LRRK2 Kinase Activity Does Not Alter Cell-Autonomous Tau Pathology Development in Primary Neurons

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Abstract.
Background: Mutations in leucine-rich repeat kinase 2 (LRRK2) are the most common cause of familial Parkinson’s disease (PD) and are also associated with genetic risk in idiopathic PD. Mutations in LRRK2, including the most common p.G2019S lead to elevated kinase activity, making LRRK2 kinase inhibitors prime targets for therapeutic development. However, the role of LRRK2 kinase activity in PD pathogenesis has remained unclear. While essentially all LRRK2-PD patients exhibit dopaminergic neuron loss, many of these patients do not have α-synuclein Lewy bodies in their brains. So, what is the neuropathological substrate of LRRK2-PD? Tau has emerged as a possible candidate due to the presence of tau pathology in the majority of LRRK2 mutation carriers and reports of hyperphosphorylated tau in LRRK2 animal models.

Objective: In the current study, we aim to address whether a mutation in LRRK2 changes the cell-autonomous seeding of tau pathology in primary neurons. We also aim to assess whether LRRK2 kinase inhibitors are able to modulate tau pathology.

Methods/Results: Treatment of primary neurons with LRRK2 kinase inhibitors leads to prolonged kinase inhibition but does not alter tau pathology induction. The lack of an effect of LRRK2 kinase activity was further confirmed in primary neurons expressing LRRK2G2019S and with two different forms of pathogenic tau. In no case was there more than a minor change in tau pathology induction.

Conclusion: Together, our results indicate that LRRK2 kinase activity is not playing a major role in the induction of tau pathology in individual neurons. Understanding the impact of LRRK2 kinase inhibitors on pathology generation is important as kinase inhibitors move forward in clinical trials.

Keywords: Leucine-rich repeat kinase 2, G2019S, genetic risk, kinase inhibition, Mapt

INTRODUCTION

Parkinson’s disease (PD) afflicts over 6 million people worldwide [1]. Multiple factors, including lifestyle, exposure to environmental pathogens, and genetic variants can influence risk of developing PD. Up to 27% of PD has been estimated to be heritable [2, 3] through both rare mutations and more common polymorphisms in the genome. The most common cause of familial PD and a common risk factor for idiopathic PD is a mutation in the gene encoding leucine-rich repeat kinase 2 (LRRK2) [4]. The most prevalent of these mutations, p.G2019S, confers a 25–42.5% risk of developing PD [5]. Although these patients show similar symptoms to idiopathic PD, neuropathologically, 21–54% of patients lack the...
hallmark α-synuclein Lewy bodies exhibited by idiopathic PD patients [6–8]. Notably, the majority of LRRK2 mutation carriers exhibit tau pathology [8, 9]. The tau pathology in idiopathic PD and LRRK2-PD is similar in conformation and distribution to AD tau and may be partially responsible for the cognitive decline seen in these patients during the disease course [8, 10]. The appearance of tau pathology in LRRK2 mutation carriers suggests that the genetic risk conferred by LRRK2 mutations may alter the development or progression of tau pathology. If LRRK2 mutations do in fact modulate tau pathology, then LRRK2 kinase inhibitors being developed for the treatment of genetic and idiopathic PD may influence tau pathology in these patients, and possibly in AD and other tauopathy patients as well.

In the current study, we seeded tau pathology in primary neuron cultures from wildtype mice and mice expressing LRRK2G2019S to determine whether tau pathology was altered in a cell-autonomous manner. We show, using two different forms of pathogenic tau, that LRRK2G2019S expression does not substantially alter the development of tau pathology. We also show that three LRRK2 kinase inhibitors show strong and sustained inhibition of LRRK2 kinase activity but have no impact on cell-autonomous tau pathology development. This study suggests that LRRK2 kinase activity does not have a substantial role in the initial development of tau pathology, but it does not preclude a role for LRRK2 in the cell-to-cell transmission of tau or long-term effects which are not recapitulated in cell culture.

