RESEARCH ARTICLE

Leucine-Rich Repeat Kinase 2 and Alternative Splicing in Parkinson's Disease

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ABSTRACT: Mutations of the leucine-rich repeat kinase 2 (*LRRK2*) gene are the most common genetic cause of Parkinson's disease (PD) and are associated with pleiomorphic neuropathology. We hypothesize that *LRRK2* mediates its pathogenic effect through alternative splicing of neurodegeneration genes. Methods used in this study included western blotting analysis of subcellular protein fractions, exon-array analysis of RNA from cultured neuroblastoma cells transfected with *LRRK2* expression vectors, and reverse-transcription polymerase chain reaction (RT-PCR) of RNA from cultured cells and postmortem tissue. Overexpression of the *LRRK2* G2019S mutant resulted in a significant (2.6-fold; $P = 0.020$) decrease in nuclear transactive response DNA-binding protein 43 levels. Exon-array analyses revealed that wild-type *LRRK2* had a significant effect on the expression of genes with nuclear ($P < 10^{-22}$) and cell-cycle functions ($P < 10^{-15}$). We replicated changes in gene expression in 30% of selected genes by quantitative RT-PCR. Overexpression of *LRRK2* resulted in the altered splicing of two genes associated with PD, with an increased inclusion of exon 10 of microtubule-associated protein tau (1.7-fold; $P = 0.001$) and exon 5 of the alpha-synuclein (*SNCA*) gene (1.6-fold; $P = 0.005$). Moreover, overexpression of *LRRK2* (G2019S) and two mutant genes associated with neurodegeneration, *TARDBP* (M337V) and *FUS* (R521H), were associated with decreased inclusion out of the dystonin (*DST*) 1e precursor exons in SK-N-MC cells. Altered splicing of *SNCA* (1.9-fold; $P < 0.001$) and *DST* genes ($\log_2$ 2.3-fold; $P = 0.005$) was observed in a cohort of PD, compared with neurologically healthy, brains. This suggests that aberrant RNA metabolism is an important contributor to idiopathic PD. © 2012 *Movement* Disorder Society

Key Words: Parkinson's disease; leucine-rich repeat kinase 2; microarray; alternative splicing; dystonin; alpha-synuclein; microtubule-associated protein tau.

Additional Supporting Information may be found in the online version of this article.

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PD (OMIM 168600) is the second-most common progressive neurodegenerative disorder, affecting approximately 2% of the population at 65 years of age.[1] The disease is characterized clinically by a combination of motor symptoms, including tremor, bradykinesia, and rigidity.[1] Neuropathologically, the disease is characterized by severe loss of dopaminergic neurons in the substantia nigra and cytoplasmic inclusions comprised mainly of α-synuclein, known as Lewy bodies.[2] The leucine-rich repeat kinase (*LRRK2*) gene codes for a large 2,527-amino-acid, guanosine triphosphatase/kinase protein[3] that has multiple physiological roles.[4–6] *LRRK2* mutations cause ~7% of familial PD cases,[7] and the most common mutation is a glycine for serine amino-acid substitution at codon position 2019 (G2019S).[8] Though most patients with *LRRK2* mutations have typical Lewy body pathology, some cases have substantia nigra degeneration alone (one with transactive response DNA-binding protein 43

[TDP-43] immunopositive inclusions), whereas others have tau-immunopositive inclusions consistent with frontotemporal lobar degenerations (FTLDs).[9] These observations suggest that *LRRK2* serves as an upstream central integrator of multiple cell-signaling pathways.[10]

