiments were carried out in an AAALAC-accredited facility and in accordance with the European Union Directive (2010/63/EU) on the protection of animals used for scientific purposes. Experiments were conducted on 28 female rhesus monkeys (*Macaca mulatta*, Xierxin, Beijing, China, mean age =  $5.4 \pm 1$  years, mean weight =  $3.8 \pm 0.4$  kg). Animal cares were supervised by veterinarians skilled in NHP healthcare and maintenance. Animals were housed in individual primate cages under controlled conditions of humidity ( $50 \pm 5\%$ ), temperature ( $24 \pm 1^\circ\text{C}$ ) and light (12h light/12h dark cycles, lights on 8:00 am), food and water were available *ad libitum*. Even though animals were housed individually, the disposition of cages allowed each animal to have visual contacts and interact with monkeys housed in the adjacent cages. Stimulations for play were provided including rubber toys and mirrors that the monkeys used to view themselves and to get a greater look around the room. A radio was played daily from 8.00am–10.00am and 3.00pm–5.00pm to provide stimulation.

### **Experimental protocol**

Ten animals were kept as untreated controls (control group). The remaining 18 animals were treated daily with MPTP hydrochloride (0.2 mg/kg, i.v., Sigma, St Louis, MO) dissolved in saline according to a previously described protocol [12, 13]. Following stabilization of the MPTP-induced syndrome, animals received either vehicle (MPTP,

n=8), or were chronically treated twice daily with 20 mg/kg levodopa for 110 days (MPTP + L-DOPA, n=10). All animals were killed by sodium pentobarbital overdose (150 mg/kg, i.v.) 1 hr after the last dose of vehicle or L-DOPA, the brains were removed quickly after death and bisected along the midline. For the histopathological study (n=5 untreated controls, 3 MPTP, 5 MPTP + L-DOPA), brains, hemispheres were post-fixed in formalin before paraffin-embedding. For the western blot study (n=5 untreated controls, 5 MPTP, and 5 MPTP + L-DOPA), the two hemispheres were immediately frozen by immersion in isopentane (-45°C) and then stored at -80°C. Coronal 300 µm-thick sections were cryostat-cut and punches of brain tissue were taken for several brain regions including the striatum and the hippocampus [14-18].

### **Brain processing**

Immunohistochemical studies were performed with procedures and antibodies used in human pathology, as described below. On 4µm thick coronal sections, a horseradish peroxidase method was used with the following antibodies : anti-tyrosine hydroxylase (TH), (monoclonal mouse antibody, 1:10000, Chemicon international, Temecula, California, USA), anti-human-specific α-synuclein clone LB509 (monoclonal mouse antibody, 1:100 ; Invitrogen by life technologies, France), anti-tau (polyclonal rabbit anti-human 1:1000; Dako, France), phosphorylation-dependant tau antibody (Tau AT8, PHF-tau monoclonal, mouse anti-human, clone AT8, 1:100; Pierce Biotechnology, USA), anti-Glial Fibrillary Acidic Protein (GFAP, polyclonal rabbit, 1:4000; Dako, France).

Sections were deparaffinized in toluene and rehydrated in graded series of ethanol. Antigen retrieval was not required for anti Tau or anti Tau AT8 labelling whereas sections labelled with GFAP were pre-treated with proteinase K (Dako, Trappes, France) and those labelled with LB509 α-synuclein were pressure-cooked in sodium citrate buffer (10 min in 0.01 M, pH 6.0). Non-specific binding was blocked with a universal blocking reagent (Biogenex) for LB509 α-synuclein, GFAP and Tau or with 5% normal goat serum in PBS for anti Tau AT8 for 30 min at room temperature. For TH labeling, non-specific binding was blocked with 3% normal horse serum in PBS for 60 min. Sections were incubated in primary antibodies overnight. Subsequently, sections were transferred in 3% H<sub>2</sub>O<sub>2</sub> in PBS for 5 min to quench endogenous peroxidase activity and treated with a ready-to-use goat anti mouse or goat anti rabbit EnVision-HRP enzyme conjugate (Dako,

France) for 30 min. For TH staining, sections were incubated with the biotinylated horse anti-mouse/rabbit IgG (1:200) secondary antibody for 2 hours and then with the avidin-biotin-peroxidase complex (ABC Elite Kit, PK-6200, Vector Labs) for further 90 min. Immunoreactions were revealed using the highly sensitive diaminobenzidine plus (DAB+) and the 3-amino-9 ethyl carbazol plus (AEC+) (both from Dako, France) as substrates chromogens. Finally, sections were counterstained with Mayer's hemalum and mounted in an aqueous medium for microscopy (Aquatex; Merck). Negative immunohistological control demonstrated the absence of signal when omitting the first antibody. Pictures were taken with a Leica microscope (Leica DM6000B) at x20 magnification.

To assess the localization of  $\alpha$ -synuclein, a double immunostaining was performed with anti-tyrosine hydroxylase (TH), (polyclonal rabbit antibody, 1:2000, Institute Jacques Boy, France) and anti  $\alpha$ -synuclein, clone LB509, (monoclonal mouse antibody, 1:100 ; Invitrogen by life technologies, France) antibodies. The same protocol for deparaffinization and antigen retrieval in sodium citrate buffer was used as above. Then, 4  $\mu$ m midbrain sections were blocked with 5% normal goat serum in PBS and sequentially incubated overnight with the primary antibodies. Secondary antibodies were Alexa Fluor 488-labeled goat anti-mouse for  $\alpha$ -synuclein, and Alexa Fluor 568-labeled goat anti-rabbit for TH (both from Invitrogen, France). To lower the intensity of autofluorescence, slides were incubated for 10 minutes with 0.1% Sudan Black B (Sigma-Aldrich, France) in 70% ethanol. After thorough washing in PBS, slides were mounted in fluorescence mounting medium (Agilent, USA). Immunofluorescence was visualized by using a Zeiss Axioplan 2 epifluorescent microscope at x40 magnification.

### **Quantitative analysis**

To minimize the inherent variability in the immunochemical procedures, all sections from all animals were processed simultaneously for a given antibody and brain region. The number of immunopositive cells was analysed using a computerized image analysis system (Mercator V6.50, Explora Nova) linked to a Leica microscope type DM 6000B. Quantitative analysis was carried out on the whole structure area and results were expressed as an average of immunopositive cells per  $\text{mm}^2$ .  $\alpha$ -Synuclein immunofluorescence intensity was assessed on 15  $\alpha$ -syn positive dopaminergic neurons in each animal. For cell fluorescence intensity, ImageJ software v1.52a was used to

measure pixel intensity and area with respect to background intensity and cell surface respectively, according to the following formula: Intensity of stained cell – (sample of background/area of sample background) X area of stained cell.

