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 (SNc) in PD. However, the mechanisms underlying this interplay are ill-defined. Here, we investigated whether LRRK2G2019S 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 AAV2/6 vectors encoding human α -synA53T and the C-terminal portion of LRRK2 (Δ LRRK2), which contains the kinase domain, with either the G2019S mutation (Δ LRRK2G2019S) alone or the D1994A mutation (Δ LRRK2G2019S/D1994A), which inactivates the kinase activity of LRRK2. The AAVs were co-injected into the rat SNc and histological evaluation was performed at 6- and 15-weeks post-injection (PI). **Results:** Most SNc neurons co-expressed Δ LRRK2 and human α -synA53T after transduction. Δ LRRK2G2019S alone produced no cell loss at 15-weeks PI, whereas as expected, transduction with AAV- α -synA53T mixed with a control AAV coding for GFP produced significant loss of DA neurons. Co-injection of AAV- Δ LRRK2G2019S and AAV- α -synA53T induced a loss of DA neurons slightly but significantly greater than that produced by co-injection of AAV- α -synA53T and AAV-GFP. We also studied the inactive form, Δ LRRK2G2019S/D1994A at 6 weeks PI. Results showed that Δ LRRK2G2019S/D1994A did not alter the early toxicity of α -synA53T, in contrast to the active form Δ LRRK2G2019S that produced a moderate but significant increase in α -synA53T toxicity.

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 activity. However, considering that the effect of Δ LRRK2G2019S upon human α -synA53T is moderate in our paradigm where pathological proteins are overexpressed, the study supports the hypothesis that the interplay between LRRK2 and α -syn also implicates non-cell-autonomous mechanisms such as those involved in neuroinflammation.

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. The neuropathological hallmarks of PD are characterized by the progressive loss of dopaminergic (DA) neurons in the substantia nigra pars compacta (SNc) and the presence of neuronal aggregates (Lewy bodies) and dystrophic Lewy neurites containing the protein α -synuclein (α -syn) [1]. There is no treatment to delay neurodegeneration. 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. Several genes have been identified as causative factors.

Duplication, triplication, and rare mutations (A53T, A30P, E46K, H50Q, G51D, A53E) in the SNCA gene encoding the α -syn protein have been found in families with dominantly-inherited PD and are associated with early-onset forms of PD, with an amplification of α -syn aggregation [2–5]. The A53T [6], A30P [7] and E46K [8] substitutions have been the most studied so far. Compelling evidence shows that α -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 [9].

Mutations in leucine-rich repeat kinase 2 (LRRK2) are the most common genetic cause of both familial and sporadic PD [10, 11]. There are also variants in the LRRK2 locus that are considered to be risk factors for developing PD. The most prevalent mutation in LRRK2 is the G2019S substitution, accounting for 5 to 6% of AD familial PD and 1 to 2% of *de novo* generic PD cases [12, 13]. 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 [14, 15]. Although G2019S patients show similar clinical manifestations as those of sporadic patients [16], several studies have shown subtle differences [17, 18]. Some have reported the presence of LBs in symptomatic LRRK2 mutation carriers and LRRK2 can be found in LBs [19], 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 [20]. 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 [21, 22]. The mechanisms underlying LRRK2 neurotoxicity are still unknown. 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 [23, 24].

The central role of α -syn in PD pathogenesis has led to the hypothesis of a functional (and possibly physical) interaction between LRRK2 and α -syn [for a review[25]. Indeed, LRRK2 toxicity may require the presence of α -syn and, conversely, the presence of variant/mutant LRRK2 could increase the risk and/or impact of α -synucleopathy in PD. The level of kinase activity of LRRK2 could thus be a modifier of α -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 α -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 α -syn toxicity [26–28].

However, the mechanisms underlying the “protoxic” effect of LRRK2 (especially the G2019S mutation) on α -syn toxicity are not known and the exact role of the kinase domain has not been completely demonstrated. Pharmacological intervention suggests that the kinase activity of LRRK2 likely contributes to the synergy (for a review [16]). In addition, it is not known whether the potentiation of α -syn toxicity by the presence of LRRK2^{G2019S} is related solely to a cell-autonomous mechanism. Alternatively, LRRK2-expressing cells that surround DA neurons, especially microglial cells, astrocytes, and cells of the immune

system likely play a role. However, pharmacological intervention with LRRK2 inhibitors, pan-cellular knock out, or transgenic animals expressing wildtype LRRK2 or mutant LRRK2 cannot easily distinguish between cell-autonomous and non-cell-autonomous mechanisms *in vivo*.