A significant proportion of the human genome is alternatively spliced, a process that allows individual genes to express multiple protein isoforms with diverse functions.[11] There is evidence that the neurodegenerative process involves the disruption of mechanisms regulating messenger RNA metabolism and alternative splicing. First, mutations have been identified in the TDP-43 (*TARDBP*) and fused in sarcoma (*FUS*) genes in familial forms of motor neuron disease.[12] These genes code for heterogeneous nuclear ribonucleoprotein (hnRNP), which is implicated in exon splicing and transcription regulation.[12] Moreover, a link between environmental insults and alternative splicing has been identified. The treatment of neuronal cells with paraquat, a pesticide to generate models of PD, has been shown to decrease the exon inclusion of a number of genes.[13] Though many gene-array studies on the pathogenic role of *LRRK2* have focused on changes in overall gene expression, we present data from exon-array analysis from cells overexpressing LRRK2 wild-type (WT) or G2019S mutant protein that demonstrate the effects of *LRRK2* on alternative splicing as well as the association of the G2019S mutation with altered splicing of key neurodegenerative genes.

Materials and Methods

Expression Constructs

Expression constructs with WT and G2019S mutant *LRRK2* complementary DNAs (cDNAs), the *TARDPB* (M337V) cDNA, and *FUS* (R521H) cDNA were made as previously reported.[14–16]

Determination of TDP-43 Levels and Subcellular Localization

Subcellular levels of TDP-43 protein were determined by western blotting, as previously reported.[17]

Lipid Raft Assay

Lipid raft and nonraft fractions were prepared from cells according to their solubility in detergent, as described previously.[18]

Exon-Array Analyses

Recombinant vectors were transfected into the human neuroblastoma line, SK-N-MC, and left for 48 hours before total RNA extraction. Microarray analysis, using the human GeneChip Exon 1.0 ST Arrays (Affymetrix, Santa Clara, California, USA), was performed by the Ramaciotti Center for Gene Function Analyses (University of New South Wales, Sydney, New South Wales, Australia).

SYBR Semiquantitative Real-Time Reverse-Transcription Polymerase Chain Reaction Validation of Gene Expression

Polymerase chain reaction (PCR) primers for the validation of each candidate gene were designed to span exon/exon boundaries to minimize the effects of genomic DNA contamination. The complete set of primers is listed in Supporting Table 1.

RT-PCR Validation of Alternative Splicing

PCR primers for each candidate gene were designed to flank the alternatively spliced exon. The complete set of primers is listed in Supporting Table 2.

PD and Neuropathologically Healthy Control Autopsy Cases for Comparison

Brain tissue (i.e., amygdala) from 10 nondemented PD cases (7 males and 3 females; age, 79 $\pm$ 2.7 years; postmortem delay, 9 $\pm$ 2.9 hours) and 8 controls (4 males and 4 females; age, 86 $\pm$ 1.4 years; postmortem delay, 11 $\pm$ 1.8 hours) was obtained with the approval from the Sydney Brain Bank and New South Wales Tissue Resource Center, which are part of the Australian Brain Bank Network of the National Health and Medical Research Council (NHMRC) of Australia.

Statistical Analysis

Mean differences in quantitative measures were compared with the use of the two-tailed Student's *t* test. Differences in proportions were determined by chi-square statistics. *P* values from validation experiments were corrected using the false-discovery-rate algorithm.[19] Mean and standard error of the mean (SEM) are given for all variables. Regression analyses were used to examine the relationship between alternative splicing levels, sex, age, and postmortem delay.

Results

Overexpression of WT and Mutant LRRK2 Affect Subcellular Levels of TDP-43

A feature of the neuropathology associated with several *LRRK2* mutations is cytoplasmic TDP-43-immunopositive inclusions.[9] This led us to examine whether LRRK2 has a role in regulating the subcellular localization of TDP-43. Transfection of *LRRK2* cDNA expression constructs resulted in overexpression of the LRRK2 protein by approximately 12-fold (as determined by real-time reverse-transcription [RT]-PCR) in