### **Sequential $\alpha$ -synuclein extraction and Western blot analysis**

Tissue was homogenized in Triton-X (TX) extraction buffer (50 mM Tris-base pH 7.6, 150 mM NaCl, 1% Triton-X-100, 2 mM EDTA) containing protease and phosphatase inhibitors [19]. The lysate was sonicated and then centrifuged (120,000 x g for 60 min at 4 °C) and the supernatant was collected (TX soluble fraction). The pellet was then washed 3 times with 1 M PBS/1% TX, centrifuged (13,000 x g for 15 min) and re-suspended in SDS extraction buffer (50 mM Tris pH 7.6, 150 mM NaCl, 1% Triton-X-100, 0.5% Na-deoxycholate, 1% SDS), sonicated, and centrifuged (120,000 x g for 60 min at 4 °C) and the supernatant was collected (TX insoluble fraction). The samples (30 ug) were run on 15% SDS-PAGE gels. Primary antibodies included antibodies against  $\alpha$ -syn [monoclonal syn-1, (1:1000; BD Biosciences)] and  $\beta$ -actin (1:2000; Millipore Sigma A5441) as a loading control. The intensity of the immunoreactive bands was estimated by densitometric quantification using ImageJ (relative density, RD) and then normalized to the corresponding  $\beta$ -actin levels.

### **Statistical analysis**

Data are presented as mean  $\pm$  SEM. Assumptions about normality of distribution and homogeneity of variances were analysed using the Shapiro-Wilk and Brown-Forsythe tests, respectively. Data were analysed using one-way ANOVA followed by post hoc Tukey tests for multiple comparisons or using Kruskal-Wallis followed by Dunn's multiple comparisons tests whenever appropriate. These analyses were completed using Graphpad Prism V 8.0. A probability level of 5% ( $p < 0.05$ ) was considered significant.

### **Data Accessibility**

The data are available from the corresponding author upon request.

## Results

### L-DOPA treatment does not affect MPTP-induced neurodegeneration and astrogliosis

We first aimed to determine if L-DOPA could have an impact on neuronal loss in the substantia nigra. As expected, MPTP intoxication induced a significant loss of TH-positive neurons in the substantia nigra ( $F_{(2,10)}=46.40$ ,  $p<0.0001$ , **Fig. 1**). The extent of TH-immunoreactive cell loss was similar between MPTP (79.9%,  $p<0.0001$ ) and MPTP + L-DOPA-treated monkeys (76.8%,  $p<0.0001$ ). Counting of Nissl-stained neurons confirmed neurodegeneration ( $F_{(2,10)}=12.46$ ,  $p<0.01$ ), both in MPTP (-66%,  $p<0.01$ ) and in MPTP + L-DOPA-treated animals (-80.4%,  $p<0.01$ ). All MPTP-treated monkeys exhibited a mild parkinsonian syndrome that was alleviated in animals receiving L-DOPA (data not shown), as previously reported [13, 20-22].

Because astrogliosis is a prominent feature of PD that is recapitulated in MPTP models [23], we next investigated if L-DOPA could affect the number of reactive astrocytes. MPTP intoxication induced significant astrogliosis in the nigrostriatal pathway compared with controls, as shown by increased densities of GFAP-positive astrocytes in the substantia nigra ( $F_{(2,10)}=10.42$ ,  $p<0.01$ , **Fig. 2A**) and putamen ( $H=9.11$ ,  $df=2$ ,  $p<0.01$ , **Fig. 2B**). Astrogliosis was also significant in MPTP + L-DOPA-treated monkeys compared with controls in the substantia nigra ( $p<0.01$ ) but not in the putamen. In the temporal cortex, MPTP + L-DOPA-treated animals displayed increased counts of GFAP-positive astrocytes compared with controls ( $F_{(2,10)}=6.54$ ,  $p<0.05$ , **Fig. 2D**). No significant effects of MPTP or MPTP + L-DOPA were found in CA1 ( $H=3.53$ ,  $df=2$ ,  $p=0.17$ , **Fig. 2C**) or in the dentate nucleus ( $H=2.34$ ,  $df=2$ ,  $p=0.32$ , **Fig. 1E**). The density of GFAP-positive astrocytes was not significantly different between MPTP and MPTP + L-DOPA-treated monkeys in any of the regions investigated, indicating that chronic L-DOPA treatment had no effect on MPTP-induced astrogliosis. Microscopic illustrations of the density of GFAP-positive astrocytes in the different regions of interest are depicted for control (**Fig. 2F-J**), MPTP (**Fig. 2K-O**), and MPTP + L-DOPA-treated (**Fig. 2P-T**) monkeys.

### MPTP and L-DOPA differentially affect $\alpha$ -synuclein and tau accumulation

MPTP intoxication and subsequent chronic L-DOPA treatment differentially affected  $\alpha$ -synuclein immunoreactivity (**Fig. 3**). In the substantia nigra, there was a significant

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difference on the density of  $\alpha$ -synuclein immuno-positive neurons between groups ( $H = 6.76$ ,  $df = 2$ ,  $p < 0.05$ , **Fig. 3A**). Post-hoc analysis indicated increased  $\alpha$ -synuclein staining in MPTP-treated monkeys compared to controls ( $p < 0.05$ ). A significant effect was found for the density of  $\alpha$ -synuclein positive fibres in the putamen ( $H = 7.47$ ,  $p < 0.05$ , **Fig. 3B**), where  $\alpha$ -synuclein immunostaining was significantly elevated in MPTP-treated monkeys compared with the MPTP + L-DOPA group ( $p < 0.05$ ). A similar effect was found in CA1 ( $H = 11.76$ ,  $p < 0.01$ , **Fig. 3C**) and post-hoc analysis indicated that MPTP-treated monkeys displayed increased  $\alpha$ -synuclein immunoreactivity compared with control and MPTP + L-DOPA groups ( $p < 0.01$ ). In the temporal cortex the density of  $\alpha$ -synuclein-immunopositive fibres was also significantly different between groups ( $H = 8.366$ ,  $df = 2$ ,  $p < 0.01$ , **Fig. 3D**), with the MPTP group displaying higher density of  $\alpha$ -synuclein positive fibres compared with the control and MPTP + L-DOPA groups ( $p < 0.05$ ). Conversely, no significant effect was found in the dentate nucleus ( $H = 3.33$ ,  $p = 0.23$ , **Fig. 3E**). Remarkably, the marked  $\alpha$ -synuclein immunostaining following MPTP was abolished in animals having received L-DOPA in all regions except in the substantia nigra (**Fig. 3Q-T**). Double immunofluorescence for  $\alpha$ -synuclein and tyrosine hydroxylase revealed no cytoplasmic accumulation of  $\alpha$ -synuclein in controls (**Fig. 4A-C**) and confirmed that  $\alpha$ -synuclein accumulation in the substantia nigra of MPTP and MPTP + L-DOPA-treated animals occurred in the remaining dopaminergic neurons (**Fig. 4D-I**). Quantification of  $\alpha$ -synuclein fluorescence intensity in remaining nigral dopaminergic neurons revealed that albeit L-DOPA was not able to clear  $\alpha$ -synuclein, immunofluorescence intensity was significantly reduced in MPTP + L-DOPA compared with the MPTP group ( $p < 0.05$ , **Fig. 4J**).

