The C-terminal fragment of LRRK2 with the G2019S substitution increases the neurotoxicity of mutant A53T α-synuclein in dopaminergic neurons in vivo

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**Research**

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Abstract

Background: Alpha-synuclein (α-syn) and leucine-rich repeat kinase 2 (LRRK2) likely play crucial roles both in sporadic and familial forms of Parkinson's disease (PD). The most prevalent mutation in LRRK2 is the G2019S substitution, which induces neurotoxicity through increased kinase activity. There is likely an interplay between LRRK2 and α-syn involved in the neurodegeneration of dopaminergic (DA) neurons in the substantia nigra (SNpc) in PD. However, the mechanisms underlying this interplay are ill-defined. Here, we investigated whether LRRK2 G2019S can increase the neurotoxicity induced by a mutant form of α-syn (A53T mutation) in DA neurons in vivo.

Methods: We used a co-transduction approach with adeno-associated virus (AAV), AAV2/6 vectors encoding human α-syn A53T and the C-terminal portion of LRRK2 (ΔLRRK2), which contains the kinase domain, with either the G2019S mutation (ΔLRRK2 G2019S) alone or the D1994A mutation (ΔLRRK2 G2019S/D1994A), which inactivates the kinase activity of LRRK2. The AAVs were co-injected into the rat SNpc and histological evaluation was performed at 6- and 15-weeks post-injection (PI).

Results: The majority of SNpc neurons co-expressed ΔLRRK2 and human α-syn A53T after transduction. ΔLRRK2 G2019S alone produced no cell loss at 15-weeks PI. Injection of AAV-α-syn A53T alone or mixed with a control AAV coding for GFP produced a significant loss of DA neurons. Co-injection of AAV-α-syn A53T with AAV-ΔLRRK2 G2019S instead of GFP slightly exacerbated that neuronal loss We also studied the inactive form, ΔLRRK2 G2019S/D1994A at 6 weeks PI. Injection of AAV-ΔLRRK2 G2019S mixed with AAV-α-syn A53T produced a neurotoxic effect that was stronger than that produced by the co-injection of AAV-DLRRK2 G2019S/D1994A and AAV-α-syn A53T.

Conclusion: Thus, these results show that mutant LRRK2 may selectively facilitate α-syn toxicity in DA neurons through a cell-autonomous mechanism involving its kinase domain. However, considering that the effect of ΔLRRK2 G2019S upon human α-syn A53T is moderate in our paradigm where pathological proteins are overexpressed, the study supports the hypothesis that the interplay between LRRK2 and α-syn may also implicate non-cell-autonomous mechanisms such as those involved in neuroinflammation and spreading of α-syn aggregated species.

Background

Parkinson's disease (PD) is a neurodegenerative disorder affecting approximately seven million people worldwide. Early in the course of the disease, the most obvious symptoms are movement-related, including shaking (resting tremor), rigidity, and slowness of movement [1,2]. The neuropathological hallmarks of PD are characterized by the progressive loss of dopaminergic (DA) neurons in the substantia nigra pars compacta (SNpc) and the presence of neuronal aggregates (Lewy bodies) and dystrophic Lewy neurites containing the protein α-synuclein (α-syn) [3]. There is currently no treatment to delay this neurodegeneration and the cause of α-syn aggregation and the preferential death of DA
neurons is unknown. PD is mainly a sporadic neurodegenerative disorder but approximately 10% of the cases are of genetic origin and several genes have been identified as causative factors [4].

Duplication, triplication, and rare mutations (A53T, A30P, E46K, H50Q, G51D, A53E) in the SNCA gene encoding the a-syn protein have been found in families with dominantly-inherited PD and are associated with early-onset forms, with an amplification of a-syn aggregation [4–7]. The A53T [8], A30P [9] and E46K [10] substitutions have been the most studied so far. Compelling evidence shows that a-syn takes center stage in PD and plays a key role via various aggregated forms, including abnormally phosphorylated aggregates that produce multiple cellular alterations, eventually leading to the death of DA neurons [11].

Mutations in leucine-rich repeat kinase 2 (LRRK2) are the most common genetic cause of both familial and sporadic PD [12,13]. There are also variants in the LRRK2 locus that are considered to be risk factors for developing PD [14,15]. The most prevalent mutation in LRRK2 is the G2019S substitution, accounting for 5 to 6% of familial PD and 1 to 2% of de novo genetic PD cases [16,17]. The cases of patients harboring the G2019S and other mutations are clinically indistinguishable from idiopathic PD cases, including the presence of Lewy bodies (LBs) in most cases [18,19]. Although G2019S patients show clinical manifestations similar to those of sporadic patients [20], several studies have shown subtle differences [21,22]. Some have reported the presence of LBs in symptomatic LRRK2 mutation carriers and LRRK2 can be found in LBs [23], although this is still a subject of debate, as other neuropathological studies have instead reported the absence of detectable LBs in a sub-population of PD patients with LRRK2 mutations [24]. In general, although LRRK2 variants or mutations are considered to be risk factors for developing PD, the onset of symptoms in LRRK2 carriers has been found to be similar to that of idiopathic PD cases [25,26]. Furthermore, the underlying mechanisms of LRRK2 neurotoxicity are still unknown. However, it is now generally accepted that the G2019S mutation increases LRRK2 kinase activity (both autophosphorylation and phosphorylation of exogenous kinase substrates) and that neurotoxicity originates from such increased activity [27,28].

The central role of a-syn in PD pathogenesis has led to the hypothesis of a functional, and possibly physical, interaction between LRRK2 and a-syn (for a review [20,29]). Indeed, LRRK2 toxicity may require the presence of a-syn and, conversely, the presence of variant/mutant LRRK2 could increase the risk and/or impact of a-synucleopathy in PD. The level of kinase activity of LRRK2 could thus be a modifier of a-syn toxicity. If this is true, the therapeutic implication would be extremely important: the regulation of LRRK2 kinase activity could be theoretically beneficial in slowing disease progression not only in individuals harboring LRRK2 mutations, but also in idiopathic PD. Recent experiments in transgenic mouse models of LRRK2 and a-syn support these hypotheses. The results of experiments in genetic models of mutant or wildtype LRRK2, in particular the effect of the pharmacological blockade of the kinase activity of LRRK2$^{G2019S}$ in these models, suggest that LRRK2 may increase a-syn toxicity [30–32].

To date, it is not known how LRRK2 (especially the G2019S mutation) exerts a protoxic effect on a-syn toxicity. In particular, the exact role of the kinase domain has not been completely demonstrated but pharmacological intervention suggests that the kinase activity of LRRK2 likely contributes to the synergy
(for a review [20]). In addition, it is not known whether the potentiation of a-syn toxicity by the presence of LRRK2G2019S is related solely to a cell-autonomous mechanism, or if LRRK2-expressing cells that surround DA neurons, especially microglial cells, astrocytes, and cells of the immune system likely play a role [33–35]

Here, we address these questions in a relevant cellular context by studying the effect of the C-terminal domain of human LRRK2 harboring the G2019S mutation (DLRRK2G2091S) or its inactive form, DLRRK2DK (mutations G2019S plus D1994A, called DK), on the neurotoxicity of human a-syn with the A53T mutation (α-synA53T). We performed experiments using adeno-associated viruses (AAVs) that lead to the overexpression of the various forms of DLRRK2 and human a-synA53T alone or in combination in DA neurons of the SNpc in adult rats. Quantitative histological evaluation showed that although DLRRK2G2091S alone induced no loss of DA neurons, it could significantly increase a-synA53T-induced neurotoxicity probably through a mechanism involving the catalytic activity of the kinase domain.

**Materials And Methods**

**Viral construction and production**

*Adeno-associated viral vectors (AAVs).* AAV6 viral particles were obtained by encapsidation of AAV2 recombinant genomes into serotype 6 AAV capsids as described previously [36]. Briefly, viral particles were produced by co-transfection of HEK-293T cells with (1) an adenovirus helper plasmid (pXX6-80), (2) an AAV packaging plasmid carrying the rep2 and cap6 genes, and (3) a plasmid encoding a recombinant AAV2 genome containing the transgene expression cassette. Seventy-two hours following transfection, viral particles were purified and concentrated from cell lysates and supernatants by ultracentrifugation on an iodixaniol density gradient followed by dialysis against PBSMK (0.5 mM MgCl2 and 1.25 mM KCl in PBS). The concentration of vector stocks was estimated by real-time-PCR following the method described by Aurnhammer et al. [37] and expressed as viral genomes per ml of concentrated stocks (Vg/ml). AAVs coding for human ΔLRRK2 (WT, G2019S, and G2019S plus D1994A mutation, i.e. “kinase dead”), α-synA53T, and GFP under the PGK1 (mouse phosphoglycerate kinase) promoter were produced.

