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Physiologically relevant factors influence tau phosphorylation by Leucine-rich repeat kinase 2

Matthew Hamm¹, Rachel Bailey^{1,2}, Gerry Shaw⁴, Shu-Hui Yen³, Jada Lewis¹, and Benoit I. Giasson¹

¹Center for Translational Research in Neurodegenerative Disease & Department of Neuroscience, College of Medicine, University of Florida, FL

³Department of Neuroscience, Mayo Clinic, Jacksonville, FL

⁴EnCor Biotechnology Inc., Gainesville, FL

Abstract

Hyper-phosphorylation and aggregation of tau are observed in multiple neurodegenerative diseases, termed tauopathies. Tau has also been implicated in the pathogenesis of Parkinson's disease (PD) and parkinsonisms. Interestingly, a subset of PD patients with mutations in the *Leucine-rich repeat kinase 2 (LRRK2)* gene exhibit tau pathology. Mutations in *LRRK2* are a major risk factor for PD, but LRRK2 protein function remains unclear. The most common mutation, G2019S, is located in the kinase domain of LRRK2 and enhances kinase activity *in vitro*. This suggests that the kinase activity of LRRK2 may underlie its cellular toxicity. Recently, *in vitro* studies suggested a direct interaction between tubulin-bound tau and LRRK2 that results in tau phosphorylation at one identified site. Here we present data suggesting that microtubules enhance LRRK2-mediated tau phosphorylation at 3 different epitopes. We also explored the effect of divalent cations as catalytic cofactors for G2019S LRRK2-mediated tau phosphorylation and found that manganese does not support kinase activity and inhibits the efficient ability of magnesium to catalyze LRRK2-mediated phosphorylation of tau. These results suggest that cofactors such as microtubules and cations in the cellular milieu may have an important impact on LRRK2-tau interactions and resultant tau phosphorylation.

Graphical Abstract

Corresponding authors: Jada Lewis BMS J485 1275 Center Drive Gainesville FL 32610; Tele: 352-273-9666; jada.lewis@ufl.edu or Benoit I Giasson BMS J483 1275 Center Drive Gainesville FL 32610; Tele 352-273-9363; bgiasson@ufl.edu.

²currently at Gene Therapy Center, University of North Carolina at Chapel Hill, Chapel Hill, NC

Conflict of Interest

Co-author Gerry Shaw is an employee of EnCor Biotechnology Inc., the company which provided monoclonal antibody MCA-4F10.

Author's Roles

MH performed the majority of experiments shown herein and drafted the original manuscript. RB performed experiments, consulted on the project and edited drafts of the manuscript. GS generated the novel monoclonal antibody MCA-4F10 for use in this project and also participated in editing drafts of the manuscript. SHY consulted heavily on overall project design, aided in experimental troubleshooting, and participated in the editing process. JL and BG are co-mentors of Matthew Hamm and participated in all experimental design as well as editing of the manuscript.

Keywords

Leucine-Rich Repeat Kinase 2; Tau; Parkinson's disease; AB_2492292; AB_2492294; AB_304676; AB_2492293; AB_2315150; AB_223647; AB_223651; AB_771432; AB_2100313; AB_2492290; rid_000081

Introduction

Tau is a microtubule (MT) binding protein that plays a role in controlling the assembly and dynamic stability of MTs (Drechsel et al., 1992; Trinczek et al., 1995). As a MT binding protein, tau influences axonal transport, growth cone development, and even nuclear processes (Klein et al., 2002; Morfini et al., 2009; Papasozomenos and Binder, 1987). The ability of tau to bind to MTs and interact with other proteins is regulated by phosphorylation of its many phospho-epitopes. While phosphorylation of tau is normal and critical to its functionality, abnormal phosphorylation of tau at specific epitopes appears to play a role in the pathogenesis of Alzheimer's disease (AD) and other tauopathies, diseases that feature abnormally aggregated, hyper-phosphorylated tau as a primary pathological feature (Hanger et al., 2009). There are also a number of neurodegenerative diseases, termed secondary tauopathies, in which the development of aggregated tau only occurs in a subset of disease cases. Parkinson's disease (PD) is one of these secondary tauopathies (Ishizawa et al., 2003; Vermersch et al., 1992, 1993); however, the origins of tau pathology in PD are still largely unknown.

A number of kinases have been implicated in both the normal and abnormal phosphorylation of tau at serine and threonine residues (Dolan and Johnson, 2010; Noble et al., 2013). Current work, including our own, suggests that Leucine-rich repeat kinase 2 (LRRK2) can also phosphorylate tau either *in vitro* or *in vivo* (Bailey et al., 2013; Kawakami et al., 2012). Interestingly, LRRK2 mutations are the most prevalent known cause of Parkinson's disease

(Dächsel and Farrer, 2010; Zimprich et al., 2004), and the discovery that LRRK2 can direct tau phosphorylation may help explain the appearance of tau pathology in some cases of PD.