Because Tau pathology is frequent in PD [24], we investigated the effects of MPTP and subsequent L-DOPA treatment on Tau accumulation. Contrary to the effect on the accumulation and extent of  $\alpha$ -synuclein, MPTP intoxication and subsequent chronic L-DOPA treatment led to only modest effects on tau immunoreactivity (**Fig. 5**). In the nigrostriatal pathway, MPTP and MPTP + L-DOPA induced a non-significant increase in the number of tau immunoreactive neurons in the substantia nigra ( $H = 3.14$ ,  $p = 0.21$ , **Fig. 5A**) and in the putamen ( $F_{(2,10)} = 1.95$ ,  $p = 0.19$ , **Fig. 5B**). No significant effects of MPTP and MPTP + L-DOPA were found in CA1 ( $F_{(2,10)} = 0.52$ ,  $p = 0.60$ , **Fig. 5C**) or in the dentate



nucleus ( $H=1.80$ ,  $df=2$ ,  $p=0.43$ , **Fig. 5E**). Conversely, there was a significant effect in the temporal cortex ( $F_{(2,10)}=4.7$ ,  $p<0.05$ ). Post-hoc analysis indicated a significantly increased number of tau immunoreactive neurons in MPTP-treated monkeys ( $p<0.05$ ) compared with controls (**Fig. 5D**). Additional investigation of the effect of MPTP and chronic L-DOPA administration on tau was performed using the AT8 antibody that recognizes tau phosphorylated at serine 202 and threonine 205 (data not shown). Counts of AT8-immunopositive neurons revealed no significant effects of MPTP and MPTP + L-DOPA in the substantia nigra ( $H=3.10$ ,  $p=0.22$ ), CA1 ( $F_{(2,10)}=0.21$ ,  $p=0.81$ ), temporal cortex ( $F_{(2,10)}=0.58$ ,  $p=0.57$ ), and dentate nucleus ( $H = 2.75$ ,  $p=0.27$ ), while in the putamen, there was a trend toward an increase of tau AT8-positive neurons in MPTP and MPTP + L-DOPA-treated animals ( $H = 4.94$ ,  $p= 0.07$ ).

To further investigate the effect of chronic L-DOPA treatment on  $\alpha$ -synuclein homeostasis, putamen and hippocampus samples from a separate cohort of control, MPTP and MPTP + L-DOPA-treated monkeys were subjected to a sequential protein extraction protocol in order to quantify triton-X (TX) soluble and TX insoluble  $\alpha$ -synuclein by western blot (**Fig. 6**).  $\alpha$ -synuclein immunoblots performed on TX-soluble and TX-insoluble fractions revealed a signal at the expected weight of 14 kDa (full length  $\alpha$ -synuclein) and a faint signal at 12 kDa (truncated  $\alpha$ -synuclein) that could be quantified upon overexposure. No high molecular weight assemblies of  $\alpha$ -synuclein were detected. In the putamen, there was no significant effect of MPTP or MPTP + L-DOPA on full-length  $\alpha$ -synuclein ( $F_{(2,12)}=1.83$ ,  $p=0.20$ , **Fig. 6A, C**), whereas a significant effect was found for the 12kDa band ( $F_{(2,12)}=20.12$ ,  $p<0.0001$ , **Fig. 6A, D**) in the TX soluble fraction. Post-hoc analysis revealed a significant decrease of  $\alpha$ -synuclein in MPTP + L-DOPA-treated animals compared with controls and with the MPTP group ( $p<0.01$ ). Interestingly, in the TX-insoluble fraction, there was a significant effect of treatment on full length  $\alpha$ -synuclein ( $F_{(2,12)}=10.05$ ,  $p<0.01$ , **Fig. 6B, E**), with MPTP + L-DOPA-treated monkeys showing significantly less  $\alpha$ -synuclein than their MPTP-treated counterparts ( $p<0.05$ ) and controls ( $p<0.01$ ). A significant effect was also observed on 12 kDa  $\alpha$ -synuclein ( $F_{(2,12)}=8.95$ ,  $p<0.01$ , **Fig. 6B, F**), with reduced 12 kDa  $\alpha$ -synuclein in the MPTP + L-DOPA compared with the MPTP ( $p<0.01$ ) and control ( $p<0.05$ ) groups.

In the hippocampus, there was a significant effect on full length  $\alpha$ -synuclein in the TX-soluble fraction ( $F_{(2,12)}=12.17$ ,  $p<0.01$ , **Fig. 6G, I**), with MPTP + L-DOPA-treated monkeys showing significantly more  $\alpha$ -synuclein than their MPTP-treated counterparts ( $p<0.01$ ) and controls ( $p<0.05$ ). No significant effect was detected for the 12kDa band ( $F_{(2,12)}=2.47$ ,  $p=0.12$ ). In the TX-insoluble fraction, no significant differences were found among the three groups for full length  $\alpha$ -synuclein ( $F_{(2,12)}=1.49$ ,  $p=0.26$ ). However, there was a significant effect of 12 kDa  $\alpha$ -synuclein ( $F_{(2,12)}=10.92$ ,  $p<0.01$ ). Post-hoc analysis revealed that 12 kDa  $\alpha$ -synuclein was significantly increased in MPTP-treated monkeys compared with controls ( $p<0.05$ ) and MPTP + L-DOPA-treated animals ( $p<0.01$ , **Fig. 5L**).

## Discussion

The spatial and temporal evolution of  $\alpha$ -synuclein accumulation over the course of PD has been well-described [3]. However very little is known about the impact of long-term exposure to DRT (i.e., the gold-standard pharmacological treatment of PD) on the propagation/distribution/accumulation of  $\alpha$ -synuclein. Since it has already been shown that intoxication with MPTP induces  $\alpha$ -synuclein aggregation in the SNpc of NHPs [8-11], we therefore investigated in MPTP-treated monkeys the fate of  $\alpha$ -synuclein and tau in several regions of the central nervous system relevant to the pathophysiology of PD and examined the consequences of chronic DRT on the pattern and extent of these aggregation-prone proteins. Therefore, characterizing the impact of DRT on their fate could aid in developing and validating reliable biomarkers to assess the progression and severity of the disease.