MATERIALS AND METHODS

Mice

All housing, breeding, and procedures were performed according to the NIH Guide for the Care and Use of Experimental Animals and approved by the University of Pennsylvania Institutional Animal Care and Use Committee. C57BL/6J (NTG, JAX 000664, RRID: IMSR_JAX:000664) and B6.Cg-Tg(Lrrk2*G2019S)2Yue/J (G2019S, JAX 012467, RRID: IMSR_JAX:012467) mice have been previously described [11]. The current G2019S BAC line was backcrossed to C57BL/6J mice for > 10 generations and bred to homozygosity at loci as determined by quantitative PCR and outbreeding. The expression level of G2019S LRRK2 was thereby stabilized in this line of mice. All experiments shown use homozygous G2019S mice.

Primary hippocampal or neuron cultures

Primary hippocampal neuron cultures were prepared as previously described [12] from postnatal day (P) 1 non-transgenic or LRRK2G2019S transgenic mice. Dissociated hippocampal or cortical neurons were plated at 17,000 cells/well (96-well plate) or 1,000,000 cells/well (6-well plate) in neuron media (Neurobasal medium (ThermoFisher 21103049) supplemented with B27 (ThermoFisher 17504044), 2 mM GlutaMax (ThermoFisher 35050061), and 100 U/mL penicillin/ streptomycin (ThermoFisher 15140122).

Recombinant tau PFFs

Purification of recombinant human tau and generation of tau X-T40 PFFs was conducted as described elsewhere [13]. The plasmid containing the gene of interest was transformed into BL21 (DE3) RIL-competent E. coli (Agilent Technologies Cat# 230245). A single colony from this transformation was expanded and tau protein was purified by cationic exchange using fast protein liquid chromatography as previously described [14]. Tau fibrillization was induced in the absence of co-factors by incubation of 40 μM recombinant T40 (4R2N tau) monomer with 2 mM DTT in Dulbecco’s phosphate-buffered saline (DPBS, Corning Cat#21-031-CV) and incubated shaking at 1,000 rpm and at 37 °C for 7 days to create passage 1 of fibrils. A second passage was set up by incubating 10% of the first passage with 90% fresh tau monomer at a final concentration of 40 μM tau. This passaging was repeated until tau was recovered in the pellet fraction. Conversion to PFFs was validated by sedimentation at 100,000 x g for 30 minutes at 22°C. Equal volumes of supernatant and pellet fraction were loaded and analyzed by Coomassie blue staining of SDS-PAGE gels.

Human tissue

All procedures were done in accordance with local institutional review board guidelines of the University of Pennsylvania. Written informed consent for autopsy and analysis of tissue sample data was obtained either from patients themselves or their next of kin. Cases used for extraction (Table 1) of PHF tau were selected based upon a high burden of tau pathology by immunohistochemical staining.
Table 1
Characterization of AD tau preparations. Descriptive information related to the cases of AD PHFs tau prepared for these studies. PMI, postmortem interval.

| Case     | Age | Sex | PMI (hours) | Total protein (µg/µL) | Tau (µg/µL) | Tau (% protein) | α-Syn (µg/mL) | Aβ 1–40 (ng/mL) | Aβ 1–42 (ng/mL) |
|----------|-----|-----|-------------|-----------------------|-------------|----------------|--------------|----------------|----------------|
| AD Case 1 | 73  | F   | 4           | 8.3                   | 2.6         | 31.3           | 0.7          | 31.2           | 111.4          |
| AD Case 2 | 62  | M   | 12          | 8.7                   | 1.4         | 16.1           | 0.5          | 53.7           | 60.1           |

**Human brain sequential detergent fractionation**

Frozen postmortem human frontal or temporal cortex brain tissue containing abundant tau-positive inclusions was selected for sequential extraction based on IHC examination of these samples as described [15] using previously established methods. These brains were sequentially extracted with increasing detergent strength as previously described [13]. After thawing, meninges were removed and gray matter was carefully separated from white matter. Gray matter was weighed and suspended in nine volumes (w/v) high salt (HS) buffer (10 mM Tris-HCL (pH 7.4), 800 mM NaCl, 1 mM EDTA, 2 mM dithiothreitol [DTT], protease and phosphatase inhibitors and PMSF) with 0.1% sarkosyl and 10% sucrose, followed by homogenization with a dounce homogenizer and centrifugation at 10,000 x g for 10 min at 4°C. The resulting pellet was re-extracted with the same buffer conditions and the supernatants from all extractions were filtered and pooled.