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

Materials And Methods

Viral construction and production

Adeno-associated viral vectors (AAVs). Plasmid constructs were packaged into AAV2/6 capsids as described previously [58]. 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 cap8 genes, and (3) the AAV2 expression vector containing the transgene. Seventy-two hours following transfection, recombinant vectors 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.* [59] and expressed as viral genomes per ml of concentrated stocks (Vg/ml). AAVs coding for human Δ LRRK2 (WT, G2019S, and “kinase dead”), α -syn A53T, and GFP were produced.

Stereotaxic injection

Adult Sprague-Dawley rats (Charles River Laboratories), weighing ~250 g, 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 adeno-associated viral vectors were injected unilaterally into the SNc, at the following stereotaxic coordinates: +3.4 mm anterior to the interaural zero and $\pm$ 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 μ l of virus at a concentration of 2.5×10^{10} viral particles per site for single injections and 2.5×10^{10} viral particles of each vector for co-injections for a total of $5 \times$

10^{10} viral particles per site, with a 34-gauge blunt-tipped needle linked to a 10- μ l Hamilton syringe by a polyethylene catheter at a rate of 0.25 μ l/min using an automatic pump (CMA-4004). The needle was left in place for five minutes and then slowly withdrawn.

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.

For immunohistochemistry, rats were transcardially perfused with 300 ml 4% paraformaldehyde in phosphate buffer at a rate of 30 ml/min. After perfusion, the brain of each rat was quickly removed and immersed in ice-cold 4% paraformaldehyde/0.1X PBS for at least 24 h, before transfer to 15% sucrose for 24 h and then 30% sucrose the next day, for cryoprotection. The brains were then cut into 40- μ m sections on a freezing microtome (CM1900, Leica, Germany). Serial sections of the striatum and midbrain were stored in antifreeze solution (30% sucrose/30% ethylene glycol in PBS) until 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_2O_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. 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. The sections were then incubated with DAB for 30 s to 1 min and mounted on slides in Eukitt mounting medium.

Cell counting

Optical fractionator sampling was carried out on a Zeiss microscope AxioPlan. 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 SNc and the number of positive neurons per section was calculated using Mercator Software (Explora Nova, France). We placed 100 $\times$ 100 μ m grids in a systematically random manner, 80 $\times$ 80 μ m apart, with a 3- μ m offset from the surface of the section. Quantification was performed on 12 serial sections spaced by 200 μ m, corresponding to the entire SNc.

The phosphorylation of α -syn on S129 (P- α -syn^{S129}) was evaluated by counting the number of P- α -syn^{S129}-positive neurons in the SNc using stereology methods. The SNc was delimited by Nissl staining and the grids (250 × 250 μ m) placed, with a space of 100 × 100 μ m. Quantification was performed on six serial sections spaced by 400 μ m, corresponding to the entire SNc. In the striatum, a threshold was applied to select only the P- α -syn^{S129}-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, Dako, 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 1 h 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 1 h 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 (DM8000, Leica, Germany).

Colocalization

The percentage of co-localization between Δ LRRK2 and α -syn was determined by counting the number of cells co-expressing both Δ LRRK2 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 Δ LRRK2 and α -syn proteins were evaluated on three coronal sections in the SNc. Twenty cells co-expressing both Δ LRRK2 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 Δ LRRK2 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.

Statistical analysis

Normal distribution of data was checked by Shapiro test. Data were analyzed by two-tailed, one-way analysis of variance (ANOVA) performed with Statistica software (Statsoft Inc., Tulsa, Oklahoma, USA) and, when appropriate, LSD post-hoc correction for multiple comparisons was applied. Unpaired Student's *t*-tests were performed for pairwise comparisons. 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 potential synergy between AAV- α -syn^{A53T} and AAV- Δ LRRK2^{G2019S} toxicity

We investigated whether human LRRK2 can increase the toxicity of human α -syn in DA neurons through cell-autonomous mechanisms. We used AAVs, which allow selective expression in neuronal cells and efficiently transduce a large volume of brain tissue due to their good diffusion properties. In a previous study [29], we showed that the C-terminal portion of human LRRK2^{G2019S} (Δ LRRK2^{G2019S}, 1330–2527 aa) retains, at least in part, the biochemical properties of full-length LRRK2^{G2019S}, including higher kinase activity than the wildtype fragment. In addition, we found that overexpression of the C-terminal portion of human Δ LRRK2^{G2019S} in the adult rat SNc, using AAVs, produced partial (~ 30%) but significant loss of DA neurons at 25 weeks post-transduction, whereas overexpression of the wildtype form of LRRK2 (Δ LRRK2^{WT}) was not toxic. Here, we used a similar approach using a slightly larger fragment (aa 1283–2527) (Fig. 1A).

