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LRRK2 Facilitates tau Phosphorylation through Strong Interaction with tau and cdk5

Mary R. Shanley,[†] Dillon Hawley,[†] Shirley Leung,[†] Nikhat F. Zaidi,[†] Roshni Dave,[†] Kate A. Schlosser,[‡] Rina Bandopadhyay,[§] Scott A. Gerber,[‡] and Min Liu*[,†]

[†]Neurology Department, Brigham and Women's Hospital, Harvard Medical School, 65 Landsdowne Street, Fourth Floor, Cambridge, Massachusetts 02139, United States

[‡]Department of Genetics and of Biochemistry, Geisel School of Medicine at Dartmouth, One Medical Center Drive HB-7937, Lebanon, New Hampshire 03756, United States

[§]Reta Lila, Weston Institute of Neurological Studies Department of Molecular Neuroscience UCL, Institute of Neurology 1, Wakefield Street, London WC1N 1PJ, U.K.

Supporting Information

ABSTRACT: Leucine-rich repeat kinase 2 (LRRK2) and tau have been identified as risk factors of Parkinson's disease (PD). As LRRK2 is a kinase and tau is hyperphosphorylated in some LRRK2 mutation carriers of PD patients, the obvious hypothesis is that tau could be a substrate of LRRK2. Previous reports that LRRK2 phosphorylates free tau or tubulin-associated tau provide direct support for this proposition. By comparing LRRK2 with cdk5, we show that wild-type LRRK2 and the G2019S mutant phosphorylate free recombinant full-length tau protein with specific activity 480- and 250-fold lower than cdk5, respectively. More strikingly tau binds to wt LRRK2 or the G2019S mutant 140- or 200-fold more strongly than cdk5. The extremely low activity of LRRK2 but strong binding affinity with tau suggests that LRRK2 may facilitate tau phosphorylation as a scaffold protein rather than as a major tau kinase. This hypothesis is further supported by the observation that (i) cdk5 or tau coimmunoprecipitates with endogenous LRRK2 in SH-SY5Y cells, in mouse brain tissue, and in human PBMCs; (ii) knocking down endogenous LRRK2 by its siRNA in SH-SY5Y cells reduces tau phosphorylation at Ser396 and Ser404; (iii) inhibiting LRRK2 kinase activity by its inhibitors has no effect on tau phosphorylation at these two sites; and (iv) overexpressing wt LRRK2, the G2019S mutant, or the D1994A kinase-dead mutant in SH-SY5Y cells has no effect on tau phosphorylation. Our results suggest that LRRK2 facilitates tau phosphorylation indirectly by recruiting tau or cdk5 rather than by directly phosphorylating tau.

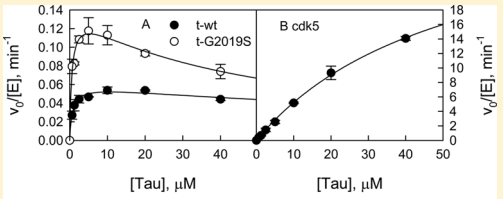

Table 3: Initial Velocity Analysis for tau Phosphorylation

	twt	tG2019S	cdk5
$k_{cat,app}$ min^{-1}	0.06 ± 0.003	0.14 ± 0.01	31.1 ± 2.0
$K_{tau,app}$ μM	0.5 ± 0.15	0.3 ± 0.1	44.4 ± 4.4
$(k_{cat}/K_{tau})_{app}$, min^{-1}μM^{-1}	0.12	0.5	0.7

Parkinson's disease (PD), characterized by tremor, rigidity, bradykinesia, and postural instability, is the second most common neurodegenerative disorder after Alzheimer's disease. Recently, leucine-rich repeat kinase 2 (LRRK2) has emerged as one of the major genetic factors of PD.[1−5]

LRRK2 belongs to the ROCO protein family which is characterized by having conserved ROC (Ras-like GTPase) and COR (C-terminal of ROC) domains. In addition to these two domains, LRRK2 also contains a kinase domain of the MAPKKK class and several protein−protein interaction domains—the N-terminal ARM, ANK, LRR, and C-terminal WD40 domains (domain structure of LRRK2: ARM-ANK-LRR-ROC-COR-MAPKKK-WD40). LRRK2 is unusual in that it encodes two distinct but functionally linked enzymes: a protein kinase and a GTPase. More than 40 mutations in LRRK2 have been found in both familial and sporadic forms of PD and have been associated with typical idiopathic, late-onset PD.[1−5] Several mutations demonstrate increased kinase activity that is correlated with increased neurotoxicity in central neurons,[6−8] suggesting a critical role of kinase activity of LRRK2 in the pathogenesis of PD.

Like most neurodegenerative diseases, PD is characterized by the formation of proteinaceous inclusions. Most PD patients with LRRK2 mutations have either α-synuclein-positive Lewy bodies[2] or tau-positive lesions.[2,9] In certain cases, TAR DNA-binding protein 43 (TDP43) inclusions were also observed.[10] The association of LRRK2, α-synuclein, and tau with PD was consistent with recent genome wide association studies (GWAS), which identified the genes coding α-synuclein, LRRK2, and tau proteins as increased risk factors of PD.[11,12] The observation that transgenic mice expressing the G2019S mutant[13,14] or the R1441G mutant[15] have increases in tau phosphorylation while *LRRK2* knockout mice have decreases in tau phosphorylation[16] provides additional evidence for the

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association of LRRK2 and tau *in vivo*. Furthermore, experimental evidence that LRRK2 phosphorylates tau *in vitro* provides direct support for the interaction between LRRK2 and tau.[17,18] But several questions remain unaddressed, including whether LRRK2 contributes to tau phosphorylation as a major tau kinase, whether the most common PD-linked mutation G2019S is more active than wt LRRK2 in phosphorylating tau, and whether LRRK2 exhibits an activity comparable to cdk5 toward tau, one of the kinases which has been widely believed to phosphorylate tau *in vivo*.[19,20]

In this study, we characterized LRRK2-catalyzed free tau phosphorylation *in vitro*, compared the kinetics of wild-type LRRK2 and the most common PD-linked G2019S mutant with cdk5, demonstrated tau phosphorylation sites catalyzed by LRRK2 and cdk5, estimated endogenous LRRK2 effect on tau phosphorylation in SH-SY5Y cells, and evaluated interactions of LRRK2, tau, and cdk5 in SH-SY5Y cells, in mouse and human brain tissue, and in PBMCs isolated from human blood samples.