Additional sarkosyl was added to the pooled supernatant to reach a final concentration of 1% and the supernatant was nucitated for 1 h at room temperature. The samples were then centrifuged at 300,000 x g for 60 min at 4°C. The pellet, which contains pathological tau, was washed once with PBS and resuspended in 100 μL of PBS per gram of gray matter by passing through a 27G/0.5-in. needle. The pellets were further suspended by brief sonication (QSonica Microson™ XL-2000; 20 pulses; setting 2; 0.5 s/pulse). The suspension was centrifuged at 100,000 x g for 30 min at 4°C. The final supernatant was utilized for all studies and is referred to as AD PHF tau. All extractions were characterized by western blotting, sandwich ELISA for tau, α-synuclein and Aβ 1–40, Aβ 1–42, and validated by immunocytochemistry in primary neurons from non-transgenic mice. For the extractions used in this study, tau constituted 16.1–35.7% of the total protein, while α-synuclein and Aβ constituted 0.011% or less of total protein.

**LRRK2 inhibitor treatments**

LRRK2 inhibitors PF-475 and PF-360 were synthesized at Pfizer, Inc. MLi-2 was obtained from Tocris Bioscience (Cat#5756). All LRRK2 inhibitors were reconstituted at 10 mM in DMSO and stored at −20°C. They were further diluted to the final concentration indicated in neuron media with DMSO as a vehicle control.

**Tau XT-40 PFF/AD PHF treatments**

**Primary neurons**

For treatment of neurons, X-T40 PFF tau and AD PHF tau were vortexed and diluted with Dulbecco’s phosphate-buffered saline (DPBS, Corning Cat#21-031-CV). They were then sonicated on high for 10 cycles of 30 s on, followed by 30 s off (Diagenode Biorupter UCD-300 bath sonicator). Tau was then diluted in neuron media to the noted concentrations and added to neuron cultures at 7 days in-vitro (DIV). Neuron cultures were harvested 14–21 days post-treatment (DPT), as noted.

**Immunoblotting**

Total protein concentration in each sample was determined by a bicinchoninic acid colorimetric assay (Fisher Cat#23223 and 23224), using bovine serum albumin as a standard (Thermo Fisher Cat# 23210). Protein was resolved on 5–20% gradient polyacrylamide gels using equal protein loading. Proteins were transferred to 0.2 μm nitrocellulose or PVDF membranes and detected with primary antibodies targeting LRRK2 (ab133474, Abcam, RRID:AB2713963, 1:500), pS935 LRRK2 (ab133450, Abcam, RRID:AB2732035, 1:400) or GAPDH (2-RGM2, Advanced Immunological, RRID:AB2721282, 1:5000). Primary antibodies were detected using IRDye 800 (Li-cor 925-32210) or IRDye 680
(Li-cor 925-68071) secondary antibodies, scanned on Li-cor Odyssey Imaging System and analyzed using Image Studio software. LRRK2 and pS935 LRRK2 values were normalized to GAPDH as an internal loading control, then further normalized to the mean of all control samples.

**Immunocytochemistry**

Primary neurons treated with X-T40 tau PFFs were fixed with 4% paraformaldehyde, 4% sucrose in phosphate-buffered saline and washed five times in PBS. Cells were permeabilized in 3% BSA + 0.3% TX-100 in PBS for 15 min at room temperature. After a PBS wash, cells were blocked for 50 min with 3% BSA in PBS prior to incubation with primary antibodies for 2 h at room temperature. Primary antibodies used were targeting pS202/T205 tau (AT8, ThermoFisher Cat#MN1020, 1:1000), MAP2 (17028, CNDR, 1:2000). Cells were washed 5x with PBS and incubated with secondary antibodies for 1 h at room temperature. After 5x wash with PBS, cells were incubated in DAPI (ThermoFisher Cat#D21490, 1:10,000) in PBS. Primary neurons treated with AD PHF tau were stained differently due to the possible presence of p-tau signal in the human-derived material added to cultures. Soluble protein was extract with 2% HDTA for 10 min at room temperature. Neurons were then fixed and stained as above, except that a primary antibody that selectively binds mouse tau (T49, CNDR, 1:2500) was utilized to detect neuronal tau inclusions.