We studied the effects of AAV- Δ LRRK2^{G2019S}, AAV- Δ LRRK2^{WT}, and AAV- Δ LRRK2^{G2019S/D1994A} alone at 15 weeks PI. We injected 4 μ l of viral vector solution in all cases. A final amount of 2.5×10^{10} viral particles per site and per vector was used. Each AAV was injected unilaterally into the SNc (2.5×10^{10} 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 SNc (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 SNc did not differ significantly between the control group (PBS) and AAV- Δ LRRK2^{WT}, AAV- Δ LRRK2^{G2019S}, or AAV- Δ LRRK2^{G2019S/D1994A} groups (Fig. 1C). Thus, these results suggest that the Δ LRRK2 fragments alone did not trigger significant neurodegeneration of DA neurons at 15 weeks PI.

We sought an injection protocol that leads to mild degeneration, in such a way that potential “pro-toxic” effect of LRRK2 constructs could be easily detected, as we wanted to investigate whether AAVs encoding

the different Δ LRRK2 constructs could increase the toxicity of AAV- α -syn^{A53T}. We conducted pilot experiments to determine the appropriate dose (titers) of AAV- α -syn alone that would lead to progressive and partial loss of DA neurons. Quantification of TH-positive cells in the SNc showed a moderate loss of DA neurons (~ 30%) at 12 and 15 weeks after transduction with AAV- α -syn^{A53T} (2.5×10^{10} particles post-injection (PI)) (Supplementary Fig. S1A-B). After transduction, DA neurons often displayed accumulation of α -syn phosphorylated at its serine 129 (p-synS129). The cells positive for p-synS129 were also positive for ThioS suggesting that these accumulations were aggregates (Supplementary Fig. S1C).

Thus, a co-injection protocol with AAV- α -syn^{A53T} and AAV- Δ LRRK2^{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^{A53T} and AAV- Δ LRRK2^{G2019S}

We next investigated whether the presence of the various Δ LRRK2 fragments (wildtype, G2019S, or G2019S/D1994A) in DA neurons could modify the toxicity of human α -syn^{A53T} using this co-transduction paradigm (with 2.5×10^{10} particles for each vector).

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

We next evaluated the toxic effects of human α -syn^{A53T} in the presence or absence of AAV- Δ LRRK2^{G2019S}. AAV- α -syn^{A53T} alone produced a significant 38% loss of TH-positive cells, as measured by unbiased stereology in the SNc at 15 weeks PI (mean count $\pm$ SEM: Control, $12,344 \pm 734$; AAV- α -syn^{A53T}, $7,555 \pm 527$). The co-injection of AAV- α -syn^{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^{A53T} alone ($6,601 \pm 360$). The co-injection of AAV- α -syn^{A53T} and AAV- Δ LRRK2^{G2019S} induced a loss (-55%) of DA neurons (mean count $\pm$ SEM: $5,585 \pm 355$) that was significantly greater than that measured in the two other groups injected with AAV- α -syn^{A53T} (Fig. 3A). We evaluated the impact of SNc cell loss on the level of dopaminergic terminals in the dorso-medial striatum using TH-immunofluorescence in both the α -syn^{A53T}/GFP and α -syn^{A53T}/ Δ LRRK2^{G2019S} groups (Fig. 4A). These measurements were performed in the dorsal striatum, which receives projections from the injected SNc region. TH immunoreactivity in the striatum in both the α -syn^{A53T}/GFP and α -syn^{A53T}/ Δ LRRK2^{G2019S} groups was 15% lower than in the

control group (PBS). This small α -syn^{A53T}-induced loss of TH-positive fibers was similar in the GFP and Δ LRRK2^{G2019S} groups (Fig. 4C).