We recently demonstrated that LRRK2 is capable of modulating the biochemical status of tau in a disease-relevant manner (Bailey et al., 2013). Specifically, we found that T149 and T153 in tau are substrates for LRRK2 phosphorylation and that phosphorylation of these sites and certain others is elevated in a transgenic mouse model of tauopathy when LRRK2 is also overexpressed. In the same paper we showed that T149 and T153 are phosphorylated in pathological inclusions characteristic of various human tauopathies including in a patient with the G2019S mutation as well as patients with various parkinsonisms. Similarly, Augustinack and colleagues have also shown that tau T153 is phosphorylated in human Alzheimer's disease cases and that this modification is a marker of the "pretangle" tau state (Augustinack et al., 2002). Together, this previous work suggests a role for LRRK2 in the development of tau pathology in a mouse model of tauopathy and an association of these epitopes with human tauopathy. In this current report, we sought to identify factors that affect tau phosphorylation by LRRK2. Here we demonstrate further evidence that tau is an *in vitro* substrate of wild-type (WT) LRRK2 and that this activity is enhanced both by the presence of the G2019S mutation in LRRK2 and by the addition of MTs. Furthermore, we demonstrate that the specific cation used in the kinase reactions has a dramatic effect on the ability of LRRK2 to phosphorylate tau. Mn^{2+} is incapable of supporting the phosphorylation of tau by G2019S LRRK2 and also inhibits Mg^{2+} -mediated LRRK2 phosphorylation of tau. This is in contrast to the phosphorylation of myelin basic protein and LRRKtide, where Mn^{2+} can be used as an effective cationic cofactor by G2019S LRRK2 to drive this reaction (Covy and Giasson, 2010; Lovitt et al., 2010). Therefore, we show for the first time that the ability of G2019S to use Mn^{2+} as a cofactor in protein phosphorylation is substrate-specific. Our data indicates that LRRK2-mediated phosphorylation can be influenced by multiple factors and the impact of these factors can be substrate-specific, at least *in vitro*. Our study suggests that these or other physiologically relevant factors may have a similar influence on LRRK2 activity *in vivo*, possibly explaining the pleomorphic presence of tau pathology in PD.

Materials and Methods

Antibodies

The novel anti-phosphothreonine 149 tau monoclonal antibody, MCA-4F10, was generated by EnCor Biotechnology Inc. (Gainesville, Florida), using the peptide DGK_pTKIATPRGAAC, where p is a phosphate group and a C-terminal cysteine was added for chemical coupling. This peptide was coupled to either maleimide activated keyhole limpet hemocyanin (KLH) or to maleimide activated bovine serum albumin (BSA). Balb/c mice were immunized with the KLH-conjugated peptide and hybridomas were generated using standard procedures by fusing spleen white blood cells with PAI cells and plating in 96 well plates. Hybridoma supernatants were screened by ELISA with the BSA peptide conjugate and positive wells were subcloned by limiting dilutions to identify monoclonal lines. One clone, MCA-4F10, was further characterized as described here. Polyclonal anti-T153 tau specific antibody was made as a service by GenScript USA Inc., as previously

described (Bailey et al., 2013). Other polyclonal tau antibodies used include pT205 (Abcam) and E1 (specific for aa 19–33, human tau) (Crowe et al., 1991), provided by Leonard Petrucelli, Mayo Clinic, Jacksonville. Other monoclonal tau antibodies used include PHF1 (specific for pS396/S404) (provided by Dr. Peter Davies, The Feinstein Institute for Medical Research, Manhasset, NY, USA), AT8 (specific for pS199/pS202/pT205), and AT270 (specific for pT181) (Innogenetics, Fisher Scientific). Anti- β -tubulin antibody MCA-4E4 (EnCor Biotechnology Inc., Gainesville, FL) was used to visualize tubulin. Anti-GST (GE Healthcare Life Science) was used to visualize GST-tagged LRRK2 and LRRKtide. Anti-pT567 Ezrin/pT564 Radixin/pT558 Moesin (Cell Signaling) was used to visualize phosphorylation of threonine on LRRKtide. All antibodies are also listed with further details in Table 1.

Generation of GST-LRRKtide

The nucleotide sequence corresponding to the amino acid sequence of LRRKtide (RLGRDKYKTLRQIRQ) with overhanging *Bam*HI and *Eco*RI restriction sites was amplified by PCR using the oligonucleotides (5'-GATCCCGACTGGGCCGAGACAAATACAAGACCCTGCGCCAGATCCGGCAG-3') and (5'-AATTCCTGCCGATCTGGCGCAGGGTCTTGTATTTGTCTCGGCCAGTCG-3') and cloned into *Bam*HI and *Eco*RI restriction sites of the bacterial expression plasmid pGEX-2T. GST-LRRKtide was expressed in *E. coli* BL21 (DE3-RIL) following induction of expression with isopropylthio- β -galactoside. Bacterial cell pellets were lysed with 1% Triton-X100 in PBS and sonicated in short bursts on ice. Protein was then batch-purified with Glutathione Sepharose 4B conjugate followed by elution with 50 mM Tris (pH 8.0), 10 mM glutathione.

Other Materials

Recombinant wild-type, G2019S, and D1994A forms of GST-LRRK2 (970–2,527) were purchased from Life Technologies. Recombinant glycogen synthase kinase 3 beta (GSK-3 β) was purchased from New England Biolabs (Ipswich, MA). Bovine brain tubulin was purchased from Cytoskeleton, Inc. (Denver, CO). 0N4R tau (corresponding to the 383 aa human transcript variant 3) cDNA cloned into the bacterial expression vector pRK172 was provided by the laboratory of Dr. Michel Goedert, Cambridge University. 0N4R tau was expressed in *E. coli* BL21 cells and purified as previously described (Hong et al., 1998).

Enzyme-linked Immuno Sorbent Assay (ELISA) for Assessment of Antibody Specificity

Method was previously described in Bailey et al, 2013. ELISA screens were performed to test specificity of MCA-4F10 antibody, using PHF1 antibody as a control. Two different types of polypeptides were used as targets in the screening. One is a synthetic peptide (DGKTKIATPRGAAC) corresponding to amino acids 146–159 of tau such that it encompasses both T149 and T153 of tau. Four forms of this peptide were used in ELISA: a non-phosphorylated version, and versions phosphorylated at T149, T153, or both T149 and T153. The second protein used is a recombinant, C-terminal fragment of human 3R tau [C' Tau] corresponding to amino acids 244–441 minus amino acids 275–305 that would be present in 2N4R tau. The C-terminal fragment was either non-phosphorylated or phosphorylated by GSK-3 β . Experiments were performed in quadruplicate.