*Lentivirus.* DNA sequences encoding GFP and the C-terminal part of human LRRK2 (kinase,K; ROC-COR-kinase, RCK; RCK plus the WD40 domain, called hereafter “DLRRK2”) were synthesised and inserted into the self-inactivated vector (SIN) backbone containing the WPRE element (W) and the murine PGK promoter. We generated lentivirus vectors LV-GFP, and LV-DLRRK2 coding for the WT form or G2019S forms of the fragments. Viral particles were produced as described elsewhere [38]. All the SIN vectors were pseudotyped with VSV glycoprotein G. Briefly, the viral particles were produced in HEK-293T cells by a four plasmid transient transfection system [39]. The supernatant was collected 48 hours later and filtered. High-titre stocks were obtained by ultracentrifugation. The pellet was re-suspended in 1% BSA in PBS, frozen and stored at −80°C. Particle content of the viral batches was determined by ELISA for the p24 antigen (Gentaur, Paris, France). LV-DLRRK2 vectors were used at a concentration of 100 ng/μl p24.
### Stereotaxic injection

**Experiments in rats.** Adult Sprague-Dawley rats (Charles River Laboratories), weighing ~250 g (Charles River, Saint Germain sur l'Arbresle, France), were housed under a 12-h light/dark cycle with *ad libitum* access to food and water, in accordance with European Community (Directive 2010-63/EEC) and French (Code Rural R214/87-130) regulations. Experimental procedures were approved by the local ethics committee and registered with the French Research Ministry (committee #44, approval #12-100, and APAFIS#1372-2015080415269690v2). For stereotaxic injections, the animals were deeply anaesthetized with 4% isoflurane, followed by a mixture of ketamine (75 mg/kg) and xylazine (5 mg/kg), and placed in a stereotaxic frame. Recombinant AAVs were injected unilaterally into the SNpc, at the following stereotaxic coordinates: +3.4 mm anterior to the interaural zero and ±2.0 mm lateral to bregma, at a depth of -7.8 mm relative to the skull, with the tooth bar set at -3.3 mm. We injected 4 µl of virus at a concentration of $2.5 \times 10^{10}$ Vg per site for single injections and $2.5 \times 10^{10}$ Vg of each vector for co-injections for a total of $5 \times 10^{10}$ Vg per site, with a 34-gauge blunt-tipped needle linked to a 10-µl Hamilton syringe by a polyethylene catheter at a rate of 0.25 µl/min using an automatic pump (CMA-4004). The needle was left in place for five minutes and then slowly withdrawn.

**Experiments in mice.** Adult male C57BL/6J mice (each weighing 25 g; Charles River, Saint Germain sur l'Arbresle, France) were used for lentiviral infections. Mice were housed (five/cage in enriched environment) in a controlled-temperature room maintained on a 12 h-hour light/dark cycle. Food and water were available ad libitum. All animal studies were conducted in accordance with French regulations (EU Directive 86/609 – French Act Rural Code R 214-87 to 131). The animal facility was approved by veterinary inspectors (authorization no. A 92-032-02) and complies with the Standards for Humane Care and Use of Laboratory Animals of the Office of Laboratory Animal Welfare (OLAW – n°#A5826-01). All procedures were approved by the ethics committee and the Research Ministry (no. 2015060417243726vl (APAFIS#770)).

LV-Htt171-82Q was used at a concentration of 150 ng/µl of p24. LVs coding for LV-DLRRK2 forms (WT, G2019S or the dead kinase G2019S/D1994A were used at a concentration of 100 ng/µl of p24.

For intracerebral infections, animal were anesthetized (100 mg/kg ketamine and 10 mg/kg xylazine). Local analgesia included subcutaneous lidocaine (5 mg/kg). A total volume of 2 µl of LV or AAV suspension was injected into the mouse striatum, as described [40], at the following stereotaxic coordinates: 1.0 mm anterior and 2.0 mm lateral to bregma, at a depth of 2.7 mm from the dura, with the tooth bar set at 0.0 mm. The mice were then left for one to two hours in a heated (30°C) ventilated box, until they had woken up and recovered fully from anesthesia. Post-surgery analgesia included acetaminophen (Doliprane) in drinking water for 48 h (1.6 mg/ml).

### Tissue processing

For all procedures, rats were first deeply anesthetized by isoflurane inhalation, followed by the intraperitoneal injection of a lethal dose of sodium pentobarbital.
Rats were transcardially perfused with 300 ml 4% paraformaldehyde (4% PFA) in phosphate buffer saline (PBS - 0.1 M phosphate buffer, 9 g/L NaCl) at a rate of 30 ml/min. After perfusion, the brain of each rat was quickly removed and immersed in ice-cold 4% PFA/PBS for at least 24 h, before transfer to 15% sucrose in PBS for 24 h and then 30% sucrose in PBS the next day, for cryoprotection. The brains were then cut into 40-μm sections on a freezing microtome (SM2400, Leica, Germany). Serial sections of the striatum and midbrain were stored in antifreeze solution (30% glycerol/30% ethylene glycol in PBS) and stored at -20°C until use.

Mice were deeply anesthetized by the intraperitoneal injection of sodium pentobarbital solution (50 µg per gram body weight). They were then transcardially perfused with 100 ml 4% PFA in PBS at a flow rate of 8 ml/min. The brains of the animals were removed, post-fixed overnight in the same solution, then cryoprotected by immersion in 30% sucrose in PBS for 36 hours. Free-floating 30-µm serial coronal sections from throughout the striatum were collected with a freezing sliding microtome. Brain slices were placed in a storage solution (30% glycerol, 30% ethylene glycol in PBS) and stored at -20°C before use.

**Immunohistological analysis and quantification**

**Immunohistochemistry**

Sections were removed from the antifreeze solution and washed in PBS. Endogenous peroxidase activity was quenched by transferring them to 1% H\textsubscript{2}O\textsubscript{2} and incubation for 30 min at room temperature (RT) and washing them three times with PBS for 10 min each. The sections were then blocked by incubation with 4.5% normal goat serum for 30 min in PBS-T (0.2% Triton X-100 in PBS) and then incubated overnight with primary antibody in 3% normal goat serum in PBS-T at 4°C with gentle shaking.

For histological evaluation using rat brain sections, the following primary antibodies were used for the present study: anti-tyrosine hydroxylase (TH) antibody: MAB318 clone LNC1, Merk-Millipore, 1:3000; anti-hemagglutinin tag (HA), Covance clone 11, 1:1000; anti-human α-synuclein, syn 211, 1:1000; anti-phospho-α-synS129, ab51253, Abcam, 1:5000]. The next day, the sections were removed from the primary antibody solution, washed three times, and incubated for 1 h at RT with the appropriate biotinylated secondary antibody in PBS-T (Vector Laboratories, Burlingame, CA, USA, 1:1000). The sections were then washed and incubated with ABC complex solution in PBS-T (1:250, reagents A and B combined in a 1:1 ratio, Vector Laboratories) for 1 h.

For histological evaluation using mouse brain sections, we used the anti-NeuN (1:2000, mouse, Millipore, Molsheim, France), anti-HA (1:500, mouse, clone 11 Covance, Princeton, NJ) and the EM48 (Chemicon, MAB5374, Temecula, CA, USA; 1 : 1000) antibodies. Sections were rinsed three times in PBS and then incubated with the appropriate anti-IgG biotinylated antibody (Vector Laboratories, Burlingame, CA) at a dilution of 1:5000, for one hour. Staining was visualized by adding avidin-biotinylated peroxidase (Vector Laboratories, Burlingame, CA) for one minute. For NeuN immunostaining, we used the MOM immunodetection kit (Vector Laboratories, Burlingame, CA).
The rat and mouse sections were then incubated with DAB for 30 s to 1 min and after dehydration mounted on slides in Eukitt mounting medium.

Cell counting

Optical fractionator sampling was carried out on a Zeiss AxioPlan microscope. Midbrain dopaminergic neurons were outlined on the basis of TH immunolabelling with reference to a coronal atlas of the rat brain (Paxinos and Watson, 6th edition). TH-positive cells were counted by unbiased stereology in the entire SNpc and the number of positive neurons per section was calculated using the Mercator Software (Explora Nova, France). We placed 100 × 100 μm grids in a systematically random manner, 80 × 80 μm apart, with a 3-μm offset from the surface of the section. Quantification was performed on 12 serial sections spaced by 200 μm, corresponding to the entire SNpc.