As reported previously, MPTP-treated animals showed large neuronal loss and pronounced astrogliosis in the SNpc (**Fig. 1&2**) [23]. Moreover, we showed that MPTP-induced astrogliosis was not limited to the substantia nigra but also occurred in the putamen (**Fig. 2B**) and the temporal cortex (**Fig. 2D**). So far, it has been demonstrated that MPTP - possibly due to its inhibitory action on the complex I of the mitochondrial respiratory chains [25, 26] - induced  $\alpha$ -synuclein accumulation in the surviving midbrain dopaminergic neurons of NHPs [8-11] and mice [27, 28]. In line with these studies, we found a significant increase in the density of  $\alpha$ -synuclein immuno-positive neurons in the SNpc of the MPTP-treated monkeys (**Fig. 3A, Fig. 4**). Moreover, we demonstrate for the first time in MPTP-treated monkeys that the density of  $\alpha$ -synuclein increased in the putamen (i.e., a motor-related/subcortical structure, **Fig. 3B**) as well as in neurons of non-motor and cortical regions, such as the hippocampal CA1 (**Fig. 3C**) region and temporal cortex (**Fig. 3D**). In PD, LBs in which  $\alpha$ -synuclein is a major constituent are widely distributed in the central nervous system, including brainstem, subcortical and cortical areas [2, 3, 29, 30]. Moreover, their distributions are most likely correlated with the motor and non-motor symptoms of PD [2, 3, 30, 31]. A major difference between idiopathic PD and the MPTP-primate models of PD is the presence of insoluble, high molecular weight assemblies of  $\alpha$ -synuclein in LBs in PD, but not in animal models of PD

[8, 11]. Here, and in line with previous results, we did not find LBs in the different brain regions of MPTP-treated animals. Nevertheless, we still demonstrated the accumulation of  $\alpha$ -synuclein in non-motor cortical regions, indicating that the widespread synucleinopathy classically observed in PD patients is somewhat recapitulated in the MPTP NHP model of PD.

Under physiological conditions,  $\alpha$ -synuclein is a soluble protein widely distributed in the brain and especially expressed in the presynaptic nerve terminals [2, 32]. However, during the disease process,  $\alpha$ -synuclein changes its conformation to form oligomers and high molecular weight insoluble aggregates. Previous studies have shown that the clearance of  $\alpha$ -synuclein in the substantia nigra is mainly related to autophagy-lysosomal pathway (reviewed in [33]), in particular through the chaperone-mediated autophagy (CMA), as  $\alpha$ -synuclein is a CMA-substrate. Autophagy is impaired in the substantia nigra in experimental models of parkinsonism (including MPTP models) and in PD, as shown with deficits in several lysosomal proteins such as LC3, LAMP1, LAMP-2a, cathepsin-D, glucocerebrosidase or ATP13A2 [34-39].

Remarkably, post-translational modifications, such as phosphorylation, ubiquitination, nitration and truncation are possible enhancers for  $\alpha$ -synuclein aggregation [40-42]. In accordance with the aggregation propensity of truncated  $\alpha$ -synuclein, our biochemical results revealed an increase in the TX insoluble fraction of the truncated (not the full-length)  $\alpha$ -synuclein in hippocampus samples of MPTP-treated monkeys. However, we did not find high molecular weight assemblies of  $\alpha$ -synuclein in our experimental conditions that might reflect aggregated forms of  $\alpha$ -synuclein as occurring in LBs. Overall, these biochemical results corroborate the histopathological results that showed that MPTP-induced  $\alpha$ -synuclein accumulation in NHPs is not limited to the substantia nigra and that the increased intraneuronal  $\alpha$ -synuclein immunoreactivity was insoluble but not in the structural form of LBs. Furthermore, the lack of effect of MPTP on total  $\alpha$ -synuclein levels (**Fig. 6**) together with the widespread increased immunoreactivity (**Fig. 3**) suggest that an anatomical redistribution of the protein may possibly occur, leading to its accumulation in cell bodies or processes.

Although  $\alpha$ -synuclein is a major constituent of LBs, other aggregation prone proteins such as tau, play a role in the pathogenesis of LBs [1, 2]. Indeed, tau is also the major structural component of neurofibrillary tangles (NFTs). It has already been showed that tau and  $\alpha$ -synuclein co-exist in LBs, and that NFTs are present around LBs [1, 43-45], thus indicating that tau aggregation and NFTs are also significant hallmarks of PD. However, whether tau accumulation, tau aggregation and NFTs occur in the MPTP animal models of PD remains relatively unexplored. Here, we only observed a slight increase in the density of tau immuno-positive neurons in the temporal cortex of MPTP-treated monkeys (**Fig. 5D**), thus indicating that MPTP intoxication induced larger effects on  $\alpha$ -synuclein than tau accumulation. Importantly, earlier *in vitro* studies demonstrated that the hyperphosphorylation of tau may promote its aggregation [46]. Moreover, tau is usually phosphorylated in LBs and NFTs [43-45]. Phosphorylation of tau at Threonine 231, Serine 202/Threonine 205 and Serine 396 has already been reported in Alzheimer disease [47, 48]. Remarkably, in the MPTP mouse model of PD, MPTP-induced tau hyperphosphorylation - which was  $\alpha$ -synuclein-dependent - was found at Serine 396/404 [27]. Moreover, an increase in tau phosphorylation at Serine 202/Threonine 205, which might occur through GSK3 $\beta$  pathway, was also recently observed in the MPTP mouse model of PD in which phosphorylated tau and  $\alpha$ -synuclein co-localized in SNpc and hippocampus and formed plaques in SNpc [48]. In contrast with this latter study, despite  $\alpha$ -synuclein accumulation, we did not find significant increases in the density of phosphorylated tau (detected using the AT8 antibody) immuno-positive neurons in the SNpc, putamen, hippocampal CA1 region, temporal cortex and dentate nucleus of the MPTP-treated monkeys. Therefore, our results suggest that although changes in  $\alpha$ -synuclein homeostasis might be necessary for phosphorylated tau accumulation and aggregation in PD brains [27],  $\alpha$ -synuclein accumulation does not necessarily lead to phosphorylated tau accumulation and aggregation.