LRRK2 Kinase Reactions

Kinase reactions were prepared in a total volume of 25 μ l. Reaction conditions consisted of 20 mM Tris/HCL (pH 7.5), 1 mM EGTA, 5 mM β -glycerophosphate, 2 mM dithiothreitol, 0.02% Polysorbate 20, 10 mM $MgCl_2$ (or $MnCl_2$) and 0.4 mM ATP. 2 μ g (1.67 μ M) recombinant, wild-type 0N4R tau or a molar equivalent of recombinant GST-LRRKtide (1.125 μ g) was added to each reaction tube. For reaction sets containing microtubules (MTs), MTs were reconstituted in polymerization buffer (15 mM Pipes, pH 7.0, 1 mM $MgCl_2$) with and without 20 μ M Taxol; either 1 μ g of re-suspended MTs or an equivalent amount of polymerization buffer without MTs was added to each reaction. 50 ng (9.6 nM) of recombinant wild-type, G2019S, or D1994A GST-LRRK2 (970–2527) was added to reactions at the end of reaction preparation. Finally, reaction tubes were incubated at 30° C for 30 minutes. Reactions were terminated by adding 25 μ l SDS-PAGE sample buffer to each reaction tube and incubating at 100° C for 5 minutes.

Microtubule Binding Protein Spin-down Assay

A MT spin-down assay was performed to assess G2019S LRRK2 binding to MTs. The experiment was performed following methods for the microtubule binding protein spin-down assay kit that is sold by Cytoskeleton Inc. (Denver, CO) (Cat. # BK029). Briefly, 50 μ l samples in general tubulin buffer (PIPES pH 7.0, 2 mM $MgCl_2$, 0.5 mM EGTA), were spun through 100 μ l of cushion buffer (PIPES pH 7.0, 1 mM $MgCl_2$, 1 mM EGTA, 20 μ M taxol, in 60% glycerol) at 45,000 RPM in a TLA-55 rotor for 40 minutes. Reaction conditions of the 50 μ l samples were as follows: 9.1 μ g MTs, 0.19 mM LRRK2, 80 mM PIPES pH 7.0, 2 mM $MgCl_2$, 0.5 mM EGTA, 20 μ M taxol, 1 mM GTP.

Western Blotting

Western blot analyses were used to assess kinase reactions. For each immunoblot, equal amounts of samples were loaded onto each lane and then resolved on SDS-PAGE gels. Samples were re-boiled at 100° C for 1 minute, cooled to room temperature, centrifuged, and re-homogenized by pipetting prior to gel loading. After SDS-PAGE gel resolution, samples were electrophoretically transferred to polyvinylidene difluoride (PVDF) membranes. PVDF membranes were blocked in Tris-buffered saline (TBS) with 5% dry-milk powder. Following block, membranes were incubated overnight with primary antibodies. Phospho-specific antibodies were diluted in TBS/5% BSA, while all remaining antibodies were diluted in TBS/5% milk. After overnight incubation, membranes were washed in TBS and then incubated for one hour in goat anti-mouse conjugated horseradish peroxidase (HRP)(Jackson ImmunoResearch), goat anti-rabbit HRP (Jackson ImmunoResearch), or rabbit anti-goat HRP (Santa Cruz Biotechnology). Immunoreactivity was visualized by applying western lighting plus-ECL to membranes and analyzing with FluorChem E system (ProteinSimple). In order to strip membranes for additional probes, membranes were incubated in stripping solution (5% SDS, 62.4 mM Tris-Base, pH 6.8, 0.7% 2-mercaptoethanol) for 15 minutes at 55° C, washed in TBS for 30 minutes, and then further processed for immunoblotting as described above.

Data Analysis

Western blots were quantified using Alpha View software, V3.4 (http://www.proteinsimple.com/software_alphaview.html#fluorchem). The largest band, as identified visually, was selected to generate a quantification area approximately the size of the band, and this same quantification size was used to measure the intensity of every band/lane on the blot. In many immunoblots, tau resolves as a doublet, which appears to be due to transient interactions with tubulin. In these cases, the entire block of signal encompassing both bands of the doublet was selected and measured for each lane. Statistical analysis was performed using Prism GraphPad, V5 (RRID:rid_000081)(<http://www.graphpad.com/scientific-software/prism/>). Figures 3 (n=5), 5 (n=5), and 7 (n=3) were analyzed by two-tailed *t*-test in order to compare pairs of data sets and figures 1 (n=4) and 7 (n=3) were analyzed by one-way ANOVA with Bonferroni's multiple comparison test post-hoc in order to compare groups of data sets.

Results

Novel monoclonal antibody MCA-4F10 recognizes tau phosphorylated at threonine 149

We previously demonstrated that LRRK2 can directly phosphorylate threonine 149 (T149) in tau (Bailey et al., 2013); however, limited amounts of the polyclonal antibody used in those experiments have hampered subsequent studies. In order to validate a new tool for these tau studies, we performed ELISAs to analyze the specificity of the novel mouse monoclonal antibody MCA-4F10 generated against a synthetic peptide corresponding to tau phosphorylated at T149. Results demonstrated that the antibody shows high affinity for a synthetic tau peptide (DGKpTKIATPRGAAC) that is phosphorylated at the T149 phospho-epitope. The antibody has minimal reactivity with the same peptide when the peptide is phosphorylated at the threonine 153 (T153) site, phosphorylated at both T149 and T153, or not phosphorylated (Fig. 1). MCA-4F10 showed little reactivity against the C-terminus fragment of tau that was phosphorylated robustly by the kinase GSK3 β , indicating that MCA-4F10 has a high affinity for tau phosphorylated at T149 but not at other tau phospho-epitopes.