The phosphorylation of α-syn on S129 (p-synS129) was evaluated by counting the number of p-synS129-positive neurons in the SNpc using stereology methods. The SNpc was delimited by Nissl staining and the grids (250 x 250 μm) placed, with a space of 100 x 100 μm. Quantification was performed on six serial sections spaced by 400 μm, corresponding to the entire SNpc. In the striatum, a threshold was applied to select only the p-synS129-positive neurons by immunostaining and quantification performed on three slices, corresponding to the beginning, middle, and end of the striatum.

Immunofluorescence

The procedure used was similar to that for immunohistochemistry, but without the incubation in 1% H₂O₂. The primary antibodies used for the immunofluorescence procedure were the same as previously described (IBA1, Wako, 1:1000). Sections were first incubated with the primary antibody overnight at 4°C. The next day, they were incubated with a fluorescent secondary antibody (Alexa Fluor 594-labeled goat anti-rabbit IgG or Alexa Fluor 488-labeled goat anti-rabbit IgG (1:1000, Life Technologies)) for 1h at RT. Sections were then washed and incubated overnight at 4°C with another primary antibody. Finally, they were incubated with a second fluorescent secondary antibody (Alexa Fluor 488-labeled goat anti-mouse IgG or 594-labeled goat anti-mouse IgG (1:1000, Life Technologies)) for 1h at RT. The sections were stained with DAPI, washed, and mounted in a fluorescence mounting medium. Images were acquired with a laser confocal microscope (SP8, Leica, Germany) or an epifluorescence microscope (DM6000, Leica, Germany).

Thioflavin-S staining

A double-staining protocol was used to verify that accumulation of positive p-synS19 inside cells could colocalize with aggregated form of α-syn. The immunostaining procedure for p-synS19 and DAPI staining was performed on floating sections before the Thioflavin-S (Thio-S) staining. Floating sections were washed in PBS and mounted on Superfrost Plus slides. Slides were place in holders and dive into 70% EtOH and 80% EtOH, for 1 min each. Then, Slides were incubated in Thio-S diluted at 1% in distilled water for 7 min. The Thio-S solution must be protected from light, filtrated before use, and should be stored at
4°C. Then, slides were washed in 80% EtOH, 70% EtOH and distilled water for 1 min each before being coverslipped with the fluorescence-mounting medium.

**Colocalization**

The percentage of co-localization between ΔLRRK2 and α-syn was determined by counting the number of cells co-expressing both ΔLRRK2 and α-syn proteins divided by the number of cells expressing α-syn alone. Images were acquired with a laser confocal microscope (SP8, Leica, Germany). On the same acquisitions, the levels of ΔLRRK2 and α-syn proteins were evaluated on three coronal sections in the SNpc. Twenty cells co-expressing both ΔLRRK2 and α-syn proteins were delineated per animal using image J software and the mean fluorescence intensity in the red and in green channels (corresponding to ΔLRRK2 and α-syn proteins, respectively) was measured in each cell.

**Fluorescence intensity measurement**

Striatal dopaminergic innervation at 15 weeks was quantified by measuring the fluorescence intensity of TH-immunoreactive terminals on three coronal striatal sections. The sections were observed by epifluorescence microscopy at a magnification of 63X and the fluorescence intensity determined using MorphoStrider software (Explora Nova, France).

**Microglia area measurement**

The area occupied by microglia was evaluated by confocal microscopy at a magnification of 20X in the dorso-medial part of the striatum and in the SN pars reticulata. A threshold was applied and the area of 20 microglia cells measured per acquisition. Three acquisitions per animal were used.

**Image analysis of lesion area.**

Observation of sections and calculation of the surface of lesion were performed using a Leica DM6000 equipped with a motorized stage and an automated image acquisition and analysis system (Mercator software, Explora Nova, La Rochelle, France). The area covered by striatal lesions resulting from LV-Htt171-82Q infection was delineated manually using a 10x objective by identifying the border of the lesion on each coronal brain section and the corresponding area was calculated. The volume of the striatal lesion was determined by the Cavalieri method and the number of EM48-positive inclusions was determined as previously described with an inter-section distance of 210 µm (i.e. we used one in every seven sections) [40–42]. Automatic detection of EM48-positive objects was performed using the Mercator software to count the number of aggregates on the entire cross-sectional area of the striatum and to measure the size of all the detected EM48-positive objects. Objects with an apparent cross-sectional area exceeding 5 µm² were reliably detected with this method.

**Statistical analysis**
Normality of data distribution was tested using the Shapiro-Wilk test and homogeneity of variance was tested with Levene’s test using a commercially available software (Statistica, 13.0; Statsoft Inc., Tulsa, Oklahoma, USA). When normality and homogeneity of variance were met, unpaired Student’s t-test was used for pairwise comparisons between groups. For comparisons of more than two groups, one-way ANOVA for multiple comparisons was carried out, with Fisher’s post hoc PLSD test. In the cases where assumption of normality and/or homogeneity of variance were not met, non-parametric tests where applied: Mann-Whitney and Kruskall-Wallis for comparison of 2 and more groups, respectively. The annotations used to indicate the level of significance are as follows: *p < 0.05, **p < 0.01, ***p < 0.001.

Results

_Determination of the experimental conditions to detect a potential synergy between AAV-a-syn<sup>A53T</sup> and AAV-ΔLRRK2<sup>G2019S</sup> toxicity_

We investigated whether human LRRK2 can increase the toxicity of human a-syn in DA neurons through cell-autonomous mechanisms. We used serotype 6 AAV capsids, which allow preferential expression in neuronal cells and leads to a high percentage of cells transduced in the injected structure, without excessive diffusion into the surrounding tissue, such as for example serotype 9 [43,44]. In a previous study [45], we showed that the C-terminal portion of human LRRK2<sup>G2019S</sup> (DLRRK2<sup>G2019S</sup>, aa 1330-2527) retains, at least in part, the biochemical properties of full-length LRRK2<sup>G2019S</sup>, including higher kinase activity than the wildtype fragment. In addition, we found that overexpression of the C-terminal portion of human DLRRK2<sup>G2019S</sup> in the adult rat SNpc, using AAVs, produced partial (~30%) but significant loss of DA neurons at 25 weeks post-transduction, whereas overexpression of the wildtype form of LRRK2 (DLRRK2<sup>WT</sup>) was not toxic [45]. Here, we used a similar approach using a slightly larger fragment (aa 1283-2527) (Fig. 1A).

We studied the effects of AAV-ΔLRRK2<sup>G2019S</sup>, AAV-ΔLRRK2<sup>WT</sup>, and AAV-ΔLRRK2DK alone at 15 weeks PI. We injected 4 µl of AAVs solution in all cases. A final amount of 2.5x10<sup>10</sup> Vg per site and per vector was used. Each AAV was injected unilaterally into the SNpc (2.5 10<sup>10</sup> Vg). In addition to the three experimental groups, a control group received injections of vehicle (PBS/pluronic acid). The integrity of the nigrostriatal pathway was assessed using unbiased stereology to count the number of DA neurons displaying tyrosine hydroxylase (TH) staining in the injected part of the SNpc (Fig. 1). Observation at low-magnification revealed no major loss of TH-positive cells in any of the groups injected with AAVs encoding the LRRK2 fragments (Fig. 1B). The total number of TH-positive cells in the SNpc did not differ significantly between the control group (PBS) and AAV-DLRRK2<sup>WT</sup>, AAV-DLRRK2<sup>G2019S</sup>, or the AAV coding the dead kinase form ΔLRRK2<sup>G2019S/D1994A</sup> (thereafter called ΔLRRK2DK) (Fig. 1C). Thus, these results suggest that the ΔLRRK2 fragments alone did not trigger significant neurodegeneration of DA neurons at 15 weeks PI.

We wanted to investigate whether AAVs encoding the different ΔLRRK2 constructs could increase the toxicity of AAV-a-syn<sup>WT</sup> AAV-a-syn<sup>A53T</sup>. Therefore, we decided on an injection protocol that would lead to
mild degeneration, such that a potential “pro-toxic” effect of LRRK2 constructs could be easily detected. We conducted pilot experiments to determine the appropriate dose (titers) of AAV-a-syn\textsuperscript{WT} and AAV-a-syn\textsuperscript{A53T} alone that would lead to progressive and partial loss of DA neurons. Quantification of TH-positive cells in the SNpc showed no significant loss of DA neurons with AAV-a-syn\textsuperscript{WT} at 15 weeks (not shown). In contrast, we found a moderate loss of DA neurons (~30%) at 12 and 15 weeks after transduction with AAV-a-syn\textsuperscript{A53T} (2.5x10\textsuperscript{10} Vg) (Fig. 1D,E). After transduction, DA neurons often displayed accumulation of a-syn phosphorylated at its serine 129 (p-synS129). The cells positive for p-synS19 were also positive for ThioS suggesting that these accumulations were aggregates (Fig. 1F).