We also counted the number of SNc cells showing P- α -synS129 immunoreactivity, a marker of α -syn aggregation, in the different groups (Fig. 3C). The number of P- α -synS129-positive cells was significantly lower in the group co-infected with AAV- α -syn^{A53T} and AAV- Δ LRRK2^{G2019S} than that of the groups infected with AAV- α -syn^{A53T} alone or in combination with AAV-GFP (Fig. 3D). We also evaluated P- α -synS129 immunoreactivity in the striatum. Small P- α -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 SNc, we found lower levels of aggregated α -syn in the striatum of rats co-infected with AAV- α -syn^{A53T} and AAV- Δ LRRK2^{G2019S} than in those infected with AAV- α -syn^{A53T} / GFP (Fig. 3E-F).

Differential effects of AAV- Δ LRRK2^{G2019S} and AAV- Δ LRRK2^{G2019S/D1994A} on AAV- α -syn^{A53T} toxicity

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

We first compared the levels of transgene expression after transduction with α -syn^{A53T} and AAV- Δ LRRK2^{G2019S} or AAV- Δ LRRK2^{DK}. Quantitative immunofluorescence analysis showed that almost the entire SNc was infected by AAV- α -syn^{A53T} when co-infected with either AAV- Δ LRRK2^{GS} or AAV- Δ LRRK2^{DK} (Fig. 5). We also re-evaluated the co-localization of the LRRK2-related transgenes and α -syn^{A53T} (Fig. 6). In total, 76% of neurons expressed both α -syn^{A53T} and Δ LRRK2 (Fig. 6C), consistent with our observations in the experiments described above (see Fig. 1). We evaluated the level of fluorescence intensity corresponding to Δ LRRK2-HA staining at higher magnification. Δ LRRK2^{GS} and Δ LRRK2^{DK} were expressed at the same level in SNc neurons (Fig. 6C). In addition, human α -syn^{A53T} protein was expressed at the same levels in neurons co-expressing Δ LRRK2^{GS} and Δ LRRK2^{DK} (Fig. 6C).

We then assessed the loss of DA neurons produced by AAV- α -syn^{A53T} when co-injected with either AAV- Δ LRRK2^{G2019S} or AAV- Δ LRRK2^{DK}. The loss of DA neurons induced by human α -syn^{A53T} was significantly lower in the presence of Δ LRRK2^{DK} than that in the presence of Δ LRRK2^{GS} (Fig. 7A-B). The number of cells with P- α -synS129 immunoreactivity was similar in the Δ LRRK2^{DK} and Δ LRRK2^{G2019S} groups.

Finally, we carried out a preliminary characterization of the status of microglial cells at this early time point (6 weeks PI), by immunohistochemistry using a validated marker (Iba1), because of the role of neuroinflammation in neurodegeneration observed in human α -syn^{A53T} models. As expected, microglial cells in rats overexpressing human α -syn^{A53T} appeared more reactive as compared to rats injected with vehicle. The quantification of immunofluorescence levels in the SN and striatum showed that human α -syn^{A53T} significantly activated microglia. However, overexpression of Δ LRRK2^{G2019S} and Δ LRRK2^{DK} did not have any impact on the microglial activation induced by the mutant human α -syn (Supplementary Fig. S2).

Finally, we investigated whether the “pro-toxic” effect of Δ LRRK2^{G2019S} on α -syn^{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 Δ LRRK2 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) [30]. Lentiviral vectors were injected into the striatum of wildtype mice to produce local cell loss within the six weeks following transduction, as previously described [31, 32]. The striatal lesions were characterized by the loss of neuronal markers DARPP32 and COX (not shown) as well as NeuN (Fig. 8A-B). Localization of the lesions in the striatum coincided with that of the expression of LRRK2 fragments detected using the HA-tag (Fig. 8C). Quantitative analysis of these histological markers showed that none of the Δ LRRK2 forms significantly modified the volume of the striatal lesions produced by mutant Htt (Fig. 8B). 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 did not indicated differences within groups (Fig. 8D). 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 [33–35]. Δ LRRK2^{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 DK forms (Fig. 8D-E).