LRRK2 binds to Microtubules in spin-down/sedimentation assays

Previous research has demonstrated that LRRK2 phosphorylates tubulin and enhances its polymerization *in vitro* (Gillardon, 2009). Here, we further confirm an interaction between polymerized tubulin, in the form of microtubules (MTs), and recombinant G2019S LRRK2 (Fig. 2). A western blot of LRRK2-MT spin-down reactions, when probed for GST-tagged LRRK2 and β -tubulin, demonstrates that the presence of MTs greatly enhances the migration of LRRK2 into the sedimented pellet fraction. This shift of LRRK2 into the pellet is indicative of a MT-LRRK2 interaction *in vitro*. Given that tau protein also interacts with MTs and has recently been shown to be a substrate for LRRK2-mediated phosphorylation (Kawakami et al., 2012, Bailey et al., 2013), these results suggest that the interaction of MTs with either LRRK2 or tau may impact LRRK2-mediated phosphorylation of tau.

In vitro phosphorylation of tau by LRRK2 is enhanced by microtubules

To investigate the effects of microtubules on the ability of LRRK2 to phosphorylate tau *in vitro*, we performed tau-kinase reactions with and without MTs and probed for the phosphorylation of epitopes that we previously demonstrated were substrates for LRRK2 (T149, T153 and Threonine 205 (T205)) (Bailey et al., 2013). It has been suggested that Taxol may compete with MTs for the tau MT binding site (Kar et al., 2003; Samsonov et al., 2004), so we also reconstituted MTs both with and without the further addition of 20 μ M Taxol. Kinase-dead (KD) LRRK2 was used as a negative control. Western blot analysis of WT LRRK2 reaction sets indicated that the presence of MTs potentiates WT LRRK2 phosphorylation of tau at T149 (Fig. 3). The addition of Taxol did not significantly influence the ability of MTs to support LRRK2-mediated phosphorylation of tau at T149 (Fig. 3). We detected no signal above background levels in kinase reactions without microtubules that were probed with MCA-4F10 (pT149) antibody. Similarly, the addition of MTs significantly enhanced LRRK2 phosphorylation of tau at T153 compared to tau only reactions. In this case, the addition of Taxol to the MT re-suspension significantly increased LRRK2-mediated phosphorylation above that seen using MT alone. LRRK2-directed phosphorylation of tau T205 was also elevated in the presence of MTs but Taxol did not significantly elevate this phosphorylation. The T205 site is part of a triple phospho-epitope in tau that is hyper-phosphorylated in human tauopathy and can be detected by the AT8 antibody (Goedert et al., 1995). We previously demonstrated that AT8 levels were elevated in tau transgenic mice expressing human WT LRRK2 compared to the tau transgenic animals alone (Bailey et al., 2013). Interestingly, we did not detect LRRK2-directed phosphorylation at this triple epitope in these *in vitro* experiments despite the addition of MTs or Taxol (Fig. 4A), indicating that T205 specifically, not the triple epitope, is phosphorylated by LRRK2.

Kawakami and colleagues have shown that LRRK2 can phosphorylate tau at threonine 181 (T181) in a MT-dependent manner (Kawakami et al., 2012); however, we failed to detect an enhanced phosphorylation of tau T181 in transgenic mice expressing human WT LRRK2 as compared to tau transgenic only mice (Bailey et al., 2013). Concurring with our previous study, in the current study we found that WT LRRK2 was unable to phosphorylate the tau T181 epitope, regardless of the presence of MTs with and without Taxol (Fig. 4A). Furthermore, we examined phosphorylation of tau at serine 396 and 404 (S396/404), a dual epitope that is commonly phosphorylated in tauopathy and which was unaltered in tau transgenic mice expressing human WT LRRK2 (Bailey et al., 2013). Using the PHF1 antibody, we found that WT LRRK2 did not phosphorylate tau at S396/404 *in vitro* even in the presence of MTs (Fig. 4A). These results support the idea that the presence of MTs does not alter the site specificity of WT LRRK2 but merely enhances phosphorylation.

We further examined the effects of MTs on LRRK2 phosphorylation of tau by analyzing reaction sets with G2019S LRRK2, a mutant form that has been shown to have elevated kinase activity (Covy and Giasson, 2010; Lovitt et al., 2010; West et al., 2005, 2007). Similarly to WT LRRK2, the presence of MTs enhanced the phosphorylation of tau by G2019S LRRK2 at T149, T153, and T205 (Fig. 5). Robust signal was visible in all reactions containing MT or MT with Taxol; whereas, reactions lacking MTs had signal that was

roughly equivalent to background levels (Fig. 5). The inclusion of Taxol in the MT reconstitution did significantly change the ability of MTs to enhance G2019S LRRK2-mediated tau phosphorylation. As opposed to the WT LRRK2 reactions, we were able to demonstrate phosphorylation of tau T181 by G2019S in the presence of MTs, although the phosphorylation does not appear to be particularly robust (Fig. 5). The presence of Taxol did not enhance LRRK2-mediated phosphorylation of tau at T181 over the influence of MTs alone. Similarly to the WT LRRK2 reactions, probes of the G2019S LRRK2 reaction set with PHF1 and AT8 antibodies were negative for signal in all LRRK2-containing reactions (Fig. 4B), indicating that a degree of site specificity is retained in the G2019S mutant. In order to demonstrate that differential LRRK2 and/or tubulin levels were not confounding our results, we subsequently performed westerns on samples analyzed for tau in Figures 5A and 5B and probed for GST-LRRK2 and tubulin (Fig. 5C). Additionally, antibodies MCA-4F10, pT153, and E1, are not commercially available; therefore, control blots including reactions with tubulin alone and LRRK2 and tubulin without tau have been included in figure 6 to demonstrate that these antibodies do not cross-react with tubulin.