Thus, a co-injection protocol with AAV-α-syn\textsuperscript{A53T} and AAV-ΔLRRK2\textsuperscript{G2019S} and the evaluation of DA cell loss at 15 weeks PI appeared to be suitable for the detection of the potential synergy of toxicity between the two pathological transgenes.

**Effects of co-transduction with AAV-α-syn\textsuperscript{A53T} and AAV-ΔLRRK2\textsuperscript{G2019S}**

We next investigated whether the presence of the DLRRK2 fragments G2019S, or G2019S/D1994A - DK) in DA neurons could modify the toxicity of human α-syn\textsuperscript{A53T} using this co-transduction paradigm (2.5x10\textsuperscript{10} Vg for each vector).

We first studied the neurotoxic effects produced by AAV-α-syn\textsuperscript{A53T} in the presence or absence of AAV-ΔLRRK2\textsuperscript{G2019S} at 15 weeks PI. We assessed the co-localization of human α-syn\textsuperscript{A53T} and LRRK2 fragments in the SNpc after co-transduction, as we wanted to investigate the combined effects of α-syn\textsuperscript{A53T} and the various LRRK2 fragments in DA neurons. Analysis by confocal microscopy showed that human α-syn expression in the SNpc was high in TH-positive neurons (Fig. 2A). On average, 70% of neurons co-expressed both human α-syn\textsuperscript{A53T} and the LRRK2 fragments (Fig. 2B).

We next evaluated the toxic effects of human a-syn\textsuperscript{A53T} in the presence or absence of AAV-ΔLRRK2\textsuperscript{G2019S} (Fig. 3A). AAV-α-syn\textsuperscript{A53T} alone produced a significant 38% loss of TH-positive cells, as measured by unbiased stereology in the SNpc at 15 weeks PI (mean count ± SEM: Control, 12,344 ± 734; AAV-α-syn\textsuperscript{A53T}, 7,555 ± 527) (Fig.3B). The co-injection of AAV-a-syn\textsuperscript{A53T} with AAV-GFP (as a control of viral load) induced a 46% loss of DA neurons, which was not statistically different from that obtained with AAV-α-syn\textsuperscript{A53T} alone (6,601 ± 360) (Fig. 3B). The co-injection of AAV-a-syn\textsuperscript{A53T} and AAV-DLRRK2\textsuperscript{G2019S} induced a loss (-55%) of DA neurons (mean count ± SEM: 5,585 ± 355) that was significantly greater than that measured in the two other groups injected with AAV-a-syn\textsuperscript{A53T} (Fig. 3B).

We also counted the number of SNpc cells showing p-synS129 immunoreactivity, a marker of α-syn aggregation, in the different groups (Fig. 3C). The number of p-synS129-positive cells was significantly lower in the group co-infected with AAV-α-syn\textsuperscript{A53T} and AAV-DLRRK2\textsuperscript{G2019S} than that of the groups infected with AAV-α-syn\textsuperscript{A53T} alone or in combination with AAV-GFP (Fig. 3D). We also normalized the number of p-synS129-positive neurons to the number of TH-positive cells which survived. In this case,
there was no difference between group, suggesting that the overexpression of AAV-α-syn\textsuperscript{A53T} and AAV-DLRRK2\textsuperscript{G2019S} does not markedly modify the presence of p-synS129-in surviving cell soma. We also evaluated p-synS129 immunoreactivity in the striatum, which receives major inputs from the SNpc. Small p-synS129 immuno-positive objects with an elongated form or with a pearl necklace-like shape, reminiscent of neurite-like aggregates were seen in the striatum (Fig. 3E). Consistent with the results obtained in the SNpc, we found significantly lower levels of p-synS129 in the striatum of rats co-infected with AAV-α-syn\textsuperscript{A53T} and AAV-DLRRK2\textsuperscript{G2019S} than in those infected with AAV-α-syn\textsuperscript{A53T} / GFP (Fig. 3E-F).

Then, we evaluated the impact of SNpc cell loss on the level of dopaminergic terminals in the dorso-medial striatum using TH-immunofluorescence in both the α-syn\textsuperscript{A53T}/GFP and α-syn\textsuperscript{A53T}/ΔLRRK2\textsuperscript{G2019S} groups. These measurements were performed in the dorsal striatum (Fig. 4A). TH immunoreactivity in the striatum in both α-syn\textsuperscript{A53T}/GFP and α-syn\textsuperscript{A53T}/ΔLRRK2\textsuperscript{G2019S} groups was 15% lower than in the control group (PBS). This small a-syn\textsuperscript{A53T}-induced loss of TH-positive bers was similar in the GFP and ΔLRRK2\textsuperscript{G2019S} groups (Fig. 4B, C).

### Differential effects of AAV-ΔLRRK2\textsuperscript{G2019S} and AAV-ΔLRRK2\textsuperscript{G2019S/D1994A} on AAV-α-syn\textsuperscript{A53T} toxicity

We next investigated whether the effect of AAV-ΔLRRK2\textsuperscript{G2019S} on AAV-α-syn\textsuperscript{A53T}-induced toxicity was dependent on the kinase activity of the LRRK2 construct. We thus compared the effect of ΔLRRK2\textsuperscript{G2019S} with that of the dead kinase form AAV-ΔLRRK2\textsuperscript{G2019S/D1994A} (thereafter called ΔLRRK2\textsuperscript{DK}). We examined an earlier time point PI (6 weeks) for these experiments. We reasoned that, although the kinase activity of ΔLRRK2\textsuperscript{DK} is “dead”, it may lead to cellular disturbances in long-term experiments because of its potential dominant-negative effect on endogenous rat LRRK2.

We first compared the levels of transgene expression after transduction with α-syn\textsuperscript{A53T} and AAV-ΔLRRK2\textsuperscript{G2019S} or AAV-ΔLRRK2\textsuperscript{DK}. Quantitative immunofluorescence analysis showed that almost the entire SNpc was infected by AAV-α-syn\textsuperscript{A53T} when co-infected with either AAV-ΔLRRK2\textsuperscript{G2019S} or AAV-ΔLRRK2\textsuperscript{G2019S/D1994A} (Fig. 5A, B). We also re-evaluated the co-localization of the LRRK2-related transgenes and α-syn\textsuperscript{A53T} (Fig. 6A, B). In total, 76% of neurons expressed both α-syn\textsuperscript{A53T} and ΔLRRK2 (Fig. 6C, upper histogram), consistent with our observations in the experiments described above (see Fig. 1). α-syn\textsuperscript{A53T} protein was expressed at the same level in SNpc neurons co-expressing either ΔLRRK2\textsuperscript{G2019S} or ΔLRRK2\textsuperscript{DK} (Fig. 6C, middle histogram). In addition, the expression of human ΔLRRK2\textsuperscript{G2019S} and that of ΔLRRK2\textsuperscript{DK} were similar in neurons (Fig. 6C, bottom histogram).

We then assessed the loss of DA neurons produced by AAV-α-syn\textsuperscript{A53T} when co-injected with either AAV-ΔLRRK2\textsuperscript{G2019S} or ΔLRRK2\textsuperscript{DK}. The loss of DA neurons induced by human α-syn\textsuperscript{A53T} was significantly lower in the presence of ΔLRRK2\textsuperscript{DK} than that in the presence of ΔLRRK2\textsuperscript{G2019S} (Fig. 7A-B). The number of cells with p-synS129 immunoreactivity was similar in the ΔLRRK2\textsuperscript{DK} and ΔLRRK2\textsuperscript{G2019S} groups as shown by quantification (Fig. 7C-D). In the striatum, a few p-synS129 immunoreactive fibers (arrow
heads), reminiscent of dopaminergic fibers, were seen in both groups expressing a-syn\textsuperscript{A53T} with no obvious apparent difference in density or size (Fig. 7E).

Finally, we carried out a preliminary characterization of the status of neuroinflammation at this early time point (6 weeks PI), by immunohistochemistry using a validated marker (Iba1), which is highly expressed in activated microglial cells. Indeed, there is a role of neuroinflammation in neurodegeneration in a-syn\textsuperscript{A53T} rodent models. As expected, microglial cells in rats overexpressing human a-syn\textsuperscript{A53T} appeared more reactive as compared to rats injected with vehicle (Fig. 8E, F). The quantification of immunofluorescence levels in the SN (Fig. 8A, B) and striatum (Fig. 8C, D) showed that human a-syn\textsuperscript{A53T} significantly activated microglia. However, overexpression of ΔLRRK2\textsuperscript{G2019S} and ΔLRRK2\textsuperscript{DK} did not have any impact on the microglial activation induced by the mutant human a-syn.