MATERIALS AND METHODS

Materials. [γ-^{33}P]-ATP was obtained from PerkinElmer (Boston, MA). Truncated wild-type LRRK2 (aa 970−2527, cat# PR8604B) and the G2019S mutant (aa 970−2527, cat# PR8764C) expressed in baculovirus system were purchased from Invitrogen. Cdk5/p25 was from Carna Bioscience (Natick, MA). Tubulin purified from porcine brain was purchased from Cytoskeleton (Danver, CO). Monomer of tubulin (MW = 55 kDa) was used for concentration calculation. Full-length human tau was purified in house as described previously.[21] Anti-LRRK2 antibody was purchased from Abcam, and antitau, anti-cdk5, and anti-PO_4-tau were all from Cell Signaling. (cat# PR8604B) and the G2019S mutant (cat# PR8764C)

Kinase buffer contains 20 mM HEPES (pH 7.4), 50 mM NaCl, 10 mM $MgCl_2$, 1 mM DTT, BSA 0.5 mg/mL, and 1 mM beta-Gly-PO_4 which is a phosphatase inhibitor.

Radiometric Filter Assay for Monitoring tau Phosphorylation. The kinase assay for tau phosphorylation was conducted in the kinase buffer containing tau, ATP, and [γ-^{33}P]-ATP. Tau and ATP were used at various concentrations as indicated in the Results section, and the ratio of ATP to [γ-^{33}P]-ATP was kept constant at all ATP concentrations (250 μM ATP/5 μCi [γ-^{33}P]-ATP). The reactions were conducted in duplicate, initiated by the addition of enzyme, and incubated at room temperature for 2 h. The reaction was stopped by the addition of 20 mM EDTA, and the mixture was transferred to a multiscreen PH filtration plate (Millipore, Billerica, MA) and washed six times with 75 mM phosphoric acid. The plate was dried, filters were removed, and the samples were counted with a scintillation counter. Background reaction was conducted in the absence of enzyme. In all cases, reaction progress curves for production of phospho-tau were linear over at last 3 h and allowed calculation of initial velocities. Examples of the reaction progress curves are shown in Supporting Information (SI Figure 1).

Cell-Based Assay for Monitoring tau Phosphorylation. SH-SY5Y cells known to express endogeneous tau were used in this study. Cells were plated in six-well plates, transfected with siRNA for 48 h, with DNA for 24 h, or treated with compounds for 24 h before cells were lysed. Cell lysates were collected and tau phosphorylation at Ser396 and Ser404 was analyzed by Western blot analysis.

Mass Spectrometry Analysis. Tau phosphorylation mapping was conducted essentially as described previously.[22] In brief: *in vitro* phosphorylated tau was resuspended in buffered (pH 8.1) SDS solution, reduced, alkylated with iodoacetamide, and separated by SDS-PAGE prior to Coomassie staining. Gel bands corresponding to tau were excised, destained, and digested with proteinase-K to peptides. The peptide mixtures were desalted using solid reverse-phase extraction cartridges, lyophilized, and analyzed by HPLC-mass spectrometry on an LTQ Orbitrap mass spectrometer. The resulting tandem mass spectra were data-searched with no enzyme specificity, requiring high mass accuracy precursor ions (± 3 ppm), and with carboxyamidomethane as a static modification on cysteine and oxidation on methionine and phosphorylation on serine, threonine, and tyrosine as dynamic modifications, using modern variants of the SEQUEST algorithm.[23,24] Phosphorylation site localization probabilities were assessed using the phosphoRS algorithm.[25] The resulting peptide spectral matches were filtered to a 1% false discovery rate with the target-decoy strategy[26] and reported.

Homogenization of Brain Tissue. Basal ganglia tissue (caudate and putamen) from three idiopathic PD and three control (neurologically normal) cases were homogenized in 10 vol of 1× TBS buffer with protease and phosphatase inhibitors (Roche) using a mechanical homogenizer. The homogenates were spun at 1000*g* for 10 min to clear nuclear and membranous debris. The TBS-soluble supernatants were collected, and protein content was measured using the Bio-Rad protein assay kit using BSA as the standard. Mouse brain tissue was homogenized similarly except that whole mouse brain was used.

Data Analysis − Basic Equations. Data were analyzed by nonlinear least-squares, using either Sigma-Plot or Grafit software packages. Standard kinetic mechanisms for two-substrate reactions and their rate equations are shown below:

Ping-Pong:

$$v = \frac{k_{cat}[E][A][B]}{K_A[B] + K_B[A] + [A][B]} \quad (1)$$

K_A and K_B are Michaelis constants.

Rapid equilibrium ordered:

$$v = \frac{k_{cat}[E][A][B]}{K_A K_B + K_B[A] + [A][B]} \quad (2)$$

K_A and K_B are substrate dissociation constants from EA and EB, respectively.

Rapid equilibrium random/steady-state ordered:

$$v = \frac{k_{cat}[E][A][B]}{\alpha K_A K_B + \alpha K_A[B] + \alpha K_B[A] + [A][B]} \quad (3)$$

For rapid equilibrium systems, K_A, K_B, αK_A, and αK_B are substrate dissociation constants from EA, EB, and EAB; for steady-state systems, K_A is substrate dissociation constant from EA, αK_A and αK_B are Michaelis constants. See Segel for definitions of mechanisms, substrate dissociation constants, and α.[27]

RESULTS

LRRK2-Catalyzed Phosphorylation of Free tau − Initial Velocity Studies. In this study we used N-terminal truncated wt LRRK2 (aa 970−2527, including domains of

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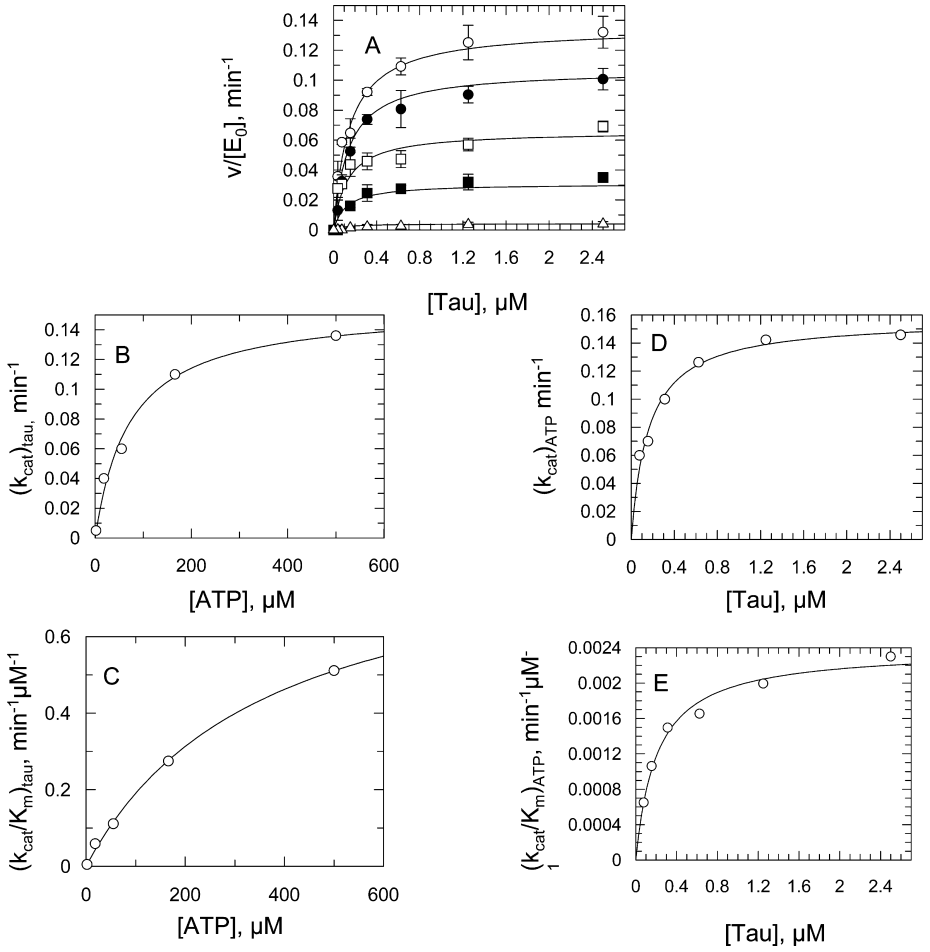