Earlier *in vitro* studies have showed that dopamine, L-DOPA, and other catecholamines (e.g., adrenaline and noradrenaline) inhibit  $\alpha$ -synuclein fibrillation [49-51] and its degradation through chaperone-mediated autophagy [52]. In aerobic conditions, these catecholamines tend to oxidize and their oxidative products appear to be even more

effective for inhibiting  $\alpha$ -synuclein fibrillation [50]. In line with these results, it has been demonstrated that the capacity of dopamine and L-DOPA to prevent the formation of  $\alpha$ -synuclein fibrils was cancelled by the addition of antioxidants in the growth medium [49]. Since quinones and aminochromes are two of the most stable classes of oxidative products derived from dopamine and L-DOPA, they could be at the source of  $\alpha$ -synuclein fibril inhibition induced by dopamine and L-DOPA in these studies [50]. Indeed, it has been proposed that inhibition  $\alpha$ -synuclein fibrillation is due to a covalent modification of  $\alpha$ -synuclein by dopamine-derived orthoquinone [49].

Although the relationship between distinct conformations of  $\alpha$ -synuclein and neurodegeneration is still unclear and debated, if  $\alpha$ -synuclein fibrillation process is involved in neuronal death [53, 54], then disaggregating  $\alpha$ -synuclein fibrils may reverse or slow down PD progression. However, despite the unique value of NHP models in translational research, the effect of DRT (L-DOPA+ carbidopa) on propagation/distribution/accumulation of pathological insoluble  $\alpha$ -synuclein in the brain of the MPTP NHP model of PD was still unexplored. In the current study, we found that  $\alpha$ -synuclein immunostaining was no longer detected in the putamen, hippocampal CA1 region, temporal cortex and dentate nucleus of the MPTP-treated monkeys with L-DOPA, thus indicating that DRT wiped out MPTP-induced alterations of  $\alpha$ -synuclein immunoreactivity in several brain regions of the central nervous system relevant to the pathophysiology of PD (**Fig. 3B-E**). However,  $\alpha$ -synuclein immuno-positive SNpc neurons were still detected in the MPTP+L-DOPA group (**Fig. 3A**), even though the intensity of  $\alpha$ -synuclein immunofluorescence was reduced in animals treated with L-DOPA (**Fig. 4**). The marked deleterious effect of MPTP on autophagy in the SNpc may have contributed to the reduced clearance of  $\alpha$ -synuclein in this region. In contrast, we did not find any effect of L-DOPA on the density of the tau immuno-positive neurons in any brain regions investigated (**Fig. 5**). In line with these results, our biochemical results revealed that L-DOPA mainly affected the TX insoluble fraction of  $\alpha$ -synuclein (**Fig. 6**) from both putamen and hippocampus samples, so that the TX insoluble fraction of the full-length and the truncated  $\alpha$ -synuclein decreased in putamen samples (**Fig. 6E and F, respectively**), while only the truncated  $\alpha$ -synuclein decreased in the hippocampus samples (**Fig. 6L**). Accordingly, it has been shown that catecholamines (probably via

their oxidative product as discussed above) not only inhibited  $\alpha$ -synuclein fibril formation but also disassembled existing fibrils generated *in vitro* and  $\alpha$ -synuclein deposits in tissue samples from SNpc of mice treated with the herbicide paraquat [50]. Similarly, it has been recently showed that administration of L-DOPA reduced phosphorylated  $\alpha$ -synuclein accumulation in the SNpc of mice injected with preformed synthetic  $\alpha$ -synuclein fibrils in the striatum [55]. Together with these studies performed in mice, our results obtained in NHPs reveal the ability of L-DOPA to mitigate  $\alpha$ -synuclein pathology, either triggered by neurotoxins [50] or by  $\alpha$ -synuclein fibrils [55]. Although further experiments are needed to understand why DRT might be less effective on  $\alpha$ -synuclein pathology in the SNpc compared to others brain regions, our results strengthen earlier *in vitro* and *in vivo* reports. Moreover, our study is the first to demonstrate *in vivo* that DRT can dramatically ameliorate  $\alpha$ -synuclein pathology in the MPTP NHP model of PD.

Our results may also have implications regarding the possible effects of DRT on the natural course of the disease that are tremendously difficult to apprehend in clinical trials. The earlier vs. later L-DOPA (ELLDOPA) trial that evaluated the effects of DRT on the progression of the disease suggested a possible neuroprotective effect of L-DOPA [56]. However, in the Levodopa in Early Parkinson's Disease (LEAP) trial, no evidence for a disease-modifying effect of L-DOPA was found [57]. What these trials show, in light of our results, is that L-DOPA does not protect from synucleinopathy as a *primum movens*. In the MPTP monkey model, the observed synucleinopathy is secondary to the neurotoxin insult and this secondary reaction is sensitive to L-DOPA. In idiopathic PD, one can assume that the primary synucleinopathy is a triggering factor and no evidence for sensitivity to L-DOPA has been shown so far.

The histopathological hallmark of Parkinson's disease (PD) is the presence of fibrillar aggregates referred to as Lewy bodies (LBs), in which  $\alpha$ -synuclein is a major constituent [2, 30]. Accordingly,  $\alpha$ -synuclein is regarded as a promising biomarker of the disease [5, 40]. Indeed,  $\alpha$ -synuclein can be measured in peripheral tissue and body fluids (e.g., cerebrospinal fluid, plasma/serum, blood and saliva) [40]. Although we are still unable to directly image aggregates of  $\alpha$ -synuclein, their structural and functional consequences can be detected by current imaging strategies (e.g., MRI, SPECT, PET) [5]. In this context and given the translational significance of the NHP studies, our results are

fundamental and indicate that patient's dopaminergic medication should be systematically taken in consideration when assessing  $\alpha$ -synuclein as a biomarker for accurate diagnosis, monitoring disease progression and response to treatment. In fact, special attention should be paid to the patient's full medical record and not only DRT since other drugs widely used in PD therapy (e.g., MAO-B inhibitors) may also influence  $\alpha$ -synuclein homeostasis.



## **Acknowledgements**

The Université de Bordeaux, Université de Poitiers, Centre National de la Recherche Scientifique and Institut National de la Santé et de la Recherche Médicale provided infrastructural support.

## **Author Contributions**

EB, POF, BD designed the research; MHC, MT, QL performed the research; POF, MT, MD analyzed the data; MD, POF, EB wrote the paper; All authors read and approved the final version of the manuscript.

## **Ethical approval**

Experiments were carried out in an AAALAC-accredited facility and in accordance with the European Union Directive (2010/63/EU) on the protection of animals used for scientific purposes. Experiments were performed after acceptance of the study design by the Institute of Lab Animal Science (Chinese Academy of Science, Beijing, China) IACUC for experiments on non-human primates.