Finally, we investigated whether the "pro-toxic" effect of ΔLRRK2\textsuperscript{G2019S} on a-syn\textsuperscript{A53T} could also be detected for other aggregating proteins. Using a different approach with lentiviral vectors in mice, we tested whether the different forms of ΔLRRK2 could modify the neurotoxicity produced by the N-terminal domain of human huntingtin (Htt) with a pathological expansion of its poly-glutamine (Q) region (Htt-N171-82Q) [46]. Lentiviral vectors were injected into the striatum of wild-type mice to produce local cell loss within the six weeks following transduction, as previously described [40,47]. The striatal lesions were characterized by the loss of neuronal markers DARPP32 and COX (not shown) as well as NeuN (Fig. 9A-B). Localization of the lesions in the striatum coincided with that of the expression of LRRK2 fragments detected using the HA-tag (Fig. 9C). Quantitative analysis of these histological markers showed that none of the ΔLRRK2 forms significantly modified the volume of the striatal lesions produced by mutant Htt (Fig. 9B). In addition, overexpression of the mutant Htt-fragment led to the accumulation of inclusions containing ubiquitin (mostly nuclear) (data not shown). Quantification of the presence of ubiquitin positive inclusions revealed no difference between groups (Fig. 9D). We also selectively detected mutant Htt aggregates using the EM48 antibody which specifically recognizes the aggregated form of the N-terminal domain of mutant Htt [41,48,49]. ΔLRRK2\textsuperscript{G2019S} significantly increased by 44% the number of EM48-positive aggregates when compared to the control group (LV-LacZ), an effect not seen with the WT or -ΔLRRK2\textsuperscript{DK} (Fig. 9E).

Our results show that the synergistic effect of ΔLRRK2\textsuperscript{G2019S} on the toxicity of human a-syn\textsuperscript{A53T} towards DA neurons depends on its kinase domain. Importantly, ΔLRRK2\textsuperscript{G2019S} overexpression did not markedly change the neurotoxic effects produced by a different aggregating protein, mutant Htt. Although the comparison between the rat model of SNpc degeneration and the mouse model of striatal lesion must be compared cautiously, our results suggest that the effect of ΔLRRK2\textsuperscript{G2019S} on a-syn\textsuperscript{A53T}-induced neurotoxicity may be due to specific molecular mechanisms rather than to a general increase in the vulnerability of the neurons.

**Discussion**
The mechanisms leading to the degeneration of DA neurons in LRRK2 mutation gene carriers with PD are unknown. It is generally accepted that the LRRK2<sup>G2019S</sup> mutation leads to increased kinase activity which could then lead to cell death [27,50–52].

In addition, a role for LRRK2 in α-syn toxicity has been suggested [30–32]. Indeed, neuropathological evaluation of the brains of PD patients with LRRK2 mutations shows in many cases the presence of <i>bona fide</i> LBs and Lewy neurites [53]. However, the role of the kinase in the crosstalk between LRRK2 and α-syn, especially how the kinase activity of LRRK2<sup>G2019S</sup> modulates α-syn neurotoxicity in the SNpc is unknown. In addition, the respective roles of cell-autonomous and non-cell-autonomous mechanisms in this interaction are largely unknown.

Here, we used an AAV-based approach to target SNpc DA neurons and investigated how the C-terminal domain of LRRK2, harboring the G2019S mutation, with increased kinase activity, could modify the loss of DA neurons induced by the overexpression of α-syn<sup>A53T</sup> in the rat SNpc. Under our experimental conditions, AAV-DLRRK2<sup>G2019S</sup> alone do not induce the loss of DA neurons whereas AAV-α-syn<sup>A53T</sup> alone can produce partial loss. The loss of DA neurons produced by co-expression of DLRRK2<sup>G2019S</sup> and α-syn<sup>A53T</sup> was significantly higher than that measured in rats injected with AAV-α-syn<sup>A53T</sup> alone or co-injected with a control vector (AAV-GFP). Conversely, overexpression of the inactive “dead kinase” form DLRRK2<sup>DK</sup>, at levels similar to those of DLRRK2<sup>G2019S</sup>, did not alter the toxicity of α-syn<sup>A53T</sup>. Quantitative characterization of microglial reactivity induced by human α-syn<sup>A53T</sup> in SNpc and striatum did not show obvious changes attributable to DLRRK2. These novel findings further support the hypothesis that the C-terminal domain of LRRK2<sup>G2019S</sup> is sufficient to augment the toxic effects of α-syn<sup>A53T</sup> through a cell-autonomous mechanism involving the catalytic activity of its kinase domain.

The histological evaluation we performed after transduction of the SNpc with AAV-α-syn<sup>A53T</sup> and AAV-DLRRK2<sup>G2019S</sup> shows that both transgenes are overexpressed in DA neurons. In both cases, approximately 70% of the SNpc was infected. After co-injection, co-localization of both transgenes in neurons of the SNpc was found in a large proportion (~77%) of SNpc neurons. Neuropathological evaluation after transduction with AAV-α-syn<sup>A53T</sup> (15 weeks PI) showed the partial loss of DA neurons, based on the detection of TH-positive cells. This likely reflects neuronal loss, as suggested in our previous work [45].

The relevance of overexpressing the C-terminal domain of LRRK2 <i>versus</i> the full-length protein is debatable and the mechanisms underlying the neurotoxic effect of DLRRK2<sup>G2019S</sup> in our models are unknown. Indeed, our DLRRK2<sup>G2019S</sup> construct lacks N-terminal domains that are known to play crucial roles in LRRK2 function. We previously showed that overexpression of the DLRRK2<sup>G2019S</sup> fragment using AAVs triggers neurodegeneration of DA neurons six months PI, whereas the DLRRK2<sup>WT</sup> fragment, expressed at similar high levels, was devoid of obvious neurotoxicity [45]. In this work, we suggested that death of DA neurons produced by DLRRK2<sup>G2019S</sup> is likely independent of the interaction with RAB10, since we found that the DLRRK2 fragment was found unable to interact with RAB10, in contrary to full-
length LRRK2 fragment [45]. Thus, other signaling pathways have to be considered. It is conceivable that the overexpression of DLRRK2<sup>G2019S</sup> leads to abnormally high phosphorylation of substrates when compared to DLRRK2<sup>WT</sup>. Indeed DLRRK2<sup>G2019S</sup> kinase activity is higher than that of DLRRK2<sup>WT</sup> [45] a phenomenon that is also observed for full-length LRRK2<sup>G2019S</sup> [54–57]. Alternatively, the “protoxic” effect of DLRRK2<sup>G2019S</sup> upon a-syn<sup>A53T</sup> could also result from molecular mechanisms unrelated to the enzymatic activity of the catalytic domains. Changes in protein-protein interactions and/or a modification of the conformation of LRRK2 fragments induced by the G2019S substitution may also play a role. In regard to this hypothesis, we now know that LRRK2 interacts with microtubules [58,59] and recent high-resolution cryo-EM studies have shown the enzymatic domain of LRRK2 (ROC-COR-Kinase) is sufficient for the interaction of LRRK2 with microtubules and their regulation [60,61]. The orientation of the kinase domain from microtubules is different between wild-type LRRK2 and LRRK2 with pathological mutations [61] and therefore in the present study, it is conceivable that the pro-toxic effects of DLRRK2<sup>G2019S</sup> are linked to microtubule-related perturbation. Further in vivo studies are required to fully address this hypothesis.

We investigated whether DLRRK2<sup>G2019S</sup> effect on a-syn<sup>A53T</sup> was specific of these two pathological proteins, or only resulted from a non-specific “protoxic” effect of DLRRK2<sup>G2019S</sup> upon neurons. If it was the case, DLRRK2<sup>G2019S</sup> should render neurons more vulnerable to different stresses, including those produced by different mutant proteins linked to neurodegenerative disorders. We used a different cellular context, the striatum, to test the effect of DLRRK2<sup>G2019S</sup> against mutant Htt with a lentiviral vector approach often used in studies aiming at testing how different gene products can modify the vulnerability of striatal projection neurons. In this mouse model, we found that the degeneration of striatal neurons induced by mutant Htt within the 6 weeks PI was not significantly changed by the overexpression of DLRRK2<sup>G2019S</sup>. However, DLRRK2<sup>G2019S</sup> overexpression significantly increased the number of mutant Htt-containing aggregates (EM48-positive) two fold when compared to DLRRK2<sup>wt</sup> and DLRRK2<sup>DK</sup>. This suggests that DLRRK2<sup>G2019S</sup>, compared to the two other fragments, produces a “biological” effect in mouse striatal neurons, although this does not appear to have an impact on the survival of striatal neurons expressing mutant Htt. Further studies are required to discover the molecular mechanisms that could explain this phenomenon. Thus, the effect of DLRRK2<sup>G2019S</sup> upon a-syn<sup>A53T</sup> neurotoxicity might be specific, although testing the effect of DLRRK2<sup>G2019S</sup> on the toxicity of other pathological proteins is necessary to fully support this hypothesis.