Our results show that the synergistic effect of Δ LRRK2^{G2019S} on the toxicity of human α -syn^{A53T} towards DA neurons depends on its kinase activity. Importantly, Δ LRRK2^{G2019S} overexpression did not significantly increase the neurotoxic effects produced by a different aggregating protein, mutant Htt, suggesting that the effect of Δ LRRK2^{G2019S} on α -syn^{A53T}-induced neurotoxicity is not due 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^{G2019S} mutation leads to increased kinase activity and that such abnormally increased kinase activity could lead to cell death [23, 36–38].

In addition, a role for LRRK2 in α -syn toxicity has been suggested [26–28]. Indeed, neuropathological evaluation of the brain of PD patients with LRRK2 mutations shows in many cases the presence of *bona fide* LBs and Lewy neurites [39]. However, the role of the kinase in the crosstalk between LRRK2 and α -syn, especially how the kinase activity of LRRK2^{G2019S} modulates α -syn neurotoxicity in the SNc is unknown. In addition, the respective roles of cell-autonomous and non-cell-autonomous mechanisms are largely unknown in this crosstalk.

Here, we used an AAV-based approach to selectively target SNc 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^{A53T} in the rat SNc. Under our experimental conditions, AAV- Δ LRRK2^{G2019S} alone did not induce the loss of DA neurons and AAV- α -syn^{A53T} alone induced a partial loss. The loss of DA cells produced by co-expression of Δ LRRK2^{G2019S} and α -syn^{A53T} was significantly higher than that measured in rats injected with AAV- α -syn^{A53T} alone or co-injected with a control vector (AAV-GFP). Conversely, overexpression of the inactive “kinase dead” form Δ LRRK2^{DK}, at levels similar to those of Δ LRRK2^{G2019S}, did not alter the toxicity of α -syn^{A53T}. Although preliminary, quantitative characterization of microglial reactivity induced by human α -syn^{A53T} in SNc and striatum did not show obvious changes attributable to LRRK2 fragments. These novel findings further support the hypothesis that the C-terminal domain of LRRK2^{G2019S} is sufficient to augment the toxic effects of α -syn^{A53T} through a cell-autonomous mechanism involving the catalytic activity of its kinase domain.

The histological evaluation we performed after transduction of the SNc with AAV and AAV- Δ LRRK2^{G2019S} show that both transgenes were overexpressed in DA neurons. In both cases, approximately 70% of the SNc was infected. After co-injection, co-localization of both transgenes was found in most neurons in the SNc. Neuropathological evaluation at 15 weeks PI after transduction with AAV- α -syn^{A53T} 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 [29].

The relevance of overexpressing the C-terminal domain of LRRK2 *versus* the full-length protein is potentially debatable. Indeed, Δ LRRK2^{G2019S} lacks N-terminal domains that are known to play crucial roles in LRRK2 function. we previously showed that overexpression of the Δ LRRK2^{G2019S} fragment using AAVs triggers neurodegeneration of DA neurons six months post-transduction, whereas the Δ LRRK2^{WT} fragment, expressed at similar high levels, was devoid of obvious neurotoxicity [29]. Cell death produced by this fragment which has a detectable kinase activity is likely independent of the interaction with RAB10, since the Δ LRRK2 fragment was found unable to interact with RAB10, in contrary to full-length LRRK2 fragment [29]. Thus, it is conceivable that the overexpression of Δ LRRK2^{G2019S} leads to abnormal/higher phosphorylation of substrates, relative to that by the overexpression of Δ LRRK2^{WT}, that is at least partially in common with that of full-length LRRK2 [40–43].

We investigated whether Δ LRRK2^{G2019S} effect on α -syn^{A53T} was specific of these two pathological proteins, or only resulted from a non-specific vulnerability of neurons expression the LRRK2 fragment. We

tested in a different cellular context the effect of Δ LRRK2^{G2019S} against mutant Htt in the striatum using a simple approach with lentiviral vectors. In this case, the degeneration of striatal neurons induced by mutant Htt within the 6 weeks PI was not significantly changed by the overexpression of Δ LRRK2^{G2019S}. Thus, the effect of Δ LRRK2^{G2019S} upon α -syn^{A53T} toxicity is likely specific of a particular interplay between these two proteins.