96-well plates were imaged on an In Cell Analyzer 2200 (GE Healthcare) and analyzed in the accompanying software. A standard intensity-based threshold was applied to MAP2 and p-tau or mouse tau channels equally across plates and the positive area was quantified. All quantification was optimized and applied equally across all conditions.

**Quantification and statistical analysis**

The number of samples analyzed in each experiment, the statistical analysis performed, as well as the p-values for all results <0.05 are reported in the figure legends. For all cell culture experiments, “n” represents the number of separate cultures (e.g., one scraped or imaged well is reported as one “n”). All cell culture data were analyzed in GraphPad Prism 7 using the noted statistical tests.

**RESULTS**

**LRRK2 kinase inhibitors show prolonged inhibition of phosphorylation in primary neurons**

Primary neuron cultures provide a rapid and accessible method for the evaluation of cellular phenotypes and the efficacy of therapeutics. Several LRRK2 kinase inhibitors have been developed which show potent inhibition of LRRK2 kinase activity, but their effect on neuropathology, particularly tau, is still unknown. We first tested whether LRRK2 kinase activity could be inhibited in primary neurons over prolonged incubation periods. To ensure kinase inhibition even when LRRK2 is overexpressed, we cultured primary neurons from LRRK2G2019S transgenic mice (B6.Cg-Tg(Lrrk2*G2019S)2Yue/J) which over-express LRRK2G2019S [11, 12]. Primary neurons were treated at 5 DIV with one of three LRRK2 inhibitors—PF-06447475 (PF-475), PF-06685360 (PF-360), and MLi-2 at 5, 30, or 120 nM concentration. After 16 days, neuronal proteins were extracted and run by western blot to determine LRRK2 kinase activity (Fig. 1). Total LRRK2 and pS935 LRRK2 were monitored as a common and reliable readout for LRRK2 kinase activity [16–19]. All three compounds were able to reduce pS935 LRRK2 levels strongly at low nanomolar concentrations with minimal disruption of total LRRK2 levels (Fig. 1A-C).

**Wildtype and LRRK2G2019S neurons exhibit robust, inhibitor-insensitive tau pathology**

Having established the ability to inhibit LRRK2 kinase activity levels over weeks, we next sought to investigate the impact of LRRK2 kinase activity on tau pathology development (Fig. 2). Tau pathology can be induced in non-transgenic neurons by seeding pathology with recombinant tau pre-formed fibrils (PFFs). PFFs were generated by shaking recombinant tau, sonicating and passaging into monomer sequentially in a manner that stochastically generates fibrillar tau without the need for a co-factor such as heparin [13]. These “X-T40” PFFs can be sonicated and added to NTG primary neurons and will seed the misfolding of endogenous tau. Neither NTG, nor LRRK2G2019S neurons have detectable levels of pS202/T205 tau without the addition of X-T40 PFFs, but PFFs induce robust tau inclusions in neurons (Fig. 2A). Interestingly, we observed a slight reduction of tau pathology in LRRK2G2019S neurons, with no apparent effect of LRRK2 kinase inhibition.
Fig. 1. LRRK2 kinase inhibitors show prolonged inhibition of phosphorylation in primary neurons. A) Primary cortical neurons from LRRK2G2019S mice were treated at 5 days in vitro (DIV) with LRRK2 inhibitors at the noted concentrations and lysate was harvested at 21 DIV. Western blot is shown for total LRRK2 and pS935 LRRK2, the latter being a proxy for LRRK2 activity. B) Quantification of total LRRK2 normalized to GAPDH and vehicle treatment (One-way ANOVA; Dunnett’s multiple comparison test: all \( p > 0.05 \), \( n = 6–9 \) samples/group). (C) Quantification of pS935 normalized to total LRRK2 and vehicle treatment (One-way ANOVA; Dunnett’s multiple comparison test: all \( ***p < 0.0001 \) as compared to vehicle treatment, \( n \) (separate cultures) = 6–9 independent samples/group).

(Fig. 2B). There was a small reduction in overall dendritic area (MAP2) with PFF treatment that was reversed with the highest dose of PF-360 (Fig. 2C).