Figure 1. Initial velocity studies of t-wt-catalyzed tau phosphorylation. (A) Tau phosphorylation was measured as a function of [tau] at [ATP] = 500 (○), 167 (●), 56 (□), 19 (■), 6.2 (Δ) μM. Each data point is the average of two independent experiments. Error bar represents the standard deviation from the mean. Each data set was fitted into the simple Michaelis–Menten equation. (B, C) ATP concentration dependencies of $(k_{cat})_{taut}$ and $(k_{cat}/K_m)_{tau}$ values. (D, E) tau concentration dependencies of $(k_{cat})_{ATP}$ and $(k_{cat}/K_m)_{ATP}$ values.

Table 1. Initial Velocity Analysis of tau and LRRKtide Phosphorylation[a]

	tau			LRRKtide	
	t-wt	t-G2019S	cdk5[b]	t-wt	t-G2019S
k_{cat}, min^{-1}	0.16 ± 0.01	0.29 ± 0.02	72 ± 6	10 ± 1	20.2 ± 2.7
K_{ATP}, μM	29 ± 3.1	32 ± 6.2	13 ± 1	105 ± 10	98 ± 11.6
K_B μM	0.09 ± 0.01	0.05 ± 0.01	10 ± 2	69 ± 7	75 ± 11
α	5.3 ± 1.3	3.6 ± 0.2	2.8 ± 0.3	1.8 ± 1.1	2.1 ± 0.4

[a]The parameter estimates were calculated by globally fitting the data into the equation reflecting rapid equilibrium random/steady-state order mechanism. Each parameter estimate is the average of two independent experiments and the error limit is the deviation from the mean. B represents either tau or LRRKtide. [b]Liu, M. et al. (2011).

LRR-ROC-COR-MAPKKK-WD40) and the G2019S mutant (aa 970–2527). They are referred to as t-wt and t-G2019S, respectively. Initial velocities were measured over a range of concentrations of tau at several fixed concentrations of ATP for t-wt (Figure 1A). The method of replots was used to examine the data carefully and judge among the three standard mechanisms—ping-pong, rapid equilibrium ordered, and random/steady-state ordered, as described previously.[28,29] The hyperbolic shape of all the replots (Figure 1B–E) confirmed that this reaction follows either a random or a steady-state ordered mechanism. Global fitting of the data to the equation reflecting the random/steady-state ordered mechanism yields parameter estimates averaged from two independent experiments: k_{cat} = 0.16 ± 0.01 min^{-1}, K_{ATP} = 29 ± 3.1 μM, K_{tau} = 0.09 ± 0.01 μM, and α = 5.3 ± 1.3, as summarized in Table 1. Similar initial velocity studies were conducted for the t-G2019S mutant (data not shown) and revealed that like t-wt, the t-G2019S mutant also follows either a random or a steady-state ordered mechanism. Global fitting of the kinetic data reveals parameter estimates averaged from two independent experiments: k_{cat} = 0.29 ± 0.02 min^{-1}, K_{ATP} = 32 ± 6.2 μM, K_{tau} = 0.05 ± 0.01 μM, and α = 3.6 ± 0.2 for t-G2019S (Table 1). The t-G2019S mutant is more active than t-wt LRRK2 in catalyzing tau phosphorylation. In other independent experiments conducted at a saturating ATP concentration, we observed strong substrate inhibition for the

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Table 2. IC_{50} Values of LRRK2 Inhibitors[a]

	tau		LRRKtide	
inhibitor	t-wt	t-G2019S	t-wt	t-G2019S
LRRK2-In-1	9 ± 2.4 nM	2.8 ± 0.3 nM	3.2 ± 0.1 nM	4.4 ± 1.0 nM
ponatinib	190 ± 30 nM (NC)	280 ± 20 nM (C)	70 ± 10 nM (NC)	200 ± 30 nM (C)

[a]IC_{50} values were determined at 50 μM ATP and 50 μM LRRKtide or 0.6 μM tau. NC represents ATP noncompetitive inhibition and C represents ATP competitive inhibition. Each IC_{50} value is the average of two independent experiments, and the error limit is the deviation from the mean.

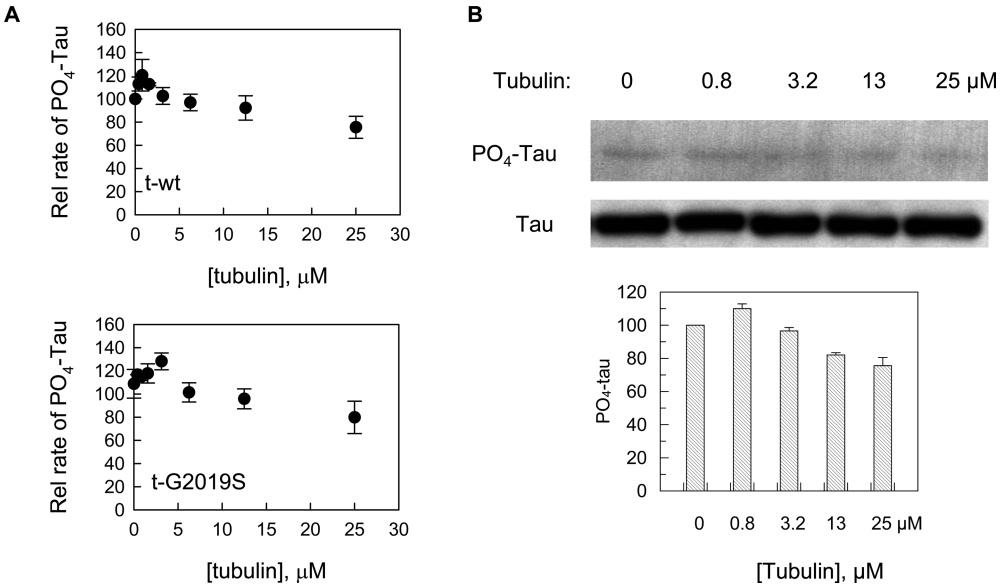

Figure 2. Effects of tubulin on LRRK2-catalyzed tau phosphorylation. (A) A radiometric filter assay—2.5 μM tau was preincubated with tubulin at different concentrations for 30 min before the kinase reaction was initiated by the addition of t-wt or t-G2019S. Samples containing corresponding concentrations of tubulin in the absence of tau were used as controls to subtract any signal contributed by tubulin phosphorylation. Each data point is the average of two independent experiments. Error bar represents the standard deviation from the mean. (B) Autoradiography analysis to evaluate the effects of tubulin on tau phosphorylation catalyzed by t-wt. Tau phosphorylation level was quantified using ImageJ software. Each data point is the average of two independent experiments. Error bar represents the standard error of the mean.