The present experimental paradigm using AAVs allowed addressing the question of the potential cell-autonomous exacerbation of α -syn^{A53T} toxicity by the kinase activity of LRRK2 directly in the SNc and only in neurons. In contrast, other viral vector models (HSV or adeno) also transduce different cell types in the striatum [44, 45]. Here, we directly investigated whether there is a functional interaction between AAV- α -syn^{A53T} and Δ LRRK2^{G2019S} in DA neurons. Our results show the existence of such an interaction, as overexpression of Δ LRRK2^{G2019S} significantly enhanced the neurotoxic effects of α -syn^{A53T} in rat SNc. 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 α -syn^{A53T} in transgenic animals [28]. In these double-transgenic mice, the authors found significant degeneration of the striatum and cortex and enhanced accumulation of α -syn aggregates. This proved the existence of a functional cross talk between α -syn and LRRK2 in neurons in vivo when the proteins are expressed at relatively high levels. Pathological transgenes were not expressed in the SNc and no DA degeneration was detected in these models. The CamKII α promoter used to drive the expression of the tetracycline transactivator (tTA), which activates the TetO promoter of the LRRK2 and α -syn^{A53T} transgenes in these mice, is likely not active in SNc DA neurons, as endogenous expression of CamKII α in neurons of the SNc is lower than that observed in forebrain neurons. In LRRK2 knockout rats, the toxicity induced by AAV coding for α -syn is lower than that in wildtype rats [27]. 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) [46]. These observations and our results suggest that the crosstalk between LRRK2 and α -syn can occur in the same neurons. Thus, our results show that synergy between LRRK2 and α -syn depends, at least in part, on cell-autonomous mechanisms. However, our results also indicate that even in an experimental condition where the two proteins are expressed in high levels (approximately 5–50 fold the level of endogenous α -syn and LRRK2 respectively), the effect of Δ LRRK2^{G2019S} on α -syn^{A53T} is moderate. Thus, it is reasonable to speculate that in a condition where physiological levels of expression of LRRK2 and α -syn, the cell-autonomous crosstalk between the two proteins might be of moderate importance in DA neurons. Non-cell autonomous mechanisms involving interaction of DA neurons with neighboring glial cells and immune cells may have more important roles in patients and transgenic animal models. For example, it has been recently shown that the seeding of α -syn aggregates by the exposure of neurons to α -syn fibrils is higher in neurons expressing mutant LRRK2 [47]. The level of LRRK2 activity in microglial cells may also regulate protoxic phenomena associated with α -syn-induced neuroinflammation [26, 48, 49]. LRRK2 plays a key role in the immune system [50]. A single nucleotide polymorphism (N2081D) in the region coding for kinase domain of LRRK2 is a major risk factors for Crohn disease, a form of inflammatory bowel disease [51].

We investigated whether $\Delta\text{LRRK2}^{\text{G2019S}}$ effect on $\alpha\text{-syn}^{\text{A53T}}$ was specific of these two pathological proteins, or only resulted from a non-specific vulnerability of neurons expression the LRRK2 fragment. We tested in a different cellular context the effect of $\Delta\text{LRRK2}^{\text{G2019S}}$ against mutant Htt in the striatum using a simple approach with lentiviral vectors. In this case, the degeneration of striatal neurons induced by mutant Htt within the 6 weeks PI was not significantly changed by the overexpression of $\Delta\text{LRRK2}^{\text{G2019S}}$. Thus the effect of $\Delta\text{LRRK2}^{\text{G2019S}}$ upon $\alpha\text{-syn}^{\text{A53T}}$ toxicity is likely specific of functional interplay between these two proteins.

The neuronal mechanisms underlying the synergy between LRRK2 and $\alpha\text{-syn}$ are ill defined. It is possible that LRRK2 - $\alpha\text{-syn}^{\text{A53T}}$ interplay involves a direct physical interaction between the two proteins [52], although, indirect effects leading to a functional interplay is more often discussed. In addition, the role of the kinase domain of LRRK2 is unclear. It is generally accepted that the higher kinase activity of $\text{LRRK2}^{\text{G2019S}}$, 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 [53]. 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 $\text{LRRK2}^{\text{G2019S}}$ have been developed and extensively characterized. These models display no (or very limited) degeneration of DA cells in the SNc. Various experimental approaches clearly demonstrate that the severity of the resulting toxicity is dependent on the level of expression of $\text{LRRK2}^{\text{G2019S}}$ [54]. There is limited evidence obtained *in vivo* that shows a relationship between the higher kinase activity of $\text{LRRK2}^{\text{G2019S}}$ and neurotoxicity to dopaminergic cells of the SNc. The overexpression of $\text{LRRK2}^{\text{G2019S}}$ (or $\Delta\text{LRRK2}^{\text{G2019S}}$) in the SNc was found to induce the loss of DA neurons using HSV and adenovirus models injected into the striatum [23, 55], as well as in our previous study with AAV- $\Delta\text{LRRK2}^{\text{G2019S}}$ injected into the SNc [56].