G2019S LRRK2 phosphorylates tau to a greater degree than wild-type LRRK2 in the presence of microtubules

G2019S LRRK2 has elevated kinase activity as compared to WT LRRK2, and we have previously demonstrated this to be the case with tau as a substrate (Bailey et al., 2013; Covy and Giasson, 2010; Lovitt et al., 2010; West et al., 2005, 2007). Here, we sought to directly compare G2019S and WT LRRK2 phosphorylation of tau specifically in the presence of MTs. Kinase reactions using G2019S and WT LRRK2 with and without MTs were analyzed with western blots using MCA-4F10, pT153, and pT205 antibodies (Fig. 7A). In the presence of MTs, G2019S LRRK2 had increased tau kinase activity over WT LRRK2 for all 3 epitopes analyzed (Fig. 7B). Comparing WT LRRK2 and G2019S LRRK2 kinase activity in reactions run with MTs reconstituted with Taxol (MTtax in Fig. 7) demonstrated very similar results to the reactions containing MTs alone. G2019S LRRK2 with MTs and Taxol phosphorylated tau significantly more than WT LRRK2 with MTs and Taxol at both the T153 and T205 sites. Significance was not achieved when comparing these groups for phosphorylation at the T149 site, but this is likely due to the high variance amongst data points representing the G2019S LRRK2 kinase reactions with MTs and Taxol (GS MTtax) for this antibody probe.

G2019S phosphorylation of tau is cofactor-type dependent

LRRKtide is a small synthetic protein that contains an epitope that is shared amongst the biological proteins Ezrin, Radixin, and Moesin, and is known to be a substrate for LRRK2 phosphorylation. Previous work has demonstrated that, unlike WT LRRK2 and other mutants, the G2019S LRRK2 mutant is capable of efficiently phosphorylating LRRKtide using either Mg^{2+} or Mn^{2+} as a divalent catalytic cofactor (Covy and Giasson, 2010; Lovitt et al., 2010). We achieved similar results when using a GST-tagged LRRKtide in our G2019S LRRK2 kinase reactions (Fig. 8A). It is noteworthy that previous studies tested other LRRK2 mutants including R1441C and I2020T and this effect was specific to the G2019S mutation, as the other mutants showed very little kinase activity in the presence of Mn^{2+} . Thus, we hypothesized that G2019S LRRK2 phosphorylation of tau using Mn^{2+} may

represent a mutation-specific, disease-relevant alteration in LRRK2 activity. To determine if G2019S LRRK2 can phosphorylate tau with Mn^{2+} , we performed kinase reactions with G2019S LRRK2, tau, MTs, and either Mg^{2+} or Mn^{2+} (Fig. 8B). Unexpectedly, we observed robust phosphorylation of tau at T149 and T153 by G2019S LRRK2 when using Mg^{2+} as the catalytic ion, but not when using Mn^{2+} (Fig. 8B & 8C). The influence of Taxol on the kinase reactions appeared negligible. We then sought to determine if Mn^{2+} simply fails to catalyze the G2019S mediated phosphorylation of tau or if it could act in an inhibitory manner to actively block the phosphorylation of tau in the presence of the normally catalytic ion Mg^{2+} . We performed LRRK2-tau reactions in which 10 mM Mg^{2+} was added in the presence of increasing Mn^{2+} concentrations. A 1:20 ratio of Mn^{2+} to Mg^{2+} was sufficient to reduce tau phosphorylation by G2019S LRRK2 and this was further inhibited at increasing concentrations of Mn^{2+} (Fig. 9).

Discussion

Although mutations in *LRRK2* are the most frequent genetic cause of Parkinson's disease, these mutations are incompletely penetrant ((Berg et al., 2005), indicating that there could be secondary events that influence disease development. Furthermore, the pathology observed in brains from individuals bearing LRRK2 mutations is pleomorphic; approximately 50% of autopsied brains from LRRK2 mutation carriers display some degree of tauopathy (Poulopoulos et al., 2012). We previously demonstrated that LRRK2 can drive phosphorylation of tau at epitopes T149 and T153 *in vitro* and that LRRK2 overexpression could exacerbate tauopathy in a mouse model (Bailey et al., 2013). We hypothesized that this data could help explain the secondary tauopathy observed in PD and sought to determine if we could identify factors that could influence LRRK2 phosphorylation of tau. Herein, we present *in vitro* evidence that MTs enhance phosphorylation of tau by both WT and G2019S mutant LRRK2 at the T149, T153, and T205 sites. Additionally, G2019S LRRK2 modestly phosphorylated tau at T181, only in the presence of MTs. Our results also demonstrate that choice of catalytic ion plays an important role in LRRK2-mediated tau phosphorylation, with Mn^{2+} acting as an inhibitor of what is normally robust Mg^{2+} -dependent LRRK2-mediated phosphorylation.