## **Conflict of interest**

The authors declare no conflict of interest.

## Figure legends

### **Figure 1: Effects of MPTP and L-DOPA on neuronal loss in the SNpc.**

Counting of TH-immunopositive neurons in the SNpc. (A) Representative images of TH immunostaining in the SNpc of control (B), MPTP (C) and MPTP + L-DOPA (D) treated monkeys. Scale bar = 200  $\mu\text{m}$ . \*\*\*\*,  $p < 0.0001$

### **Figure 2: Effects of MPTP and L-DOPA on astrogliosis**

Astrogliosis, as determined by the density of GFAP immunopositive astrocytes in the SNpc (A), putamen (B), CA1 (C), temporal cortex (D) and dentate nucleus (E). Representative images of GFAP immunostaining in the SNpc (F, K, P), putamen (G, L, O), CA1 (H, M, R), temporal cortex (I, N, S) and dentate nucleus (J, O, P). Scale bar = 50  $\mu\text{m}$ . \*,  $p < 0.05$ ; \*\*,  $p < 0.01$  compared with the control group.

### **Figure 3: Effects of MPTP and L-DOPA on $\alpha$ -synuclein accumulation**

$\alpha$ -Synuclein accumulation, as determined by the density of  $\alpha$ -synuclein immunopositive neurons in the SNpc (A), fibers in the putamen (B), CA1 (C), and temporal cortex (D) and neurons dentate nucleus (E). Representative images of  $\alpha$ -synuclein immunostaining in the SNpc (F, K, P), putamen (G, L, O), CA1 (H, M, R), temporal cortex (I, N, S) and dentate nucleus (J, O, P). Scale bar = 50  $\mu\text{m}$ . \*,  $p < 0.05$  vs control group; \*\*,  $p < 0.05$  vs MPTP group, \*\*\*\*,  $p < 0.01$  vs control and MPTP+L-DOPA groups.

### **Figure 4: Phenotypic identity of $\alpha$ -synuclein accumulating neurons in the SNpc**

Double immunofluorescence for  $\alpha$ -synuclein (A, D, G) and tyrosine hydroxylase (B, E, H) and overlay (C, F, I), in control (A-C), MPTP (D-F) and MPTP+L-DOPA (G-I) treated monkeys. Scale bar = 30  $\mu\text{m}$ . Quantification of  $\alpha$ -synuclein fluorescence intensity in remaining nigral dopaminergic neurons of MPTP and MPTP + L-DOPA treated animals (J). \*,  $p < 0.05$ .

### **Figure 5: Effects of MPTP and L-DOPA on tau accumulation**

Tau accumulation, as determined by the density of tau immunopositive neurons in the SNpc (**A**), putamen (**B**), CA1 (**C**), temporal cortex (**D**) and dentate nucleus (**E**). Representative images of tau immunostaining in the SNpc (**F**, **K**, **P**), putamen (**G**, **L**, **O**), CA1 (**H**, **M**, **R**), temporal cortex (**I**, **N**, **S**) and dentate nucleus (**J**, **O**, **P**). Scale bar = 50  $\mu$ m. \*,  $p < 0.05$  compared with the control group.

**Figure 6: Effects of MPTP and L-DOPA on  $\alpha$ -synuclein homeostasis and insolubility**

$\alpha$ -Synuclein immunoblot levels following sequential protein extraction of putamen (**A**, **B**) and hippocampus (**G**, **H**), in triton soluble (**A**, **G**) and insoluble fractions (**B**, **H**) and quantification of full-length (14 kDa) and truncated (12 kDa)  $\alpha$ -synuclein in the putamen (**C-F**) and hippocampus (**I-L**).  $n=5$  per group. Data represent mean  $\pm$  SEM. \*,  $p < 0.05$  ; \*\*,  $p < 0.01$ .