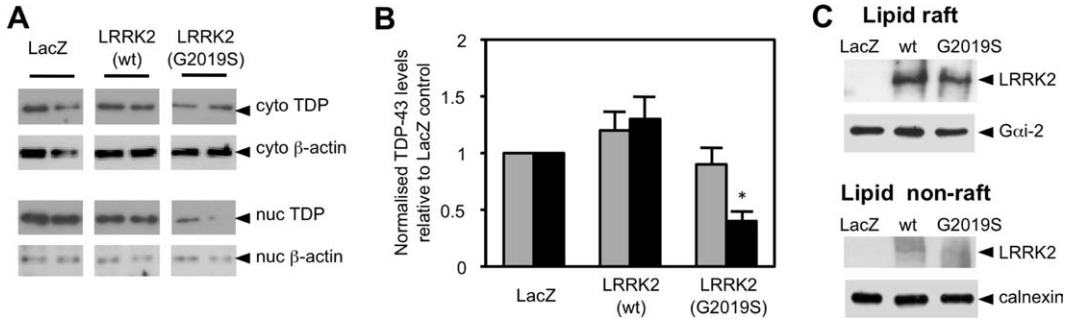

FIG. 1. Modulation of *LRRK2* expression by transfection of expression constructs with the full-length WT or G2019S mutant *LRRK2* cDNA led to altered TDP-43 levels. (A) Western blotting analysis shows endogenous TDP-43 levels in the cytoplasmic (cyto-) and nuclear (nuc-) fractions of transfected cells overexpressing *LRRK2*, compared to LacZ. The house-keeping β-actin protein was used to normalize protein levels. (B) Chemiluminescent band intensities were quantified, and the levels of cytoplasmic (grey columns) and nuclear (black columns) TDP-43 proteins are presented relative to the LacZ control transfection. Mean and SEM from five transfections. Significance of $P < 0.05$ is indicated (*). (C) Western blotting analysis of lipids from raft and nonraft fractions show that both the WT and G2019S LRRK2 protein did not differ markedly in their distribution between raft and nonraft fractions. Gαi-2 (raft specific) and calnexin (nonraft specific) were used to normalize protein levels.

transfected SK-N-MC cells, relative to cells transfected with the LacZ construct (data not shown). Cells transfected with *LRRK2* G2019S mutant constructs resulted in a significant (2.6-fold; $P = 0.020$) decrease in nuclear TDP-43 levels, compared to LacZ-transfected cells (Fig. 1A,B). However, no significant changes were observed for TDP-43 in cytoplasmic fractions (Fig. 1A,B). Cells transfected with WT *LRRK2* construct did not result in significant changes of TDP-43 in the cytoplasmic or nuclear fractions (Fig. 1A,B), relative to the LacZ control.

The LRRK2 protein has been previously shown to localize to lipid rafts, which are glycolipoprotein microdomains within cellular membranes and are thought to mediate a number of processes, including protein trafficking.[5] We examined whether the WT and G2019S mutant LRRK2 proteins colocalize to lipid rafts by western blotting after the extraction of lipid raft and nonraft components (Supporting Materials; Fig. 1C). We confirmed that both WT and mutant proteins localize mainly to the lipid raft fractions, as previously reported.[5] However, we did not observe any difference in the ability of the G2019S mutant to alter its affinity with lipid rafts after we normalized protein levels using the Gαi-2 lipid-raft–specific marker and the calnexin lipid non-raft-specific marker[18] (Fig. 1C), suggesting that the alterations in nuclear TDP-43 levels were not mediated by a change in lipid raft localization of the mutant protein.

Overexpression of WT and Mutant LRRK2 Resulted in Significant Overlap in Number of Differentially Expressed Genes