t-G2019S mutant at [tau] > 5 μM and weak substrate inhibition for t-wt at [tau] > 20 μM (Figure 3A). It is worth noting that, in the initial velocity studies (such as data in Figure 1A), the highest tau concentration was set at 2.5 μM for both enzymes. This concentration is well-above $K_{tau,app}$ (0.3 μM for t-G2019S and 0.5 μM for t-wt determined at a saturating ATP concentration) and allows accurate determination of kinetic parameters for t-wt and the t-G2019S mutant. Under this condition, substrate inhibition was excluded, which has simplified data analysis that otherwise will be very complicated for the two-substrate reaction.

We noticed that using tau as a substrate, the values of k_{cat} for both t-wt and the t-G2019S mutant are quite low compared with our published data using LRRKtide as a substrate, a widely used peptide substrate of LRRK2.[30] To make certain that the low specific activity of LRRK2 in tau phosphorylation is not attributed to enzyme inactivation, we evaluated both t-wt LRRK2 and the mutant with LRRKtide. The kinetic parameters of LRRK2-catalyzed LRRKtide phosphorylation are shown in Table 1. They are very consistent with our previously reported values, ruling out the possibility that LRRK2 enzymes used in this study were inactive.

Inhibition of LRRK2-Catalyzed tau Phosphorylation in Vitro. Two LRRK2 inhibitors, ponatinib (originally identified as a DFG-out inhibitor of Abl kinase[31]) and LRRK2-In-1,[32] were tested for t-wt and the t-G2019S mutant in tau phosphorylation. The $K_{i,app}$'s are presented in Table 2 along with those for LRRKtide phosphorylation. Both ponatinib and LRRK2-In-1 inhibit the two enzyme forms with similar potency, and their potency is independent of the substrate identity. Previously using LRRKtide as the phosphoryl acceptor, we showed that ponatinib inhibits t-wt and the t-G2019S mutant in different inhibition mechanisms—it inhibits t-wt as an ATP noncompetitive inhibitor as expected for a DFG-out inhibitor but inhibits the t-G2019S mutant in an ATP-competitive manner.[33] In this study using protein tau as a substrate, identical results were reproduced for ponatinib, suggesting that the inhibition mechanism of ponatinib is independent of substrate identity.

Effects of Tubulin on tau Phosphorylation. It was reported previously that tubulin-associated tau but not free tau is phosphorylated by LRRK2.[17] In order to evaluate the effect of tubulin on full-length tau phosphorylation, here we preincubated 2.5 μM tau with different concentrations of tubulin for 30 min before the kinase reaction was initiated by the addition of t-wt or the t-G2019S mutant. Samples containing tubulin at corresponding concentrations in the absence of tau were used as controls to subtract any signal contributed by tubulin phosphorylation. In this study, phosphorylation of tau and tubulin was monitored by the radiometric filter assay as described in the Materials and Methods section. First, we were able to replicate the finding that LRRK2 phosphorylates tubulin *in vitro*.[16] However, tubulin

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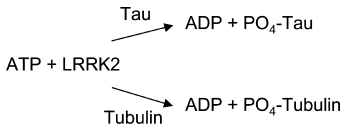
binds to LRRK2 (either t-wt or t-G2019S) much weaker than tau, with $K_{tubulin,app} \gg 25\ \mu M$ compared with $K_{tau,app}$ of 0.5 μM.

Next, the effects of tubulin on tau phosphorylation were estimated. As shown in Figure 2A, small increases in tau phosphorylation (1.3-fold increase) were observed at low tubulin concentration ([tubulin] < 3 μM or ratio of [tubulin]/[tau] $\leq$ 1.2). Further increases in tubulin concentration led to inhibition of tau phosphorylation with an IC_{50} > 25 μM, consistent with the weak interaction between tubulin and LRRK2 determined from tubulin phosphorylation. The effects of tubulin on tau phosphorylation observed in the filter assay were confirmed by the autoradiography analysis (Figure 2B) where tau phosphorylation at different tubulin concentrations was directly monitored on gel. Under certain reaction conditions, tubulin undergoes polymerization, which might lead to decreased phosphorylation of tau at higher tubulin concentrations. To rule out this possibility, absorbance assay monitoring tubulin polymerization at OD_{340}[34,35] was conducted for the sample containing 2.5 μM tau and the highest tubulin concentration used in this study (25 μM). No absorbance changes were observed for up to 3 h (data not shown), indicating that under our reaction condition, tubulin polymerization was not measurable even in the presence of tau.

The inhibition in tau phosphorylation observed at high tubulin concentrations could be due to the competitive nature between tubulin and tau, both being phosphoryl acceptors of LRRK2. As depicted in Scheme 1, when the alternative substrate tubulin is introduced into tau reaction, it gives rise to an alternative reaction pathway and generally leads to a common product ADP and two alternative products PO_4-tau and PO_4-tubulin. When PO_4-tau is measured, the effect of the alternative substrate tubulin is inhibition because of the competitive nature of the two substrates tau and tubulin. Our results suggest that tubulin does not significantly facilitate free tau phosphorylation. The discrepancy between our observation and a previous report that tubulin-associated tau but not free tau is phosphorylated by LRRK2[17] could be due to the difference in experimental conditions, buffer components, and recombinant tau proteins.