The present experimental paradigm using AAVs allowed us to address the question of the potential cell-autonomous exacerbation of a-syn<sup>A53T</sup> toxicity by the kinase activity of LRRK2 directly in the SNpc and only in neurons. In contrast, other viral vector platforms that could potentially host the full-length LRRK2 ORF [i.e. vectors derived from Herpes Simplex Virus (HSV) or adenovirus] also transduce other cell types in the striatum [62,63]. Here, we directly investigated whether there is a functional interaction between AAV-α-syn<sup>A53T</sup> and DLRRK2<sup>G2019S</sup> in DA neurons. Our results show the existence of such a “functional” interaction, as overexpression of DLRRK2<sup>G2019S</sup> significantly enhanced the neurotoxic effects of a-
syn\(^{A53T}\) in rat SNpc. Lin et al. showed that the overexpression of LRRK2 (wildtype or with the G2019S mutation) in forebrain neurons (striatum and cerebral cortex) increased the toxicity of a-syn\(^{A53T}\) in transgenic animals [64,65]. In these double-transgenic mice, the authors found significant degeneration of the striatum and cortex and enhanced accumulation of a-syn aggregates. This proved the existence of a functional crosstalk between a-syn and LRRK2 in neurons \textit{in vivo} when the proteins are expressed at relatively high levels. Pathological transgenes were not expressed in the SNpc and DA degeneration was not evidenced in these models [66]. The CamKIIα promoter used to drive the expression of the tetracycline transactivator (tTA), which activates the TetO promoter of the LRRK2 and a-syn\(^{A53T}\) transgenes in these mice, is likely not active in SNpc DA neurons, as endogenous expression of CamKIIα in neurons of the SNpc is lower than that observed in forebrain neurons ([66] and see also the Allen Brain Atlas, http://mouse.brain-map.org/experiment/show/79490122). In LRRK2 knockout rats, the toxicity induced by AAV coding for a-syn is lower than that in wildtype rats [31]. Daher et al. found no synergy between the transgenes following the crossbreeding of other transgenic models in which the promoters driving LRRK2\(^{G2019S}\) and α-syn\(^{A53T}\) expression were different (Prion and CMV respectively) [67]. Indeed, data from the latter work indicate that the expression level of human LRRK2 transgene is low in the SNpc (see Figure 2 in [67]). Neurons that express a-syn\(^{A53T}\) are apparently sparse in the SNpc compared to the known density of DA neurons in this structure (see Figure 5 in [67]). These observations and our results suggest that the crosstalk between LRRK2 and a-syn can occur if the two proteins are localized in the same neurons. Thus, our results show that synergy between LRRK2 and a-syn depends, at least in part, on cell-autonomous mechanisms.

In our experimental condition, the interplay between DLRRK2\(^{G2019S}\) and a-syn\(^{A53T}\) is detected while the two proteins are expressed in high levels. We could not precisely compare the overexpression levels of the transgenes that are of human origin with the endogenous rat proteins. It can be only grossly estimated that expression of human DLRRK2\(^{G2019S}\) and α-syn\(^{A53T}\) is likely 5-50 fold higher than those of the rat endogenous proteins. This estimation is based on the previous experiments where other mouse transgenes (DCLK3, Crym, abhd11os, Capucin) were overexpressed with lentiviral vectors or AAVs [40,47,48,68,69]. Thus, it cannot be ruled out that in the presence of physiological levels of expression of LRRK2 and a-syn, the cell-autonomous crosstalk between the two proteins might be of moderate importance in DA neurons.

In our AAV-based model, LRRK2 fragments are expressed only in neurons, which allowed us to investigate the cell-autonomous mechanisms of LRRK2 / a-syn interplay. The other comparable experimental approaches that investigated this interplay were carried out in in models where LRRK2 is expressed in all cells (See Daher and collaborators [31]). In the transgenic animal models and in patients LRRK2 is expressed in cells of different types (i.e. neurons, microglia, oligodendrocytes, astrocytes). In these conditions, non-cell autonomous mechanisms involving interaction of DA neurons with neighboring glial cells and immune cells may have also important roles. For example, it has been recently shown that the seeding of a-syn aggregates by the exposure of neurons to a-syn fibrils is higher in neurons expressing mutant LRRK2 [70]. More generally, LRRK2 mutation may change the potential propagation of
aggregated a-syn species in the brain [71]. The level of LRRK2 activity in microglial cells may also regulate prototoxic phenomena associated with α-syn-induced neuroinflammation [30,34,72]. LRRK2 plays a key role in the immune system [73]. A single nucleotide polymorphism (N2081D) in the region coding for the kinase domain of LRRK2 is a major risk factors for Crohn's disease, a form of inflammatory bowel disease [74].

The neuronal mechanisms underlying the synergy between LRRK2 and a-syn are ill-defined. It is possible that LRRK2 - a-syn\textsuperscript{A53T} interplay involves a direct physical interaction between the two proteins [75] though, indirect effects leading to a functional interplay is more often discussed. The present results indicate that DLRRK2\textsuperscript{G2019S} does not markedly change p-synS129 immunoreactivity at 6 and 15 weeks PI. This suggests that the “pro-toxicity” produced by the overexpressing the mutant LRRK2 fragment may not be related to a change in a-synA53T bioavailability/expression levels, although a more complete biochemical study is required to further support this hypothesis. In addition, the role of the kinase domain of LRRK2 is unclear. It is generally accepted that the higher kinase activity of LRRK2\textsuperscript{G2019S}, relative to that of wild-type LRRK2, leads to neurodegeneration through increased phosphorylation of substrates, possibly through multifactorial cellular changes, including the disruption of microtubule assembly, mitochondrial defects, and alterations in protein translation [76]. However, whether the increased kinase activity of LRRK2 mutations plays a key role is still a subject of debate. As already mentioned, various transgenic rodent models expressing LRRK2\textsuperscript{G2019S} have been developed and extensively characterized. These models display no (or very limited) degeneration of DA cells in the SNpc. Various experimental approaches clearly demonstrate that the severity of the resulting toxicity is dependent on the level of expression of LRRK2\textsuperscript{G2019S} [77]. However, there is limited evidence obtained \textit{in vivo} that shows a relationship between the higher kinase activity of LRRK2\textsuperscript{G2019S} and neurotoxicity to dopaminergic cells of the SNpc. The overexpression of LRRK2\textsuperscript{G2019S} (or DLRRK2\textsuperscript{G2019S}) in the SNpc was found to induce the loss of DA neurons using HSV and adenovirus models injected into the striatum [27,78], as well as in our previous study with AAV-DLRRK2\textsuperscript{G2019S} injected into the SNpc [79].

Only a few studies have directly addressed the role of the kinase domain in the interaction between a-syn and LRRK2 toxicity. It was shown that neuroinflammation and neurodegeneration produced by the transduction of the SNpc with AAV-a-syn is significantly attenuated in LRRK2 KO rats relative to that in wildtype littermates. In these experiments, the role of the kinase activity was not assessed [30]. More recently, Daher \textit{et al.} showed that the toxicity of AAV-α-synuclein in the SNpc was higher in transgenic LRRK2\textsuperscript{G2019S} than wildtype rats. Interestingly, treatment of both genotypes with the LRRK2 inhibitor PF-06447475 reduced the toxicity of a-syn [31]. This suggests that the exacerbation of a-syn toxicity by LRRK2\textsuperscript{G2019S} could result from elevated catalytic activity of the kinase. Since it has been observed that some LRRK2 inhibitors, including PF, can reduced cellular levels of the protein [80], it is possible that protection by PF-06447475 against the toxicity triggered by injection of AAV-a-syn in LRRK2\textsuperscript{G2019S} mice may result from the reduction of LRRK2 levels rather than actual inhibition of the catalytic activity of the kinase. Indeed, it has been suggested that the level of expression of the LRRK2 protein could play a determinant role in mutant LRRK2 rather than the kinase activity [77]. However, new generation of
inhibitors with protective effects do not reduce LRRK2 levels (see for review ([81])). In our work, the inactive protein DLRRK2^{DK} did not alter the toxicity of AAV-α-syn^{A53T}, whereas expression of DLRRK2^{G2019S} increased the toxicity of AAV-α-syn^{A53T} towards DA neurons and we were able to verify that the difference between the toxicity of DLRRK2^{G2019S} and DLRRK2^{DK} was not related to a difference in protein levels as evaluated by confocal microscopy.