**Tau from AD brains induces inhibitor-insensitive tau pathology in wildtype and LRRK2G2019S neurons**

Recent structural biology studies have established that recombinant tau fibrils are not the same as human brain-derived tau fibrils [20]. Further, the structure of tau fibrils differs between diseases [20]. We therefore sought to induce pathology using human-derived tau. The majority of tau pathology in LRRK2 mutation carriers is Alzheimer’s disease (AD) type tau, as recognized by a selective AD tau antibody [8]. We utilized a biochemical sequential detergent extraction of gray matter from AD patient brains to obtain an enriched fraction of paired helical filament (PHF) tau (Table 1, Fig. 3A) [13]. This extraction method yielded a final purity of 16.1–31.3% tau, with 0.01% or less α-synuclein and amyloid β (Table 1). The purified tau fraction retains the pathogenic conformation present in human disease and induces the misfolding of tau in primary neurons and mice without the overexpression of tau [13]. AD tau therefore serves as a valuable tool to assess tau pathology in different genetic backgrounds. Primary hippocampal neurons from NTG or LRRK2G2019S mice were treated with vehicle or LRRK2 inhibitors at 5 DIV followed by treatment with DPBS or AD tau at 7 DIV (Fig. 3B). Notably, neither NTG nor LRRK2G2019S neurons form tau inclusions without the addition of PHF tau. However, 14 days post-treatment with AD tau, neurons form detergent-insoluble tau inclusions from endogenous mouse tau, which do not differ by neuron genotype. Further, LRRK2 kinase inhibition had no effect on tau pathology in these neurons (Fig. 3B,3C).

Together, these results suggest that LRRK2G2019S and LRRK2 kinase inhibition have a minimal effect on the cell-autonomous production of tau pathology in neurons. However, primary neuron cultures do not allow for the assessment of the impact of LRRK2 on tau pathology over long time periods, effects on cell-to-cell spread, or non-cell autonomous effects.
Fig. 2. Wildtype and LRRK2<sup>G2019S</sup> neurons exhibit robust, inhibitor-insensitive tau pathology. A) Primary hippocampal neurons from NTG or LRRK2<sup>G2019S</sup> mice were treated with vehicle or LRRK2 inhibitors at the noted concentrations at 5 DIV. They were further treated with X-T40 tau PFFs at 1.5 μg/mL at 7 DIV and fixed and stained for pS202/T205 tau (AT8, magenta) and MAP2 (gray) at 21 DIV. Scale bar = 50 μm.

B) Quantification of the pS202/T205 tau area normalized to MAP2 area and further normalized to NTG-DMSO-PFF. LRRK2<sup>G2019S</sup> neurons showed a small, genotype-level significant reduction in tau pathology, and each genotype showed no tau pathology without addition of X-T40 tau PFFs (Two-way ANOVA; genotype effect ***p < 0.0001, Dunnett’s multiple comparison test within genotype: NTG: DMSO-PFF vs. DMSO ***p < 0.0001; G2019S: DMSO-PFF vs. DMSO ***p < 0.0001; All other values were not statistically significant). C) Quantification of the MAP2 area also showed a small genotype-level significant change as well as a reduction related to PFF treatment. Interestingly, the highest dose of PF-360 also elevated MAP2 area slightly (Two-way ANOVA; genotype effect ***p = 0.001, Dunnett’s multiple comparison test within genotype: NTG: DMSO-PFF vs. 120 nM PF-360-PFF **p = 0.0027; G2019S: DMSO-PFF vs. DMSO ***p < 0.0001; DMSO-PFF vs. 120 nM PF-360 *p = 0.0216; All other values were not statistically significant). n (separate cultures) = 7-8 independent samples/group. Data are represented as mean ± SEM with individual data points plotted.
Fig. 3. Tau from AD brains induces inhibitor-insensitive tau pathology in wildtype and LRRK2<sup>G2019S</sup> neurons. A) Schematic diagram of AD PHF tau extraction from human brain tissue and subsequent treatment of primary neurons. B) Primary hippocampal neurons from NTG or LRRK2<sup>G2019S</sup> mice were treated with vehicle or LRRK2 inhibitors at 300 nM at 5 DIV. They were further treated with AD PHF tau at 1 μg/mL at 7 DIV, and both fixed and stained for insoluble mouse tau (T49, magenta) and DAPI (gray) at 21 DIV. Scale bar = 15 μm. C) Quantification of the insoluble mouse tau area/nuclei number in each condition. No insoluble tau was present in the absence of PHF treatment and there was no overall effect of genotype (Two-way ANOVA; genotype effect <i>p</i> = 0.837, Dunnett’s multiple comparison test within genotype: NTG: DMSO-PHF vs. DMSO <i>∗∗∗∗</i> <i>p</i> < 0.0001; DMSO-PHF vs. MLi-2 <i>∗∗∗∗</i> <i>p</i> < 0.0001; G2019S: DMSO-PHF vs. DMSO <i>∗∗∗∗</i> <i>p</i> < 0.0001; DMSO-PHF vs. MLi-2 <i>∗∗∗∗</i> <i>p</i> < 0.0001; all other values were not statistically significant, n (separate cultures) = 15 independent samples/group). Data are represented as mean ± SEM with individual data points plotted.