Scheme 1. Alternative Substrate Probe



Comparison of LRRK2 with cdk5/p25 in tau Phosphorylation. Our data demonstrate that LRRK2 could phosphorylate free tau *in vitro*. However, the question whether LRRK2 exhibits an activity comparable to cdk5 remains unaddressed. We first compared the kinetic parameters of LRRK2 with those of cdk5 determined by us previously[29] as shown in Table 1. Strikingly we found that tau interacts with t-wt LRRK2 or the t-G2019S mutant significantly more tightly than it binds to cdk5, as evidenced by the 140−200-fold difference in substrate binding affinity (Table 1). Because of the cost of purchased LRRK2 enzyme, confirmation of such strong interaction between LRRK2 and tau by biophysical analyses such as ITC in this study is not feasible. But such analyses in the future will be required to confirm these observed kinetic constants. Next we directly compared t-wt and the t-G2019S mutant with cdk5 by measuring tau phosphorylation at a saturating [ATP] as shown in Figure 3A,B. At first glance, t-wt and the t-G2019S mutant are dramatically less active than cdk5,

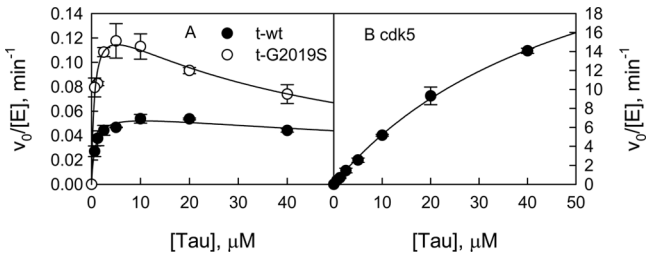


Figure 3. Comparison of tau phosphorylation catalyzed by (A) LRRK2 or (B) cdk5. Tau phosphorylation was measured at a saturating concentration of ATP. Each data point is the average of two independent experiments. Error bar represents the standard deviation from the mean.

as evidenced by $k_{cat,app}$ 220−520-fold lower than that of cdk5. A closer look at values of $(k_c/K_{tau})_{app}$, a kinetic parameter for enzyme catalytic efficiency, revealed that at very low tau concentrations, the t-G2019S mutant has similar catalytic efficiency compared with cdk5, 0.5 $min^{-1}\ \mu M^{-1}$ for the t-G2019S mutant and 0.7 $min^{-1}\ \mu M^{-1}$ for cdk5 (Table 3). This

Table 3. Initial Velocity Analysis for tau Phosphorylation

	twt	tG2019S	cdk5
$k_{cat,app}$, min^{-1}	0.06 ± 0.003	0.14 ± 0.01	31.1 ± 2.0
$K_{tau,app}$, μM	0.5 ± 0.15	0.3 ± 0.1	44.4 ± 4.4
$(k_{cat}/K_{tau})_{app}$, $min^{-1}\ \mu M^{-1}$	0.12	0.5	0.7

observation raises the question of whether LRRK2 facilitates cdk5-catalyzed tau phosphorylation by initiating tau phosphorylation at very low tau concentrations or by activating cdk5.

To evaluate whether LRRK2 is an activator of cdk5, we compared tau phosphorylation ([tau] = 0.5 μM) catalyzed by t-wt LRRK2, t-G2019S, or cdk5 alone with tau phosphorylation catalyzed by mixtures of t-wt and cdk5 or t-G2019S and cdk5. As shown in Figure 4A, tau phosphorylation was increased when both cdk5 and LRRK2 were present compared with cdk5 alone. However, the increase is not significant with p values >0.07. The increase is a direct contribution of LRRK2 on tau phosphorylation rather than a result of cdk5 activation by LRRK2. Next we evaluated whether LRRK2 could facilitate cdk5-catalyzed tau phosphorylation by initiating tau phosphorylation. We prephosphorylated tau by wt or the G2019S mutant for 2 h at 0.5 μM tau, which is well below its K_m for cdk5 (10 μM). Next, the reaction was stopped by LRRK2 selective inhibitor LRRK2-In-1, and tau phosphorylation was reinitiated by the addition of cdk5. The inhibitor LRRK2-In-1 does not inhibit cdk5 at all (Figure 4B). No significant increases in tau phosphorylation were observed for tau pretreated with LRRK2, suggesting that prephosphorylated tau by LRRK2 is not a better substrate for cdk5 (Figure 4B). It is worth noting that the LRRK2 enzymes used in this study is truncated but not full-length LRRK2; therefore in this study we only characterize the interaction between cdk5 (or tau) and truncated LRRK2.

Tau Phosphorylation Sites Revealed by Mass Spectrometry Analysis. The phosphorylation sites of full-length tau catalyzed by LRRK2 and cdk5 were analyzed by mass spectrometry (Table 4). These phosphorylation sites were not present in identical MS analysis of recombinant full-length tau

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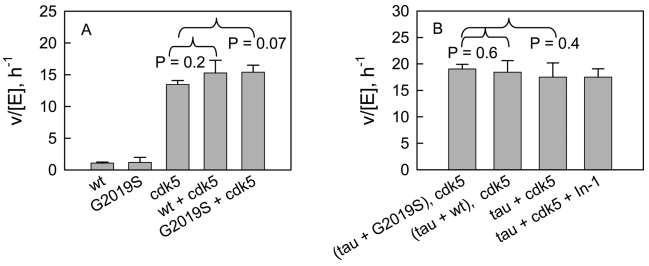

Figure 4. Effects of LRRK2 on cdk5-catalyzed tau phosphorylation. (A) Comparison of tau phosphorylation catalyzed by t-wt, t-G2019S, or cdk5 alone, or by the mixture of t-wt and cdk5 or t-G2019S and cdk5. The increase in tau phosphorylation when both cdk5 and LRRK2 are present compared with cdk5 alone is not significant as evidenced with $P > 0.07$. (B) Comparison of prephosphorylated tau with tau as substrates of cdk5. Prephosphorylated tau: tau was prephosphorylated by t-G2019S or t-wt (the left two bars in panel B) for 2 h, and the reaction was stopped by LRRK2 inhibitor LRRK2-In-1. Next, cdk5 was added to the samples of prephosphorylated tau (the left two bars in panel B), tau (the third bar in panel B), or tau with LRRK2-IN-1 (the most right bar in panel B) to initiate or reinitiate tau phosphorylation for another 1 h. Each data point is the average of two independent experiments. Error bar represents the standard deviation from the mean.

alone. The mass spectrometry analysis revealed four tau phosphorylation sites for the G2019S mutant, three of which are overlapped with those sites catalyzed by cdk5, including two phosphorylation sites associated with AD (Ser396 and Ser404). Thr149 is a unique site which was only phosphorylated by LRRK2 (both t-wt and t-G2019S mutant) but not by cdk5. The mass spectrometry analysis revealed three unique tau phosphorylation sites for wt LRRK2, including one phosphorylation site associated with AD, Ser68. So far, the major kinase(s) that phosphorylates this site has not been identified.

More major tau phosphorylation sites catalyzed by the G2019S mutant were reported in other study, including T153, T169, T205, T263, and T231.[18] The difference in tau phosphorylation sites reported here and that study might be due to the different recombinant tau proteins used: full-length tau vs tau isoform 0N3R and due to different experimental conditions.