Only a few studies have directly addressed the role of the kinase in the interaction between $\alpha\text{-syn}$ and LRRK2 toxicity. It was shown that neuroinflammation and neurodegeneration produced by the transduction of the SNc with AAV- $\alpha\text{-syn}$ 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 [26]. More recently, Daher *et al.* showed that the toxicity of AAV- $\alpha\text{-synuclein}$ in the SNc was higher in transgenic $\text{LRRK2}^{\text{G2019S}}$ than wildtype rats. Interestingly, treatment of both genotypes with the LRRK2 inhibitor PF-06447475 (PF) reduced the toxicity of $\alpha\text{-syn}$ [27]. This suggests that the exacerbation of $\alpha\text{-syn}$ toxicity by $\text{LRRK2}^{\text{G2019S}}$ could result from elevated catalytic activity of the kinase. However, LRRK2 inhibitors, including PF, destabilize LRRK2, leading to reduced cellular levels of the protein [57]. Thus, protection by PF against combined $\alpha\text{-syn}/\text{LRRK2}^{\text{G2019S}}$ toxicity may result from the reduction of LRRK2 levels rather than actual inhibition of the catalytic activity of the kinase. Here, we found that overexpression of the inactive protein $\Delta\text{LRRK2}^{\text{G2019S/D1994S}}$ did not alter the toxicity AAV- $\alpha\text{-syn}^{\text{A53T}}$, whereas expression of $\Delta\text{LRRK2}^{\text{G2019S}}$ increased the toxicity of AAV- $\alpha\text{-syn}^{\text{A53T}}$ towards DA neurons. In our experimental system where both proteins have high expression levels, we were able to verify that the difference between the

toxicity of $\Delta\text{LRRK2}^{\text{G2019S}}$ and $\Delta\text{LRRK2}^{\text{DK}}$ was not related to a difference in protein levels. Quantitative confocal analysis showed that the levels of the two proteins were similar in DA neurons *in vivo*. Thus, it is likely that the difference between the effects of $\Delta\text{LRRK2}^{\text{WT}}$ and $\Delta\text{LRRK2}^{\text{G2019S}}$ is mainly due to their different kinase activities.

Conclusions

Our results suggest that the C-terminal domain of $\text{LRRK2}^{\text{G2019S}}$ containing the ROC-COR, Kinase and WD40 domains is sufficient to potentiate the toxicity of human $\alpha\text{-syn}^{\text{A53T}}$ in DA neurons *in vivo* and that this effect is kinase-dependent. This cell-autonomous mechanism may additively or synergistically acts with other non-cell-autonomous mechanisms, especially those involving neuro-inflammation to trigger the death of DA neurons in PD. Our findings further support the view that inhibition of the kinase activity of LRRK2 might be beneficial to slow $\alpha\text{-syn}$ -dependent mechanisms in PD.

Abbreviations

ΔLRRK2 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

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

SNc 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

WT Wild-type

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.

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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 $\pm$ 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 μ m.

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 $\pm$ 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 μ m; high magnifications, 50 μ m.

Figures

Figure 1

Immunohistochemistry for tyrosine hydroxylase (TH). A The C-terminal fragment of LRRK2, called Δ LRRK2, was generated in different forms, WT: G2019S, or dead kinase (DK). B-C, The three fragments were cloned into an AAV and unilaterally injected into the rat SNc. Fifteen weeks PI, TH immunohistochemistry was performed (B). The number of TH-positive neurons was evaluated by stereology (C), with no obvious toxicity due to the LRRK2 fragments at 15 weeks PI. Results are expressed

as means $\pm$ the standard error of the mean (SEM). N = 10 animals/group. ANOVA and PLSD post hoc test. n.s.: not significant. Scale bars: 750 μ m and 400 μ m.