**Conclusions**

Our results show that the C-terminal domain of LRRK2^{G2019S} containing the ROC-COR, Kinase and WD40 domains is sufficient to potentiate the toxicity of human a-syn^{A53T} in DA neurons in vivo and suggest that this effect depends on the kinase domain. This cell-autonomous mechanism may act additively or synergistically with other non-cell-autonomous mechanisms, especially those involving neuro-inflammation to trigger the death of DA neurons in PD.

**Declarations**

*Authors consent*

All authors gave their consent to Emmanuel Brouillet for publication of the present results.

*Ethics approval and consent to participate Manuscripts reporting studies involving human participants, human data or human tissue must:*

Not applicable

*Consent to publication*

Not applicable

*Competing interest*

The authors have no competing financial interests to declare.

*Availability of data and material*

The different LRRK2 vectors maps and raw data of analyses can be obtained from the corresponding author on request. The authors would be glad to share on a collaboration basis the plasmids for producing the different vectors coding for the LRRK2 fragments described herein.

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**Authors’ contributions**

Noémie Cresto discussed the design of the experiments, performed validation of viral vectors, stereotaxic surgery, histological evaluation (unbiased stereological analyses) of injected rats and statistical analyses on most of the experiments. She significantly contributed to the preparation of most of the figures.

Camille Gardier performed validation of viral vectors in vitro, contributed to histological evaluation of injected rats, and was involved on the writing of the manuscript.

Marie-Claude Gaillard participated to the design of the rat experiments, supervised technical aspects requiring RT-qPCR analysis in the course of the studies, supervised molecular biology of the different constructs used in the study.

Francesco Gubinelli discussed the design of the experiments, performed histological evaluation (unbiased stereological analyses and confocal microscopy) and contributed to edit the manuscript.

Pauline Roost contributed to the discussion on the experimental design, performed histological analyses (unbiased stereological analyses).

Daniela Molina carried out all the quantitative histological evaluation corresponding to the experiments using mutant huntingtin.

Charlène Josephine and Noelle Dufour produced the different AAV vectors and lentiviral vectors respectively, and all the quality control processes.

Gwenaëlle Auregan contributed to the stereotaxic surgery in the rat substantia nigra and mouse striatum.

Martine Guillermier contributed to the entire process to obtain Ethical authorizations, performed the stereotaxic surgery in the rat substantia nigra and mouse striatum, and the optimization of anaesthesia for the different experiments.

Suéva Bernier, supervised anesthesia of rats and the stereotaxic surgery in rats.

Caroline Jan supervised the histological works (histochemistry and confocal).

Pauline Gipchtein significantly contributed to the histological processing (perfusion, sectioning, immunohistochemistry, mounting).
Philippe Hantraye contributed to scientific discussions on the design of the different experiments, and helped to write the manuscript.

Marie-Christine Chartier-Harlin discussed many conceptual and methodological aspects of the study and contributed to the writing of the manuscript.

Gilles Bonvento set up the entire neurosurgery room for stereotaxy, contributed to scientific discussions on the design of the different experiments and the interpretation of results, and helped to write the manuscript.

Nadja Van Camp significantly helped in many preliminary neuroimaging experiments that were required to perform all these surgery experiments.

Jean-Marc Taymans discussed LRRK2 constructs design, provided suitable advice on many reagents in the course of the study, and contributed to write the manuscript.

Karine Cambon supervised the behavioral observations carried out in the study, discussed crucial aspects of the experimental design and cell counting methodology.

Géraldine Liot supervised C.G. for the in vitro studies to validate the different expressing plasmids and viral vectors.

Alexis-Pierre Bemelmans supervised the conception, validation and production of viral vectors and participated in the writing of the manuscript.

Emmanuel Brouillet designed the present study, helped performing neurosurgery, supervised all the experiments, and the different types of analyses and wrote the manuscript.

All authors read and approved the final manuscript.

**Abbreviations**

**∆LRRK2** ROC-COR-kinase plus the WD40 domain

**AAV** adeno-associated virus

**ANK** Ankyrin

**ANOVA** analysis of variance

**ARM** Armadillo

**ATP** Adenosine triphosphate
BSA Bovine serum albumin
BCA bicinchoninic acid assay
CamKII Calmodulin-kinase II
COR C-terminal of ROC
DA Dopaminergic
DAB Diaminobenzidine
DLU Density light unit
DK double-mutant G2019S/D1994A dead kinase
GFP Green fluorescent protein
GS G2019S mutation
GWAS Genome-wide association studies
HA Hemagglutinin tag
K Kinase domain of LRRK2
LB Lysis buffer (50 mm Tris, pH 8.0, 150 mm NaCl, 1 mm EDTA, 0.5% Triton X-100, 1% NP40, protease inhibitors)
LRR Leucin-rich repeats
LRRK2 Leucin-rich repeats kinase 2
p-synS129 a-synuclein phosphorylated at serine 129
PAGE Poly-Acrylamide Gel Electrophoresis
PBS Phosphate buffer saline
PBS-T Phosphate Buffer Saline with 0.2% Triton X-100
PD Parkinson’s disease
RCK Kinase domain plus the ROC-COR domain
ROC Ras-of-complex protein
SDS Sodium-dodecyl-Sulfate
**SNpc** Substantia nigra *pars compacta*

**SNPs** single-nucleotide polymorphisms

**TBS** Tris Buffer Saline

**TBS-T** Tris Buffer Saline with 0.1% Tween-20

**TH** Tyrosine hydroxylase

**Vg** Viral particle

**WT** Wild-type

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Supplementary Materials Legends

Supplementary Figure 1. Results of the pilot experiments carried out to set up the AAV-α-synA53T model, characterized by partial degeneration of DA neurons in the SNc of adult rats. (A, B) AAV-α-synA53T produced partial loss of TH-positive neurons in the SNc at 12 and 15 weeks post injection. Stereological
cell count in B shows significant loss compared to control rats of the same age (injected with PBS). Neurons in the SNc after infection were found to be immune-positive for the phosphorylated form of α-syn at serine 129 (P-synS129) and thioflavin S (ThioS) fluorescence suggesting aggregation of α-synS129. Results are expressed as the mean ± the standard error of the mean (SEM). One-way ANOVA and post hoc Fisher's PLSD test. *, p<0.01. Scale bar in C, 10 µm.

**Supplementary Figure 2.** Evaluation of microglial activation (microglia fluorescent area) based on IBA1 immunofluorescence in the SNc (A-B) and striatum (C-D). Results are expressed as the percentage of staining of the control group (PBS). Results are expressed as the mean ± the standard error of the mean (SEM). N = 8 animals/group. ANOVA and PLSD post hoc test (SNc) and Kruskal-Wallis and Mann-Whitney test (striatum). *, p < 0.05; **, p<0.01. Scale bar: low magnification, 200 µm; high magnifications, 50 µm.

**Figures**
Figure 1

Immunohistochemistry for tyrosine hydroxylase (TH). A The C-terminal fragment of LRRK2, called ∆LRRK2, was generated in different forms: the wild type form (WT), the pathological form with G2019S substitution (GS), or dead kinase form of G2019 with the D1994A mutation (DK). B-C, The three fragments were cloned into an AAV and unilaterally injected into the rat SNpc. Fifteen weeks post-injection (PI), TH immunohistochemistry was performed (B). The number of TH-positive neurons was
evaluated by stereology (C). D, E, the quantification of TH-positive cells in the SNpc showed a moderate loss of DA neurons (~30%) at 15 weeks after injection of AAV-α-synA53T (2.5x10¹⁰ Vg) compared to control PBS injection. F, At 15 weeks PI, AAV-α-synA53T leads to accumulation of α-syn phosphorylated at its serine 129 (p-synS129) in SNpc neurons (in red). The high levels p-synS19 immunoreactivity were also positive for ThioS (in green) suggesting that these accumulations were aggregates. Results are expressed as means ± the SEM. N=8-12 animals/group. ANOVA and PLSD post hoc test. n.s.: not significant. Scale bars: B, 750 µm; D, 400 µm; F, 10 µm.