LRRK2 Interacts with cdk5 or tau in Cells and *in Vivo*. The interaction between tau and LRRK2 *in vitro* provides strong evidence for the direct association between tau and LRRK2. However, the extremely low activity of LRRK2 makes it difficult to rationale LRRK2 as a major tau kinase. It is likely that LRRK2 serves as a scaffold protein by forming a complex with tau, cdk5, or other kinases, and therefore facilitates tau phosphorylation by recruiting tau or cdk5. To test this hypothesis, we conducted coimmunoprecipitation in inducible HEK293FT cells that stably express wt LRRK2 or the G2019S mutant. Cdk5 was found to coimmunoprecipitate with both wt LRRK2 and the G2019S mutant (Figure 5A). Next we evaluated the interaction of endogeneous LRRK2, cdk5, and tau in SH-SY5Y (Figure 5B) and mouse brain tissue (Figure 5C). Not surprisingly, cdk5 or tau coimmunoprecipitates with LRRK2 in SH-SY5Y cells. Using fresh mouse brain tissue, we were able to observe LRRK2/cdk5 interaction when LRRK2 was the bait protein and LRRK2/tau interaction when tau was the bait protein. We also evaluated the interaction of LRRK2 with tau using frozen homogenates isolated from human brain tissue of idiopathic PD patients and healthy controls. Instead of a protein band of MW around 280 kDa as seen in cultured cells and fresh mouse brain tissue, a protein band of LRRK2 with MW around 120 kDa was observed (Supporting Information, SI Figure 2), suggesting that LRRK2 was degraded into fragments in our frozen homogenates of human brain tissue. Consequently no interaction between LRRK2 and tau was observed. Our results suggest that fragments of LRRK2 disrupt the interaction with tau.

In order to further confirm LRRK2/tau and LRRK2/cdk5 interaction *in vivo*, we used fresh PBMCs isolated from human blood samples of healthy controls. As shown in Figure 5D, cdk5 or tau coimmunoprecipitates with LRRK2 in PBMCs.

Table 4. Tau Phosphorylation Sites Analyzed by Mass Spectrometer

phosphorylation sites	enzymes			sequence	XCorr[c]	MMD[c]	localization[e]
S68[a,b](S68)[c]	wt LRRK2			SDAKpSTPTAEDVTAPLV	4.02	1.05	0.92
T149 (T466)	wt LRRK2	G2019S		ADGKpTKIATPR.G	2.93/2.97 (wt/G2019S)	0.87/0.34 (wt/G2019S)	1.00/1.00 (wt/G2019S)
T153 (T470)			cdk5	KIApTPRGAAPPGQKG	2.76	0.07	1.00
T245 (T562)			cdk5	RTPPKSPSSAKSRLQpTA	2.36	0.74	1.00
Y310 (Y627)	wt LRRK2			QIVpYKPVDLSK	1.61	0.75	1.00
S324 (S641)	wt LRRK2	G2019S	cdk5	TSKCGpSLGNIHH	2.16/2.11/2.16 (wt/G2019S/cdk5)	1.08/1.08/1.17 (wt/G2019S/cdk5)	1.00/0.89/0.99 (wt/G2019S/cdk5)
T386 (T703)	wt LRRK2			NAKAKpTDHGAEIVYKS	1.98	0.48	1.00
Y394[a,b] (Y711)			cdk5	VpYKSPVVSG	2.10	1.37	0.98
S396[a] (S713)	wt LRRK2	G2019S	cdk5	KTDHGAEIVYKpSPVVSG	1.64/1.6/2.10 (wt/G2019S/cdk5)	0.70/0.34/1.37 (wt/G2019S/cdk5)	0.92/0.89/0.98 (wt/G2019S/cdk5)
S422[a] (S739)			cdk5	DMVDpSPQLAT	1.74	1.19	1.00
S404[a] (S721)	wt LRRK2	G2019S	cdk5	KTDHGAEIVYKSPVVSGDTpSPRH	3.35/3.06/3.78 (wt/G2019S/cdk5)	0.96/0.96/0.44 (wt/G2019S/cdk5)	0.94/1.00/1.00 (wt/G2019S/cdk5)
S409 (S726)	wt LRRK2		cdk5	VYKSPVVSGDTSPRHLpSN	2.39/2.86 (wt/cdk5)	0.90/1.09 (wt/cdk5)	0.90/0.38 (wt/cdk5)

[a]Tau phosphorylation sites directly identified in AD patients' brains. [b]Kinases that phosphorylate this site were not identified previously. [c]Tau phosphorylation sites are numbered in two ways: (i) based on sequence of tau isoform (2N4R) such as Ser396/Ser404 and (ii) based on full length tau such as Ser713/Ser721 shown with parentheses. [c]Sequest primary search score. [d]Mass measurement deviation from theoretical (in parts-per-million). [e]Localization probability as assessed by PhosphoRS.

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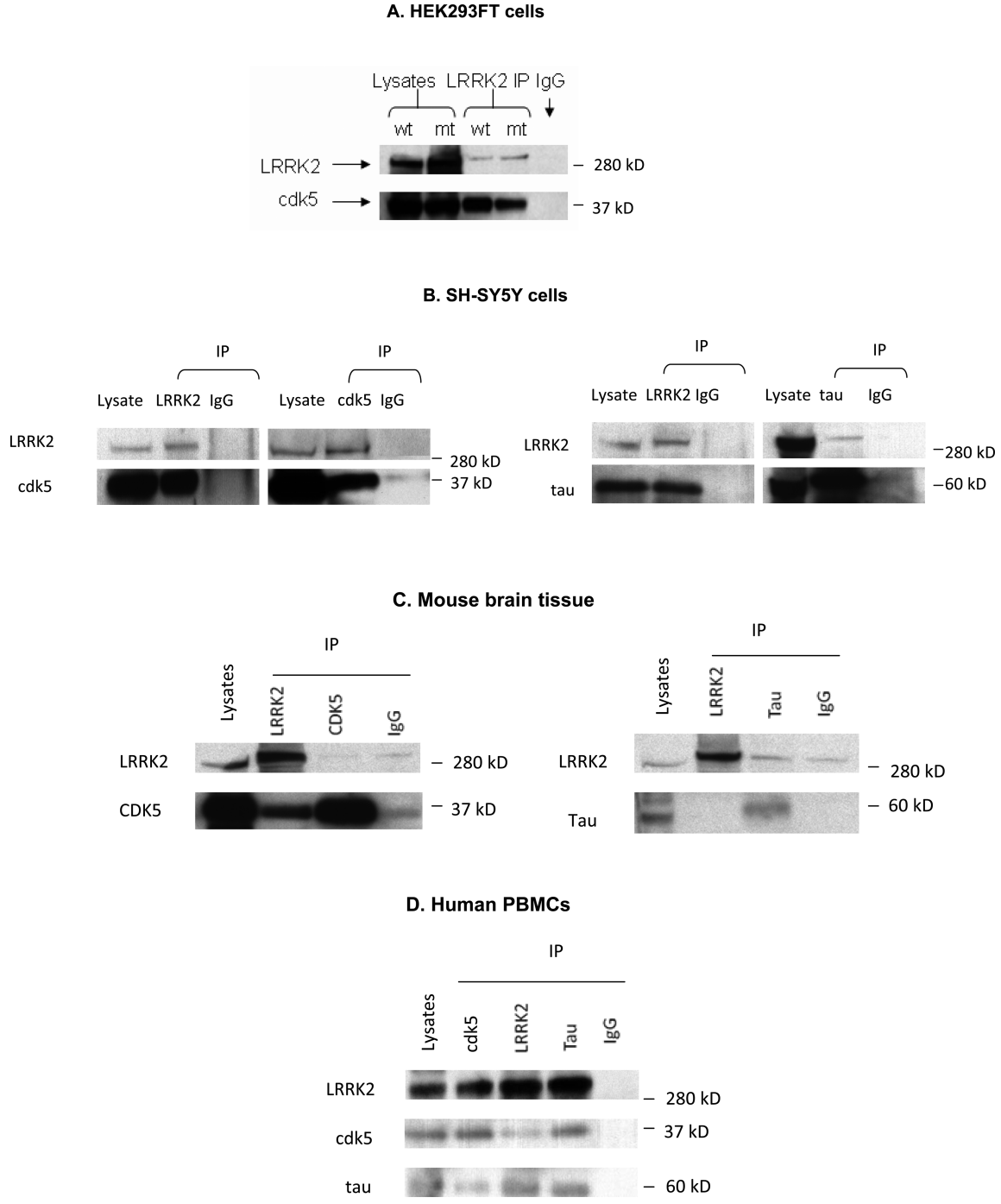