Figure 2

Histological evaluation of transgene expression in the SNpc at 15 weeks post-injection. (A) Evaluation of α -syn (in green) transduction in the SNpc. TH staining, in red. Scale bar: 500 μ m. (B) Measurement of the number of neurons expressing both α -syn and $\Delta\text{LRRK2GS}$. The higher magnification shows cytoplasm localization of $\Delta\text{LRRK2GS}$. Results are expressed as means $\pm$ the standard error of the mean (SEM). Scale bars: 40X = 60 μ m, 63X zoom 4 = 10 μ m.

Figure 3

Immunohistochemistry for tyrosine hydroxylase (TH) and phospho- α -synS129 aggregates at 15 weeks post-injection. (A-D) Evaluation of the number of TH-positive and P- α -synS129-positive neurons by stereology in the SNpc. Scale bars: 750 μm , 400 μm . (E-F) Determination of the percentage of the area occupied by P- α -synS129-positive staining in the striatum at 15 weeks PI. Results are expressed as

means $\pm$ the standard error of the mean (SEM). N = 10 animals/group. ANOVA and PLSD post hoc test. *P < 0.05, ***P < 0.001. Scale bars: 200 μ m, 50 μ m.

Figure 4

Immunohistochemistry for tyrosine hydroxylase (TH). (A) Evaluation of striatal fiber loss in the dorso-medial striatum by TH-immunofluorescence. Scale bar: 1000 μ m. (B-C) Quantification of the mean grey value at a magnification of 5X. Results are expressed as means $\pm$ the standard error of the mean (SEM).

N = 10 animals/group. ANOVA and PLSD post hoc test. **P < 0.01 (A-B) scale bar: 1000 μ m. (B-C) scale bar: 100 μ m.

Figure 5

Measurement of transduction volume. A, confocal images to delineate the SNpc based on TH staining (in red), reported in the green channel, corresponding to the human α -syn protein when co-expressed with Δ LRRK2G2019S (GS) and the dead kinase form of Δ LRRK2. Scale bar: 1000 μ m. B, Quantification of the fraction (%) of the SNpc expressing human α -syn protein after co-transduction with Δ LRRK2G2019S or Δ LRRK2DK. Note that the level of overexpression of human α -syn protein is similar between the two conditions. Results are expressed as means $\pm$ the standard error of the mean (SEM). N = 8 animals/group. No statistical difference, Student t test.

Figure 6

Co-localization and expression of Δ LRRK2 and α -syn. A-B, Double immuno-fluorescence of Δ LRRK2 and α -syn at low and high-magnification, showing co-expression of both transgenes at similar levels by most cells. Scale bars: 200 μ m, magnifications = 50 μ m and 10 μ m. C, Quantification of the percentage of co-localization and protein levels (mean fluorescence) for two different groups. Results are expressed as

means ± the standard error of the mean (SEM). N = 8 animals/group. No statistical difference, Student t test.

Figure 7

Immunohistochemistry for tyrosine hydroxylase (TH) and phospho- α -synS129 aggregates at six weeks post-injection. (A-D) Evaluation of the number of TH-positive and P- α -synS129-positive neurons in the

SNc by stereology. Results are expressed as means $\pm$ the standard error of the mean (SEM). N = 10 animals/group. ANOVA and Fisher PLSD post hoc test. *, P < 0.05. Scale bar: 750 μ m, 400 μ m.

Figure 8

Effect of the various forms of Δ LRRK2 on the toxicity of mutant Htt. Mice received a bilateral intrastriatal injection of a mixture of two lentiviral vectors (LV): LV-Htt171-82Q with LV-LacZ (CT, control), LV- Δ LRRK2WT (WT), LV-LRRK2G2019S (GS) or LV- Δ LRRK2G2019S/A1994E (DK). Six weeks after injection,

brains were processed for histological evaluation by immunohistochemistry (ICH) for the HA-tag, NeuN, ubiquitin and mutant Htt aggregates using the EM48 antibody to evaluate Htt171-82Q-dependent neurotoxicity. (A) Camera Lucida representation of the rostro-caudal extension of striatal lesions produced by the mutant Htt fragment as seen using NeuN ICH. Dark gray 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 Δ LLRK2 groups. (D, E) Histograms showing quantification of mutant Htt inclusions (ubiquitin) and aggregates (EM48). Note the absence of major effects of Δ LLRK2 constructs on mutant Htt toxicity, except for a mild increase of the number of EM48 aggregates in the Δ LLRK2G2019S group. Results are expressed as the mean $\pm$ the standard error of 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 μ m.

Supplementary Files

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