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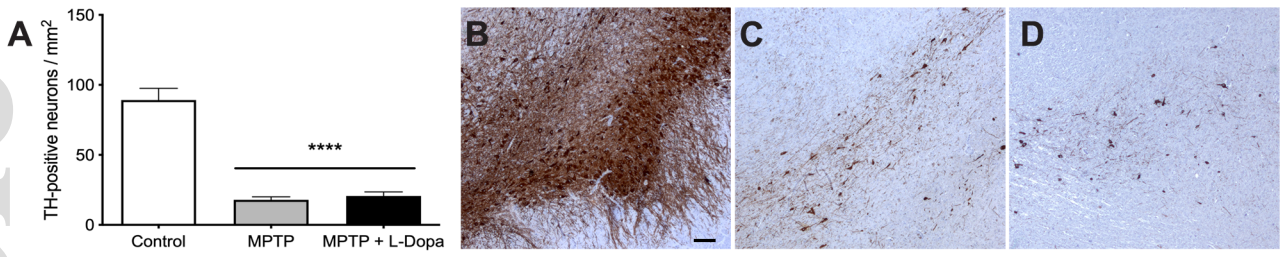
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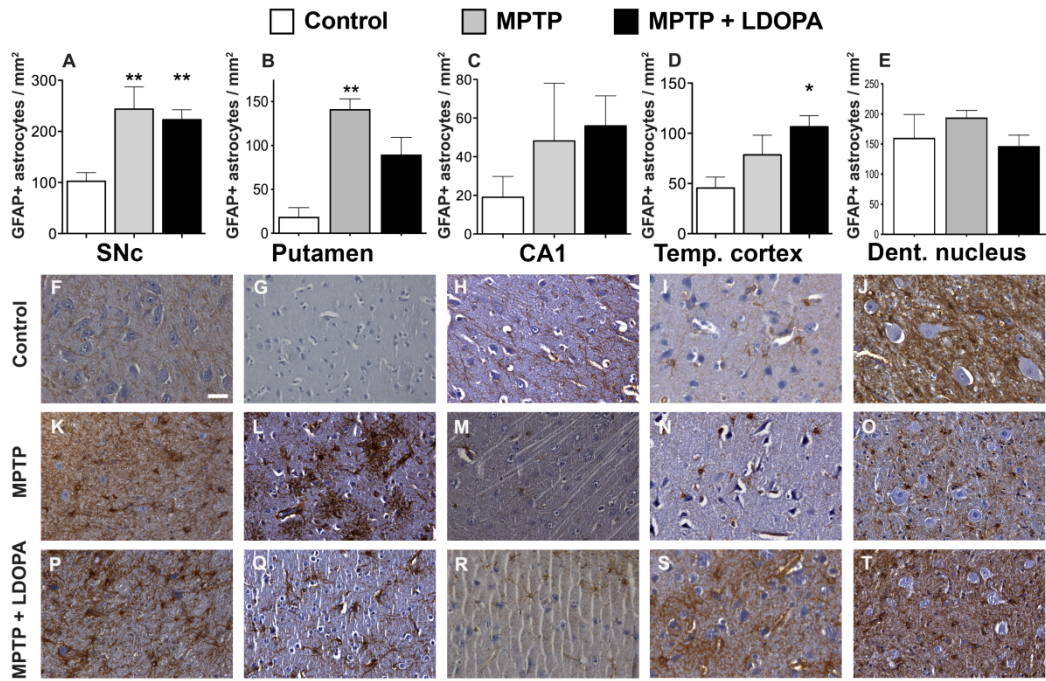
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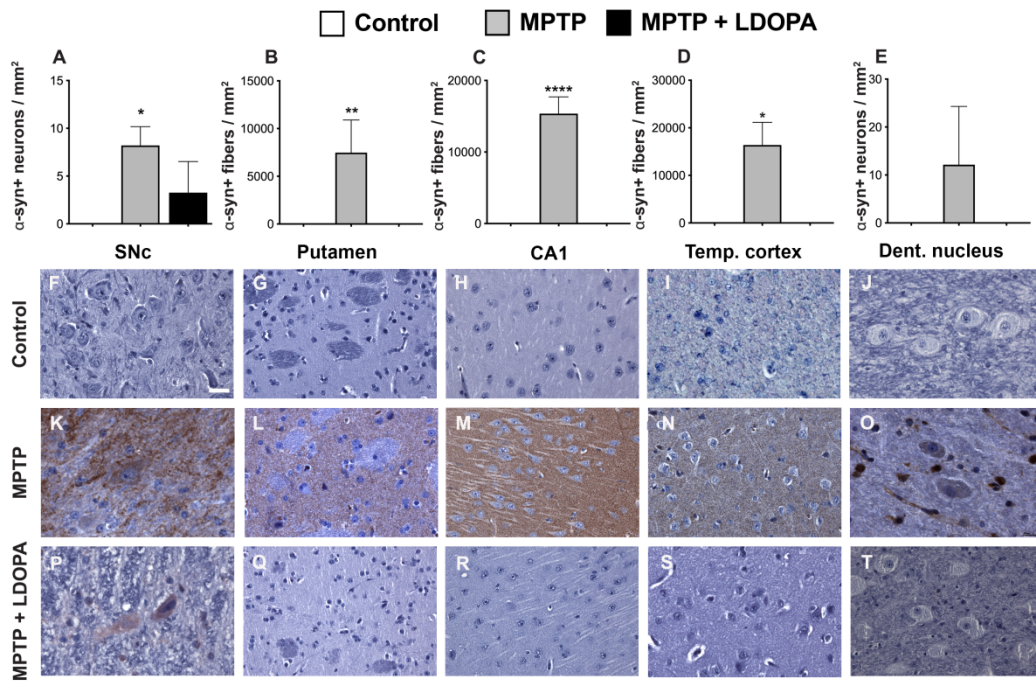