Previously, we identified LRRK2-based modulation of the T149, T153, and T205 phospho-epitopes of tau in a combination of *in vitro* and *in vivo* experiments. Specifically, we used mass spectrometry to identify sites on tau phosphorylated *in vitro* and we observed an elevation of phosphorylated tau T149 and T205 in the insoluble tau fraction of mice bearing transgenic mutant tau and WT LRRK2 as compared to mice bearing the mutant tau alone. It is also noteworthy that we saw a reduction of phosphorylation at the T181 epitope in this same mouse model. Recent evidence suggests that the interaction of tubulin with LRRK2 and tau may be pivotal for the phosphorylation of tau by LRRK2 at T181 (Kawakami et al., 2012); however, the authors provided no details regarding the influence of WT versus mutant LRRK2 on this phosphorylation event and no other phospho-epitopes were identified as targets of LRRK2 kinase activity. Our earlier findings, paired with those of Kawakami et al., suggest that MTs may influence the phosphorylation of tau at the T149, 153, and 205 sites as well. In this work we provide *in vitro* evidence that MTs enhance the phosphorylation of tau at T149, T153 and T205 by both WT and G2019S mutant LRRK2

and show an MT-dependent elevation of phosphorylation at the T181 site, but only with G2019S LRRK2.

Consistent with previous studies (Covy and Giasson, 2010; Lovitt et al., 2010; West et al., 2005, 2007), in this paper we demonstrate that G2019S LRRK2 is capable of effectively phosphorylating LRRKtide using either Mg^{2+} or Mn^{2+} as a catalytic ion. This has been attributed to the relatively similar catalytic rates or V_{max} for G2019S LRRK2 in the presence of Mg^{2+} -ATP and Mn^{2+} -ATP using either LRRKtide or myelin basic protein (MBP) as protein substrates; in comparison, WT LRRK2 is much more active in the presence of Mg^{2+} -ATP compared to Mn^{2+} -ATP due to a much lower catalytic rate when using Mn^{2+} as the cation cofactor (Covy and Giasson, 2010; Lovitt et al., 2010). Strikingly, G2019S LRRK2 was not able to phosphorylate tau at T149 or T153 in the presence of Mn^{2+} , as compared to robust phosphorylation of these sites in the presence of Mg^{2+} alone. The paucity of tau phosphorylation by G2019S LRRK2 phosphorylation in the presence of Mn^{2+} -ATP demonstrates that the ability of this enzyme to maintain its catalytic activity in the presence of Mn^{2+} is dependent on the protein substrate. The reason for this cation-specific variability in G2019S LRRK2 kinase activity is not clear, but it is likely that tau and Mn^{2+} -ATP are not simultaneously capable of accessing the kinase active site or that this combination of substrates may be sterically unsuited for phosphate transfer. It is also a possibility that the binding of Mn^{2+} -ATP to tau simply alters the conformation of tau in a manner that hinders LRRK2 interaction. These results led us to the notion that Mn^{2+} may actively inhibit G2019S phosphorylation of tau, even when Mg^{2+} is present. To test this hypothesis, we incubated G2019S LRRK2 with tau, Mg^{2+} , and varying sub-stoichiometric concentrations of Mn^{2+} . We found that the presence of Mn^{2+} inhibits Mg^{2+} -dependent G2019S LRRK2 phosphorylation of tau. This inhibitory effect of Mn^{2+} on G2019S LRRK2 phosphorylation of tau in the presence of Mg^{2+} -ATP is similar to Mn^{2+} inhibition of WT LRRK2 for other substrates and is consistent with the higher affinity (i.e. low K_m) of LRRK2 for Mn^{2+} -ATP compared to Mg^{2+} -ATP (Covy and Giasson, 2010; Lovitt et al., 2010).

Our finding that the ATP divalent cation cofactor has a dramatic effect on the ability of G2019S LRRK2 to phosphorylate specific substrates (i.e. tau), but not others such as LRRKtide, suggests that perhaps the pathogenic mechanism of this mutation may involve altered substrate specificity and that other proteins may be similarly affected. For example, it is possible that G2019S LRRK2 may phosphorylate substrates that are not modified by WT LRRK2. We have previously speculated that the ability of G2019S LRRK2 to maintain its catalytic activity in the presence of Mn^{2+} , with WT LRRK2 having a much lower activity, could be a pathogenic mechanism if inhibition of LRRK2 by Mn^{2+} serves as a cellular sensor of increased Mn^{2+} levels (Covy and Giasson, 2010). Our data does not exclude the possibility that tau may be involved in such a pathway, but this specific regulation for tau would not be adversely affected by G2019S LRRK2. This *in vitro* data argues against our original hypothesis that the G2019S mutation broadly enhances Mn^{2+} -dependent phosphorylation of all LRRK2 substrates. The substrate specific differences in these results may still suggest a pathologically important alteration in G2019S activity. If the presence of Mn^{2+} facilitates the phosphorylation of certain G2019S LRRK2 substrates but inhibits LRRK2-mediated tau phosphorylation, then differential exposures to manganese could affect the affinity and kinetics of LRRK2 for its various substrates, driving other substrates

to be favored over tau at higher concentrations of Mn^{2+} . For reasons that are still largely unclear, high levels of environmental manganese exposure can result in PD-like symptomology. This substrate-dependent change induced by the G2019S mutation may be a mechanism through which environmental exposures to manganese influence the pathological progression of G2019S LRRK2 carriers. Nevertheless, more research is necessary to fully probe for a link between Mn^{2+} levels and G2019S toxicity. Taken together, our data demonstrate phosphorylation of tau by LRRK2 at select epitopes is enhanced in the presence of MTs and suggest that altered use of different catalytic ions by G2019S LRRK2 may impact its role in pathogenesis. MTs and catalytic ions are just two of many potentially influential factors when considering the physiological function and dysfunction of LRRK2, and further exploration of these factors and others in more complex model systems should shed more light on the role of LRRK2 and tau in PD pathogenesis.