**Figure 2**

Histological evaluation of the expression of the transgenes in the SNpc at 15 weeks post-injection. (A) Evaluation of α-syn (in green) transduction in the SNpc after co-injection of AAV-α-synA53T with
ΔLRRK2G2019S (ΔLRRK2GS) was determined using delineation of SNpc with TH staining (red). Scale bar: 500 µm. (B) Measurement of the number of neurons expressing both α-synA53T and ΔLRRK2GS from confocal images. The higher magnification shows cytoplasm localization of ΔLRRK2G2019S. Results are expressed as means ± SEM. Scale bars: 40X = 60 µm, 63X zoom 4 = 10 µm.
Immunohistochemistry for tyrosine hydroxylase (TH) and p-synS129 positive cells and axons at 15 weeks post-injection of AAV-α-synA53T with AAV-GFP or ΔLRRK2G2019S (ΔLRRK2GS). A, Photomicrographs of rat brain sections labelled for TH immunohistochemistry at the level of the SNpc at low (left) and higher (right) magnifications. B, unbiased stereological counts of TH-positive cells in the SNpc. C, Photomicrographs of rat brain sections labelled for p-synS129 immunohistochemistry at the level of the SNpc at low (left) and higher (right) magnifications. D, upper histogram: number of p-synS129-positive (p-synS129+) neurons in the SNpc; lower histogram: ratio of the number of p-synS129+ neurons normalized to the number of TH-positive neurons measured in B. E, Photomicrographs of rat brain sections labelled by p-synS129 immunohistochemistry at the level of the striatum at low (top) and higher (bottom) magnifications. Note the presence of sparse positive objects with a necklace-like organization. F, Determination of the percentage of the field of view (area) occupied by p-synS129+ staining in the striatum at 15 weeks PI. Results are expressed as means ± SEM. N=10 animals/group. ANOVA and PLSD post hoc test. *,P < 0.05; **,P < 0.01; ***, P < 0.001. Scale bars: 750 µm left panel and 400 µm right panel in A and C; 200 µm top images and 50 µm bottom images in E.
Figure 4

Determination of Tyrosine hydroxylase (TH) levels in the striatum of rats injected with AAV-α-synA53T with AAV-GFP or ΔLRRK2G2019S in the right SNpc. A, photomicrographs at low magnification showing immunofluorescence for TH (red) and GFP (green) in the striatum at 15 weeks post-injection. Scale bar: 1000 µm. B, photomicrographs at two different magnifications (5X and 63X) showing TH-related immunofluorescence levels in the striatum of rats injected either with PBS, or AAV-α-synA53T with AAV-GFP as a control or ΔLRRK2G2019S (GS). Scale bar in B: X5, 1000 µm, X63, 100 µm. C, Quantification of
flourescence levels in the striatum. Quantification was performed at 5X magnification. Results are expressed as means ± SEM. N=10 animals/group. ANOVA and PLSD post hoc test. **P < 0.01.

**Figure 5**

Measurement of the SNpc volume transduced by AAV-ΔLRRK2G2019S and the dead kinase form ΔLRRK2G2019S/D1994A. A, Confocal images to delineate the SNpc based on TH staining (in red), reported in the green channel, corresponding to the α-syn immunofluorescence when co-expressed with ΔLRRK2G2019S (ΔLRRK2GS) or ΔLRRK2DK, the dead kinase form ΔLRRK2G2019S/D1994A (ΔLRRK2DK). Scale bar: 1000 µm. B, Quantification of the fraction (%) of the SNpc expressing α-syn protein after co-transduction with ΔLRRK2G2019S or ΔLRRK2G2019S/D1994A. Results are expressed as means ± SEM. N=8 animals/group. No statistical difference, Student-t test.
Figure 6

Co-localization and expression of ΔLRRK2 and α-synA53T 6 weeks after the co-injection of AAV-α-synA53T with either AAV-ΔLRRK2G2019S or AAV-ΔLRRK2G2019S/D1994A. A and B are photomicrographs showing the results of immuno-fluorescence detection of ΔLRRK2G2019S (ΔLRRK2GS) or ΔLRRK2G2019S/D1994A (ΔLRRK2DK – red channel) and α-syn (green channel). Left and right images were obtained at low (A) and high (B) magnification respectively. Note that a majority of
neurons express both transgenes. Scale bars: 200 µm for top images in A; 50 µm for bottom images in A; and 10 µm in B. C, Quantiﬁcation of the percentage of co-localization (upper histogram), α-syn ﬂuorescence levels (middle histogram) and ΔLRRK2 ﬂuorescence levels (bottom histogram). Results are expressed as means ± SEM. N=8 animals/group. No statistical difference between groups, Student-t test.

Figure 7
Immunohistochemistry for tyrosine hydroxylase (TH) and p-synS129 positive cells and axons 6 weeks after the injection of AAV-α-synA53T with either AAV-GFP, ΔLRRK2G2019S or the dead kinase form ΔLRRK2G2019S/D1994A. A, Photomicrographs of rat brain sections labelled for TH immunohistochemistry at the level of the SNpc at low (left) and higher (right) magnifications. B, unbiased stereological counts of TH-positive cells in the SNpc. C, Photomicrographs of rat brain sections labelled for p-synS129 immunohistochemistry at the level of the SNpc at low (left) and higher (right) magnification. D, upper histogram: number of p-synS129-positive neurons in the SNpc; lower histogram: ratio of the number of p-synS129-positive neurons normalized to the number of TH-positive neurons measured in B. E, Photomicrographs of rat brain sections labelled by p-synS129 immunohistochemistry at the level of the striatum at low (top) and higher (bottom) magnifications. F, Determination of the percentage of the field of view (area) occupied by p-synS129-positive staining in the striatum at 6 weeks PI. Results are expressed as means ± SEM. N=10 animals/group. ANOVA and Fisher PLSD post hoc test. *P < 0.05. Scale bars: 750 µm left panel and 400 µm right panel in A; 200 µm top images and 50 µm bottom images in E.
Figure 8

Microglial activation induced by α-synA53T is not modified by overexpression of ΔLRRK2 fragments. Histological evaluation was performed 6 weeks after the injection of PBS or AAV-a-synA53T alone, or AAV-a-synA53T mixed with either AAV-GFP, ΔLRRK2G2019S (ΔLRRK2GS) or the dead kinase form ΔLRRK2G2019S/D1994A (ΔLRRK2DK). Cells positive for IBA1 were detected by immunofluorescence and confocal microscopy and their cross-section area determined by image analysis. A, Photomicrographs of
rat brain sections revealed for IBA1 immunoreactivity in the SNpc at low (upper images) and high (lower images) magnification in the different groups. B, quantification of the mean cross-sectional area of IBA1-positive cells. C, Low (upper images) and high (lower images) magnification photomicrographs of rat brain sections revealed for IBA1 immunoreactivity in the striatum in rats injected into the SNpc with PBS or AAV-a-synA53T with AAV coding for ΔLRRK2 constructs. D, Quantification of the mean cross-sectional area of IBA1-positive cells. E, Low (upper images) and high (lower images) magnification photomicrographs of rat brain sections revealed for IBA1 immunoreactivity in the SNpc in rats injected with AAV-GFP or AAV-a-synA53T alone. F, Quantification of the mean cross-sectional area of IBA1-positive cells. Results are expressed as the mean percentage +/- SEM of the staining of the control group (PBS in A-D, AAV-GFP in E and F). N=8 animals/group in B-D, n=5-7 animals/group in F. In B, ANOVA and PLSD post hoc test (SNpc); D, Kruskal-Wallis and Mann-Whitney test (striatum). F, Unpaired Student t-test. *, p < 0.05; **, p<0.01., ***, p<0.0001. Scale bar: low magnification, 200 µm; high magnifications, 50 µm.
Effect of the various forms of ΔLRRK2 on the toxicity of mutant Htt Mice received a bilateral intrastratal injection of a mixture of two lentiviral vectors (LV): LV-Htt171-82Q with LV-LacZ (CT, control), LV-ΔLRRK2WT (WT), LV-LRRK2G2019S (GS) or LV-ΔLRRK2G2019S/D1994A (DK). Six weeks after injection, brains were processed for histological evaluation to evaluate Htt171-82Q-dependent neurotoxicity using immunohistochemistry (IHC) for the HA-tag, NeuN, and mutant Htt aggregates using an ubiquitin
antibody and the EM48 antibody. A, Camera Lucida representation of the rostro-caudal extension of striatal lesions produced by the mutant Htt fragment as seen using NeuN IHC. Grey spots in the striatum represent area with loss of NeuN staining (lesions). B, Histograms of the volumes of the striatal lesions for the four groups. C, Photomicrographs of the mouse striatum showing that lesions superimposed with HA tag-positive areas in the 3 ΔLRRK2 groups. D and E shows the quantification of mutant Htt inclusions (ubiquitin positive volume) and aggregates (number of EM48-positive aggregates). Note the absence of major effects of ΔLRRK2 constructs on the size of mutant Htt-induced lesions, and a significant increase in the number of EM48 aggregates in the ΔLRRK2G2019S group. Results are expressed as the mean ± SEM. N = 10-12/group. One-way ANOVA and post hoc Fisher's PLSD test. *, p<0.05, Scale bars: A, 1 mm; C, 500 µm.

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