Figure 5. Interaction of LRRK2, tau, and cdk5 in cells and *in vivo*. (A) Cdk5 coimmunoprecipitates with LRRK2 in HEK293FT cells overexpressing wt LRRK2 or the G2019S mutant. (B) Interaction of endogenous LRRK2, cdk5, and tau in SH-SY5Y cells. (C) Interaction of endogenous LRRK2, cdk5, and tau in mouse brain tissue. (D) Interaction of endogenous LRRK2, cdk5, and tau in PBMCs isolated from healthy human blood samples. All the experiments were repeated at least three times. For C and D, experiments were repeated using >3 different samples.

Measuring CNS components in PBMCs offers a simple and less invasive source to find molecular signatures of PD and other neurodegenerative diseases based on mounting evidence that PBMCs directly participate in neurodegenerative processes.[36,37] Consequently, evaluating tau, LRRK2, alpha-synuclein, and UCHL1 in PBMCs as biomarkers of PD and other neurodegenerative diseases have been reported in multiple studies.[38−41] Here we observed LRRK2/tau and LRRK2/cdk5 interaction in PBMCs for the first time. Our data strongly suggest that LRRK2 interacts with cdk5 and tau in cells and *in vivo*.

LRRK2 Facilitates tau Phosphorylation in a Manner Independent of Its Kinase Activity. We next evaluated effects of endogenous LRRK2 on tau phosphorylation in SH-SY5Y cells. We monitored two major tau phosphorylation sites, Ser396 and Ser404, which are phosphorylated by wild type LRRK2, the G2019S mutant, or cdk5 *in vitro*. As shown in Figure 6A, knocking down LRRK2 by its siRNA significantly reduces tau phosphorylation at both Ser396 and Ser404.

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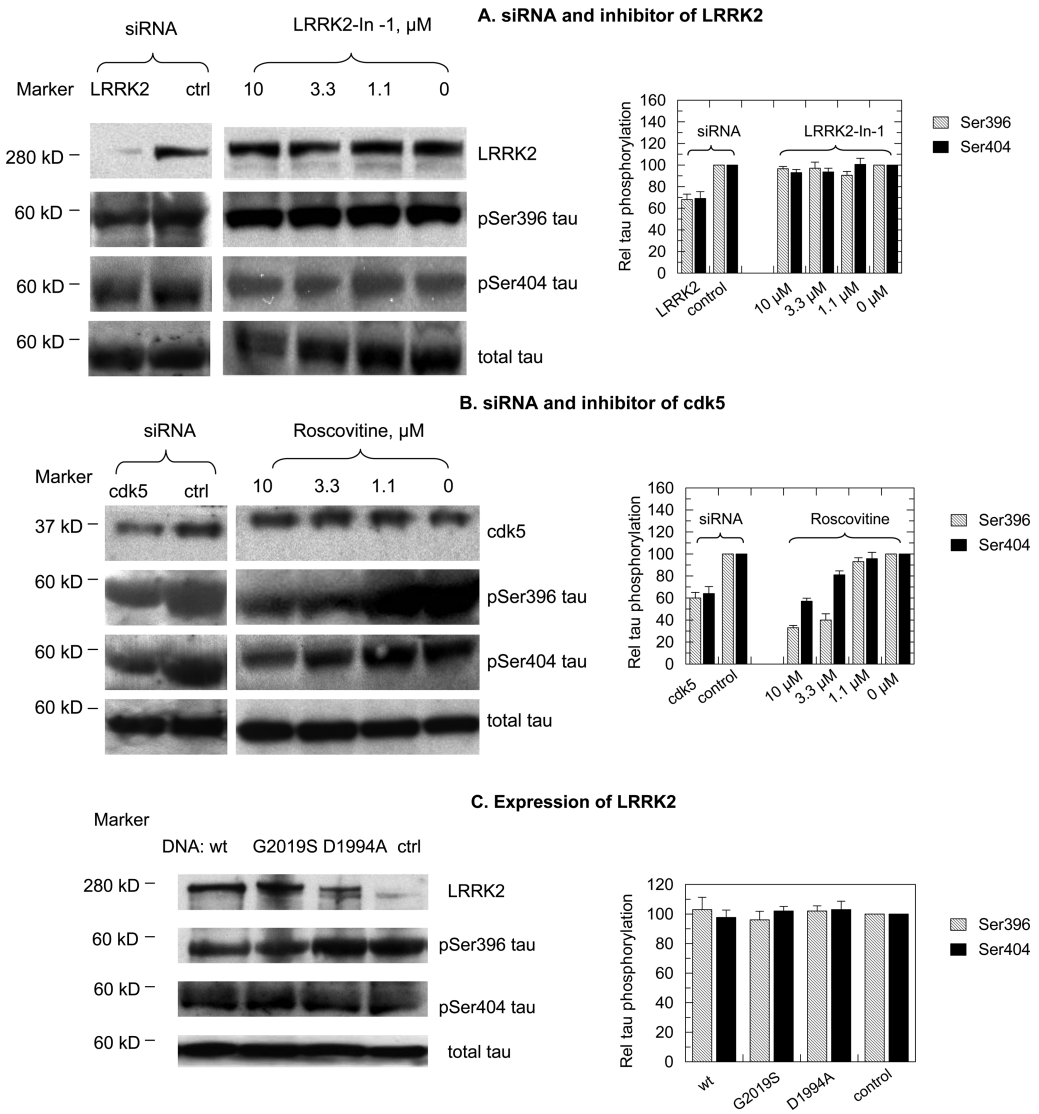

Figure 6. Effects of LRRK2 on tau phosphorylation in SH-SY5Y cells. (A) Knocking down LRRK2 by its siRNA reduces tau phosphorylation at Ser396 and Ser404, whereas LRRK2 inhibitor LRRK-In-1 leads to no changes in tau phosphorylation at these two sites. (B) Cells treated with siRNA of cdk5 or its inhibitor roscovitine have reduced tau phosphorylation at Ser396 and Ser404. (C) Overexpressing wt LRRK2, the G2019S, or the D1994A kinase dead mutant has no effect on tau phosphorylation at Ser396 or Ser404. All the experiments were conducted with $n > 3$. Ratio of PO_4-tau/total tau (rel tau phosphorylation) was used for quantification, where each data point is the average of rel tau phosphorylation determined from multiple independent experiments. Error bar represents the standard error of the mean.