Work from Gasser and Gillardon indicates that LRRK2 is capable of phosphorylating MTs as well, and that this phosphorylation may affect neurite outgrowth (Caesar et al., 2013; Gillardon, 2009). This information, taken with our data and that of Kawakami and colleagues, suggests a complex relationship between LRRK2, tau and MTs. MT stabilization is crucial to the healthy functioning of the neuron and tau is a key controller of this stabilization. Thus, it is possible that dysregulation of LRRK2 activity may have downstream effects on both MT and tau phosphorylation and consequently on the way in which the latter two proteins interact. Disruption of normal functioning of either MT or tau alone may prove deleterious to neuronal health, and hyper-phosphorylated tau is implicated in tau-aggregate based toxicity. Given that LRRK2 mutations are most predominantly implicated in PD, it seems likely that the interaction between LRRK2, tau, and MTs may be one avenue through which LRRK2 dysregulation contributes to PD pathogenesis.

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Abbreviations in paper (in order of 1st appearance)

PD	Parkinson's disease
LRRK2	Leucine-Rich Repeat Kinase 2
MT	microtubule
WT	wild-type
AD	Alzheimer's disease

KLH	keyhole limpet hemocyanin
BSA	bovine serum albumin
GSK3β	Glycogen Synthase Kinase 3 beta
TBS	Tris-buffered saline
HRP	horseradish peroxidase
T149	Threonine 149
T153	Threonine 153
T205	Threonine 205
KD	kinase-dead
T181	Threonine 181
MTtax	microtubules reconstituted with Taxol
MBP	myelin basic protein

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Significance Statement

In this paper, we demonstrate that microtubules enhanced phosphorylation of tau by LRRK2 at three sites and show that in contrast to other substrates manganese cofactor cannot drive the phosphorylation of tau by G2019S LRRK2. As LRRK2 and tau have both been implicated in Parkinson's disease, LRRK2-mediated phosphorylation of tau has been identified as a potential effector of Parkinson's pathogenesis. The identification of modulators of LRRK2-mediated tau phosphorylation provides important insights into this interaction.

FIGURE 1. Mouse monoclonal antibody MCA-4F10 specifically recognizes tau phosphorylated at T149

ELISAs of tau peptides with MCA-4F10 antibody and previously characterized PHF1 antibody. Each ELISA analysis was performed in quadruplicate. The novel monoclonal antibody reacted strongly with tau peptides DGKpTKIATPRGAAC phosphorylated at T149 (pT149) and had little reactivity with the same peptides when they were phosphorylated at T153 (pT153), dually phosphorylated at T149/T153 (pT149/pT153 dual), or not phosphorylated (non-phospho). Antibody MCA-4F10 also did not react with a recombinant C-terminal tau fragment phosphorylated *in vitro* with GSK3 β (C' tau/GSK3B).

Comparatively, antibody PHF1 reacted strongly with GSK3 β -phosphorylated tau C-terminal.

*** $P < 0.001$ [one-way ANOVA (optical density) with post hoc Bonferroni multiple comparison test]

Figure 2. G2019S LRRK2 binds to microtubules

A MT-binding/spin-down assay was performed as described in “Materials and Methods”. Included are the supernatant (S) and pellet (P) fractions from assays containing only MT, only G2019S LRRK2 (G), or both MTs and G2019S LRRK2. Immunoblots with antibodies to GST, to recognize GST-G2019S LRRK2, and β -tubulin were performed to determine the distribution of either protein. The presence of MTs greatly increased the amount of GST-G2019S LRRK2 in the pellet fraction, demonstrating an interaction with MTs.

FIGURE 3. Wild-type LRRK2 phosphorylates 0N4R tau at several specific epitopes in the presence of microtubules

(A) Western blot analyses of *in vitro* kinase-tau reactions in the presence of 10 mM Mg^{2+} . 0N4R Tau was incubated with WT LRRK2 (W) or kinase-dead LRRK2 (KD) as a negative control. The LRRK2-tau reactions contain microtubules (MT), MTs reconstituted with Taxol (MT with Taxol), or no MTs. All reactions were performed in triplicate and loaded onto gels such that each gel contained 3 sets of reactions; representative blots are shown. Each panel represents a separate western blot that was first probed with specific phospho-antibodies as indicated and then stripped and re-probed with total tau antibody, E1. The mobilities of molecular mass markers are identified on the left of each panel. (B) Densitometric quantification of western blots shown in A. The signal of each phospho-probe was normalized to the E1 probe for the same lane. Two-tailed *t*-tests were performed comparing each group of data points. All cases of significance are demarcated; **P* < 0.05, ****P* < 0.001.

FIGURE 4. Paucity of phosphorylation of 0N4R tau at the PHF1 and AT8 epitopes by wild-type or G2019S LRRK2

(A) Western blot analyses with PHF-1 (pS396/pS404), AT8 (pS199/pS202/pT205) and AT270 (pT181) antibodies showing that the phospho-epitopes recognized by these respective antibodies are not phosphorylated by WT (W) LRRK2 even in the presence of microtubules. (B) AT8 and PHF-1 immunoblots of reactions containing G2019S (G) LRRK2 were also negative. Reactions with kinase-dead (KD) LRRK2 were added as negative controls for each blot and reactions with GSK3β (β) were included as positive controls.

FIGURE 5. G2019S LRRK2 phosphorylates 0N4R tau at multiple epitopes in the presence of microtubules

(A) Western blots analyses of in vitro kinase-tau reactions in the presence of 10 mM Mg^{2+} . WT tau was incubated with G2019S LRRK2 (G) or kinase-dead LRRK2 (KD) as a negative control. All reactions were run in triplicate and loaded onto gels such that each gel contained 3 sets of reactions; representative blots are shown. Each panel represents a separate western blot that was first probed with a phospho-antibody as indicated and then stripped and re-probed with total tau antibody, E1. (B) Densitometric quantification of western blots shown in A. The signal of each phospho-probe was normalized to the E1 probe for the same lane. Each group of data points were compared to one another by two-tailed *t*-test; however, none of the comparisons demonstrated significant differences. (C) Western blots of samples analyzed in 4A and B, probed with GST to detect GST-G2019S LRRK2 and β -tubulin antibodies as a loading control.