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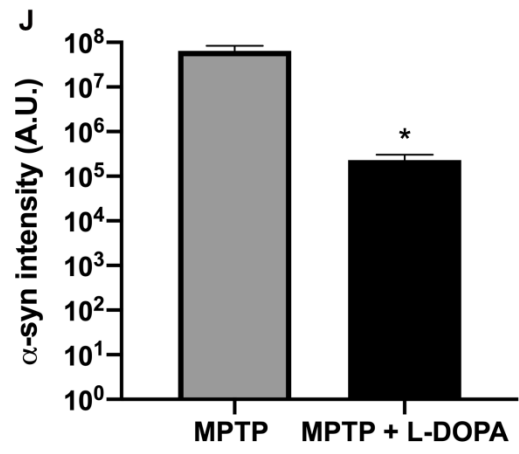
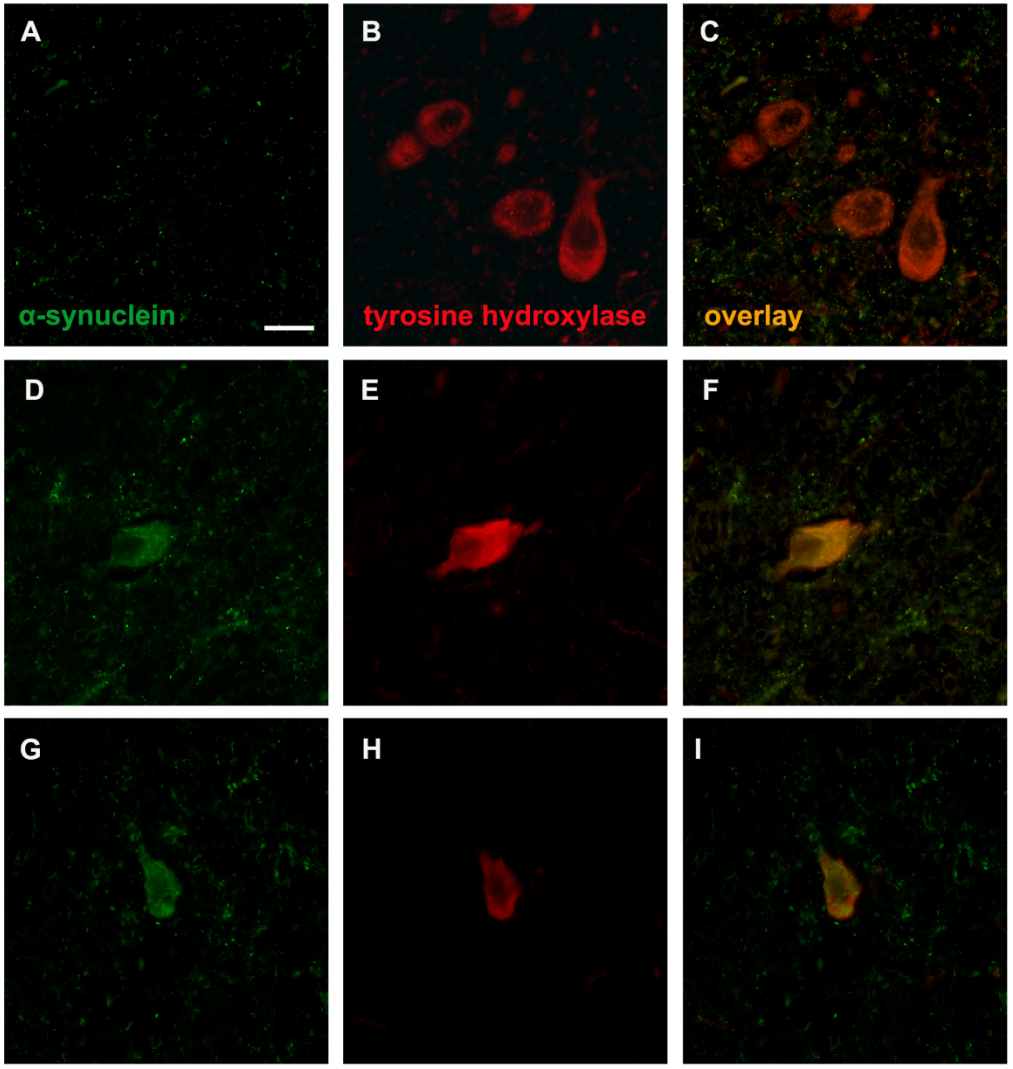
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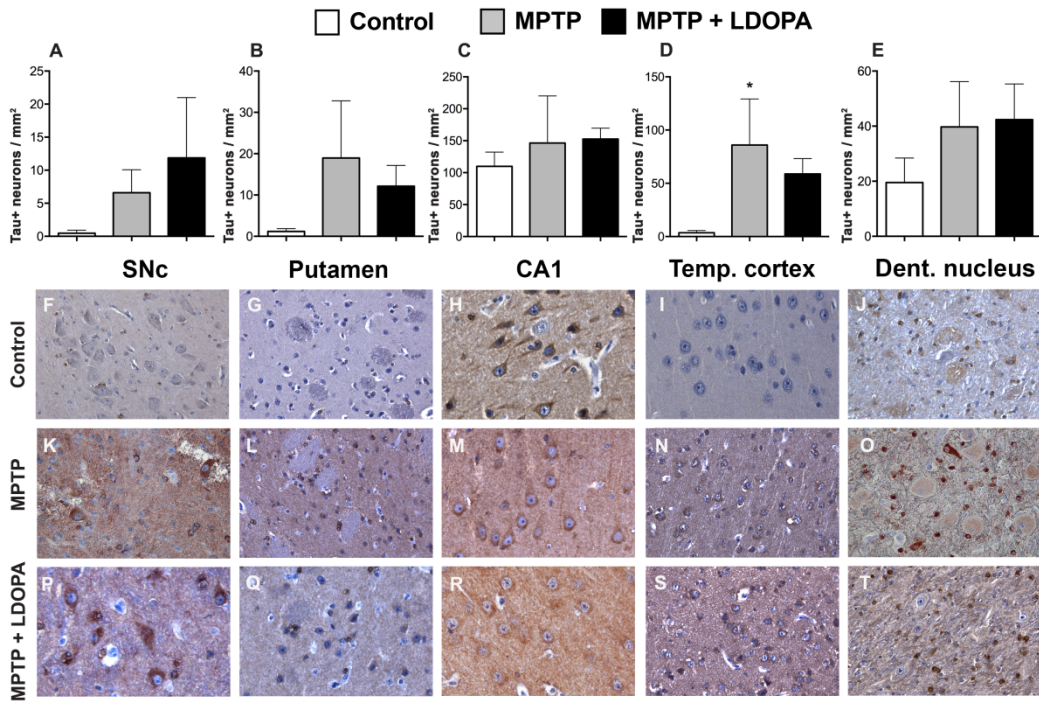
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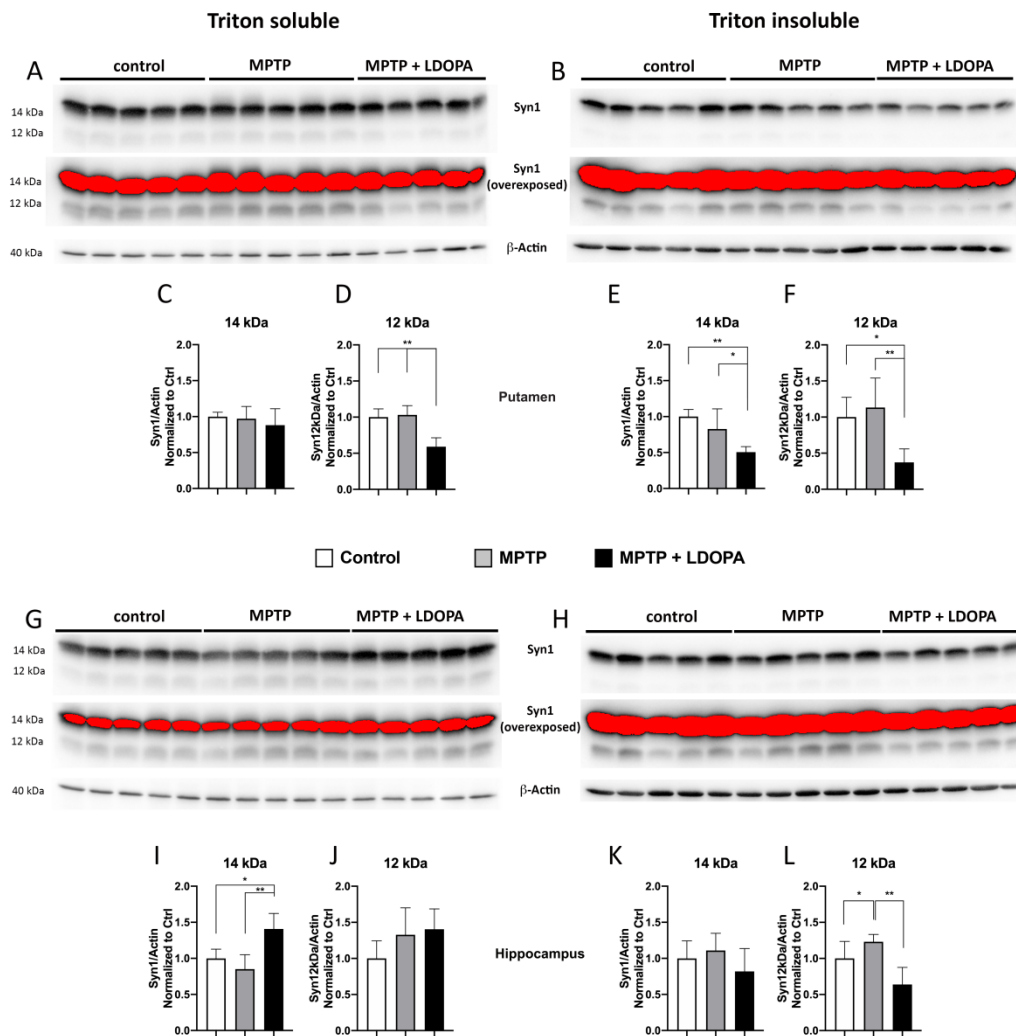
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