However, inhibiting its kinase activity by LRRK2-In-1 (Figure 6A) or ponatinib (data not shown) does not affect tau phosphorylation at either site. In contrast, knocking down cdk5 or inhibiting its kinase activity by roscovitine reduces phosphorylation at both sites significantly (Figure 6B). Previously, it was reported that overexpressing wild type LRRK2 or the G2019S mutant in SH-SY5Y cells slightly increases PO_4−Ser396 by 1.3- or 1.4-fold, respectively.[42] However, we did not observe changes in tau phosphorylation in these two sites when wt LRRK2, the activating G2019S mutant, or the kinase-dead D1994A mutant was transiently overexpressed in SH-SY5Y cells (Figure 6C). Our data strongly suggest that LRRK2 facilitates tau phosphorylation but in a kinase activity-independent manner.

DISCUSSION

The normal physiological role of tau is thought to be involved in the structural integrity of microtubules, a process regulated by phosphorylation. Phosphorylation at specific residues of tau favors its detachment from the microtubules[43] and eventually leads to the formation of neurofibrillary tangle (NFT). Although NFT pathology is extensive and prevalent in Alzheimer's disease (AD),[19,44] tau has not been identified as a risk factor of AD. Interestingly, recent GWAS studies have linked tau as a risk factor of PD where tau pathology is not consistently observed.

Most PD patients with LRRK2 mutations have either α-synuclein-positive Lewy bodies or tau-positive lesions.[2] The mechanism by which LRRK2 contributes to tau phosphorylation is controversial. There has been evidence that LRRK2 directly phosphorylates tubulin-associated tau at Thr181[17] and free tau at Thr149 and Thr 153,[18] two phosphorylation sites that have not been largely explored. There is also evidence supporting an indirect role of LRRK2 in tau phosphorylation: LRRK2 activates GSK-3β and leads to increased tau phosphorylation;[42] the G2019S mutant forms a complex with

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GSK-3β and facilitates the recruitment of GSK-3β for tau phosphorylation.[45]

In this study, we found that both t-wt LRRK2 and the t-G2019S mutant phosphorylate free human tau protein *in vitro*. Direct comparison of the kinetic parameters for LRRK2 and cdk5 revealed two startling findings. First, the mutant t-G2019S phosphorylates tau with a specific activity at least 140-fold lower than that of cdk5. The G2019S mutant, the most common LRRK2 mutation, is the only mutation that has been consistently shown to increase LRRK2 kinase activity by 2−10-fold, depending on the identity of the phosphoryl acceptors.[46−48] We and others reported that it stabilizes the active conformation of LRRK2 kinase domain and leads to increased kinase activity.[49,50] Even for such an activating mutation of LRRK2, its specific activity is dramatically lower than that of cdk5, it is therefore hard to believe that other LRRK2 mutations contribute to tau phosphorylation mainly through direct interaction as an enzyme.

The puzzle how LRRK2 contributes to tau phosphorylation is partially answered by the other startling finding: tau interacts with t-wt LRRK2 or the t-G2019S mutant with a substrate affinity 140−200-fold tighter than it when tau binds to cdk5. Under normal cellular conditions, only 2% of tau stays in free form, while the majority of tau is associated with tubulin. It is possible that wt LRRK2 or its PD-linked mutations recruits free tau and its kinases and therefore facilitates tau phosphorylation. This hypothesis was further supported by our observation that (i) tau or cdk5 coimmunoprecipitates with endogenous LRRK2 in SH-SY5Y cells, in wild type mouse brain tissue and in human PBMCs isolated from healthy controls; (ii) knocking down endogenous LRRK2 in SH-SY5Y cells reduces tau phosphorylation at Ser404 and Ser396; (iii) however, inhibiting LRRK2 kinase activity or overexpressing wt, G2019S, or KD mutant has no effect on tau phosphorylation at these two sites. Our data suggest that LRRK2 mainly contributes to tau phosphorylation at Ser396 and Ser404 indirectly by recruiting cdk5 or tau with a small contribution from directly catalyzing tau phosphorylation. Consistent with previous report, we also found that GSK-3β coimmunoprecipitates with endogenous LRRK2 in mouse brain (data not shown). It is therefore likely that LRRK2 also contributes to tau phosphorylation through recruiting GSK-3β. It is worth noting that our conclusion is based on the analysis of Ser396/404 epitopes but not others identified in the mass spectrometry analysis here or elsewhere.[18] It is possible that LRRK2 does phosphorylate other residues of tau as a main tau kinase, but those sites have not been explored.

This is the first study that infers a strong interaction between tau and LRRK2 and also the first instance that shows interaction between cdk5 and LRRK2 in cells and *in vivo*. Our data suggest that LRRK2 facilitates tau phosphorylation by recruiting tau or cdk5, it is therefore logical to expect that this process will be increased in PD patients with LRRK2 mutations. However, what is puzzling us the most is why tau pathology was only observed in certain LRRK2 carriers. The mechanisms that lead to tau pathology are more complicated than we currently understand. It is possible that hyperphosphorylated tau is a common feature in LRRK2 carriers, and tau pathology is steered by different environmental factors.

Most PD patients with LRRK2 mutations have either α-synuclein-positive Lewy bodies or tau-positive lesions.[2] Tau-positive lesions is more evident in PD patients with the I2020T mutant than patients carrying other LRRK2 mutations.[9] Hyperphosphorylated tau was also found in brain tissues of transgenic mice overexpressing the R1441G or the G2019S mutant of LRRK2.[14,15] All these lines of observation suggest a critical role of LRRK2 in tau phosphorylation-related neuronal pathology. However, a recent study reported that LRRK2 R1441G and tau P301S double transgenic mice did not exacerbate tau hyperphosphorylation or aggregation observed in tau P301S mice.[51] Whether a pathophysiological relationship only exists between LRRK2 and wild type tau or LRRK2 and tau have independent roles in the development of PD is currently not clear. Clarification of this issue in the future will be important for advancing our understanding of the pathogenesis of PD.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.biochem.5b00326.

Progress curve of tau phosphorylation; interaction of endogenous LRRK2 and tau in human brain tissue (PDF)

■ AUTHOR INFORMATION

Corresponding Author

*Phone: (617)768-8658. Fax: (617)768-8606. E-mail: mliu@rics.bwh.harvard.edu.

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■ ABBREVIATIONS

PD, Parkinson's disease; LRRK2, leucine-rich repeat kinase 2; cdk5, cyclin-dependent kinase 5; PBMCs, peripheral blood mononuclear cells

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