FIGURE 6. Phospho-tau antibodies MCA-4F10 and pT153, as well as total tau antibody E1, do not recognize tubulin

Western blots of *in vitro* G2019S (G)LRRK2-in the presence of combinations of MTs and Taxol as indicated above each lane as well as similar reactions without the presence of G2019S LRRK2. A Reaction with kinase-dead (KD) LRRK2 is also included as a control. In A and B, the top panels are immunoblots probed with antibodies MCA-4F10 (pT149) and pT153, respectively, then stripped and probed with secondary antibody alone (middle panels) to show the dearth of remaining signal. Membranes were then re-probed with E1 total tau antibody (bottom panels. (C) An immunoblot of the same reactions shown in A and B, probed only for E1.

FIGURE 7. G2019S LRRK2 phosphorylates tau significantly more than WT LRRK2 at certain phospho-epitopes

(A) Western blots analyses of *in vitro* kinase-tau reactions in the presence of 10 mM Mg^{2+} . 0N4R WT tau was incubated with WT LRRK2 (W), or G2019S LRRK2 (G). The LRRK2-tau reactions contain MTs (MT), MTs reconstituted with Taxol (MTtax), or no microtubules. All reactions were run in triplicate and loaded onto gels such that each gel contained 3 sets of reactions; representative blots are shown. Each panel represents a separate western blot that was first probed with a phospho-antibody as indicated and then stripped and re-probed with E1. The mobilities of molecular mass markers are identified on the left. (B) Densitometric quantification of western blots shown in A for reactions including MTs or MTs with Taxol. The signal of each phospho-probe was normalized to the E1 probe for the same lane. The four groups of data points in each plot were compared to one another by two-tailed *t*-test; **P* 0.05, ***P* < 0.01, ****P* < 0.001.

FIGURE 8. G2019S LRRK2 phosphorylates WT tau in the presence of Mg^{2+} but not Mn^{2+}
(A) Western blot analyses of *in vitro* GST/LRRKtide-LRRK2 kinase reactions. GST-tagged LRRKtide was incubated with GST-tagged G2019S LRRK2 in the presence of either 10 mM Mg^{2+} or Mn^{2+} . Image represents a blot that was probed with anti-moesin pT558 and then stripped and re-probed with anti-GST. Results indicate that unlike 0N4R tau, LRRKtide is capable of being phosphorylated by GS LRRK2 under conditions that use Mn^{2+} as the catalytic ion. (B) Western blot analyses of *in vitro* G2019S LRRK2-Tau kinase reactions. All reactions contain either 10 mM Mg^{2+} or 10 mM Mn^{2+} , as indicated above each lane. Reactions were run in triplicate but only one representative set is shown here. Each phospho-antibody/E1 probe set represents a separate western blot that was first probed with a phospho-antibody as indicated and then stripped and re-probed with E1. (C) Densitometric quantification of western blots shown in B. The signal of each phospho-antibody was normalized to the E1 probe for that blot. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ [one-way ANOVA with post hoc Bonferroni multiple comparison test].

FIGURE 9. Mn^{2+} inhibits Mg^{2+} catalyzed-LRRK2 phosphorylation of tau

(A) Western blot analyses of tau phosphorylation at T149, T153, and T205 of *in vitro* G2019S LRRK2-tau kinase reactions. All reactions contain 10 mM Mg^{2+} but varying concentrations of Mn^{2+} (0–5 mM) as demarcated on the top of each blot. Each phospho-antibody/E1 probe set represents a western blot that was first probed with tau phospho-antibodies and then stripped and re-probed with E1.

Table 1

All antibodies used in experiments

Antibody	Immunogen	Vendor, cat #, RRID, host, clonality	Concentration
MCA-4F10 (tau pT149)	DGKpTKIATPRGAAC	EnCor Biotech., MCA-4F10, AB_2492292, mouse, monoclonal	1:1000
anti-tau T153	DGKTKIApTPRGAAC	Genscript, N/A, AB_2492294, rabbit, polyclonal	1:1000
anti-tau pT205	Synthetic peptide containing tau pT205	Abcam, ab4841, AB_304676, rabbit, polyclonal	1:1000
E1 (human tau aa 19–33)	Synthetic peptide representative of human tau aa 19–33	Lab of Leonard Petrucelli, N/A, AB_2492293, rabbit, polyclonal	1:80000
PHF1 (tau pS396/pS404)	Detergent-extracted human PHF tau	Lab of Peter Davies, N/A, AB_2315150, mouse, monoclonal	1:3000
AT8 (tau pS199/pS202/pT205)	Partially purified human PHF-tau	Life Tech., MN1020, AB_223647, mouse, monoclonal	1:3000
AT270 (tau pT181)	Partially purified human PHF-tau	Life Tech., MN1050, AB_223651, mouse, monoclonal	1:2000
Anti-GST	Schistosomal GST	GE healthcare, 27-4577-01, AB_771432, goat, polyclonal	1:1000
Anti-pT567 Ezrin/pT564 Radixin/pT558 Moesin	Synthetic peptide containing Ezrin T567	Cell Signaling, 3141, AB_2100313, rabbit, polyclonal	1:1000
Beta tubulin MCA-4E4	Tubulin purified from pig brain	EnCor Biotech., MCA-4E4, AB_2492290, mouse, monoclonal	1:5000