DISCUSSION

PD is a heterogenous clinical syndrome. In contrast, patients with a similar genetic risk factor often have more homogenous clinical symptoms, and therefore are a good group in which to investigate underlying disease mechanisms [21]. It is therefore surprising that disease pathology is so heterogeneous in LRRK2-PD patients, with 21–54% of patients lacking the pathognomonic Lewy body inclusions [6–8]. These neuropathological findings make it unlikely that LRRK2 leads to neuron loss only through α-synuclein aggregation. This supposition is bolstered by the lack of α-synuclein pathology in LRRK2 mutant mice [22, 23].

In contrast, most LRRK2 PD patients harbor some degree of tau pathology [6, 8, 9]. While LRRK2 mutations are rare in primary tauopathies, such as progressive supranuclear palsy (PSP) [24, 25], a recent genome-wide association study found that genetic variance in the <i>LRRK2</i> locus is associated with survival in PSP patients [26]. Cell [27] and animal [28–30] models expressing mutant LRRK2 have consistently been reported to accumulate hyperphosphorylated tau. Several reports have also shown that under certain conditions LRRK2 can directly phosphorylate tau [31–34]. These studies suggest that LRRK2 mutations may be predisposing neurons to the development of tau pathology. However, in the current study, we found no evidence in primary neurons that LRRK2 kinase activity can cause direct development of tau pathology, as evidenced by the lack of phosphorylated tau in the absence of tau seeding. Further, elevated LRRK2 kinase activity or LRRK2 kinase inhibition did not substantially alter the development of tau pathology following pathogenic tau seeding. Therefore, on the cell-autonomous level, LRRK2 kinase activity does not seem to play a major role in tau pathology development.

A limitation of this work is the nature of neuron cell culture. While neuron culture allows the rapid assessment of kinase inhibition and cellular
phenotypes, cultures largely consist of homogenous neuron types with minimal glial growth. This precludes assessment of multiple neuron types, network spread of pathology, and non-cell autonomous effects of glia or peripheral immune cells. LRRK2 is highly expressed in the innate immune system and LRRK2 mutations have been associated with dysregulation of immune inflammatory pathways [35]. It is therefore possible that LRRK2G2019S impacts intact organisms through modulation of inflammation. Another possible mechanism by which LRRK2 could facilitate tau pathology progression is through enhanced neuronal release of tau, a mechanism which would make little difference in a cell culture dish. Enhanced synaptic vesicle release is one phenotype which has been associated with LRRK2G2019S expression in mice. Because tau is released from neurons [36–38] in an activity-dependent manner, changes in physiology by LRRK2 could also change pathology spread in a non-cell autonomous manner. One study in mice [39] showed that viral overexpression of tau in LRRK2G2019S mice led to elevated tau in sites distal from the injection site, supporting enhanced spread of tau pathology with a LRRK2 mutation. Finally, multiple studies crossing tau transgenic mice to LRRK2 transgenic mice have found little effect of LRRK2 on tau pathology [39, 40], suggesting that when tau pathology develops synchronously across the brain, there is little effect of LRRK2 on pathology.

In conclusion, evidence suggests that tau pathology is not regulated in a cell-autonomous manner by LRRK2 kinase activity. However, there is substantial evidence from human case studies, cell and animal models, that LRRK2 is somehow promoting tau pathology spread. Future studies should investigate the mechanisms of tau pathology spread and how they relate to LRRK2 kinase activity.

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CONFLICT OF INTEREST

The authors have no conflict of interest to report.

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