We compiled a list of genes that are differentially regulated by the overexpression of either WT or G2019S mutant *LRRK2* cDNAs, compared to LacZ control (Supporting Data 1 in Supporting Materials). We observed that the overexpression of WT *LRRK2* affected the regulation of 20-fold more genes than mutant LRRK2, despite similar levels of transgene expression. Biological pathway analyses[20] showed that the WT *LRRK2* gene list showed a significant enrichment of genes encoding nuclear proteins ($P < 2 \times 10^{-22}$) and genes involved in cell cycle ($P < 6 \times 10^{-16}$) and cell division ($P < 2 \times 10^{-15}$). Conversely, no significant enrichment of any biological pathways was observed for the *LRRK2* mutant gene list. We chose nine target genes for validation by real-time RT-PCR (Table 1). These were genes that were specific to the WT *LRRK2* gene list (e.g., *CHORDC1*, *CHMP2B*, *TARDBP*, *BRCA1*, *MAPK8*, *DICER1*, and *TMEM100*), which are genes known to be involved in neurodegeneration (e.g., *TARDBP*[12] and *CHMP2B*[21]), and two genes whose expression were discordantly regulated by the WT and mutant *LRRK2* (e.g., *NDUFA4* and *CD1C*). We were able to significantly replicate three genes (*TARDBP*, transmembrane protein 100 [*TMEM100*], and *DICER1*) in which the changes in direction of gene expression were also consistent with the microarray data (Table 1). Of interest, we were able to validate a significant effect of WT *LRRK2* ($\log_2$ 3.2-fold; $P = 0.005$) on the expression of *DICER1*, a key regulator of microRNA biogenesis.[22]

Overexpression of Mutant LRRK2 Is Associated With Altered Splicing of Two Major PD Genes

We also compiled a list of genes whose exons were differentially spliced by the overexpression of WT or mutant *LRRK2* (Supporting Data 2 in Supporting Materials) cDNAs. We observed that WT *LRRK2* overexpression resulted in a 2-fold increase in the number of differentially spliced genes, compared to the mutant protein. Moreover, there was a significant increase in the proportion of genes that were differently spliced versus those that were differentially expressed in cells transfected with the G2019S mutant (760:80), compared to WT *LRRK2* (1,678:1,678) (Pearson's chi square = 452; $P < 0.0001$) (Fig. 2A). Biological pathway analyses did not reveal any

TABLE 1. Validation by real-time RT-PCR of candidate genes associated with LRRK2 overexpression

Gene	Biological Process*	*LRRK2* (WT)		*LRRK2* (G2019S)	
		Log_2 Fold Change (Microarray *P* Value)	Log_2 Fold Change (RT-PCR-Corrected *P* Value)	Log_2 Fold Change (Microarray *P* Value)	Log_2 Fold Change (RT-PCR-Corrected *P* Value)
CHORDC1	Chaperone-mediated protein folding	2.2 (0.006)[a]	1.6 (0.529)	1.4 (0.218)	3.9 (0.220)
CHMP2B	Cellular membrane organization	1.4 (0.023)[a]	2.7 (0.335)	1.2 (0.251)	2.5 (0.465)
TARDBP	Transcription regulation, mRNA processing	1.3 (0.014)[a]	2.9 (0.040)[a]	1.1 (0.168)	3.0 (0.073)
BRCA1	Cell cycle, DNA repair	1.3 (0.015)[a]	2.8 (0.080)	1.1 (0.224)	1.2 (0.465)
MAPK8	Histone deacetylase regulator activity, regulation of protein localization	1.2 (0.003)[a]	2.0 (0.324)	1.1 (0.241)	0.5 (0.888)
TMEM100	Integral to membrane	1.2 (0.042)[a]	3.8 (0.005)[a]	1.0 (0.792)	2.9 (0.073)
DICER	RNA-mediated gene silencing	1.2 (0.006)[a]	3.2 (0.005)[a]	1.1 (0.047)[a]	2.2 (0.073)
NDUFA4	Mitochondrial electron transport, NADH to ubiquinone	1.2 (0.115)	2.6 (0.064)	−1.4 (0.038)[a]	2.1 (0.090)
CD1C	Antigen processing and presentation	−1.1 (0.115)	0.4 (0.675)	1.1 (0.048)[a]	0.2 (0.888)

*Terms derived from gene ontology databases.[20]
[a]Significance at $P < 0.05$.
Abbreviation: NADH, nicotinamide adenine dinucleotide.

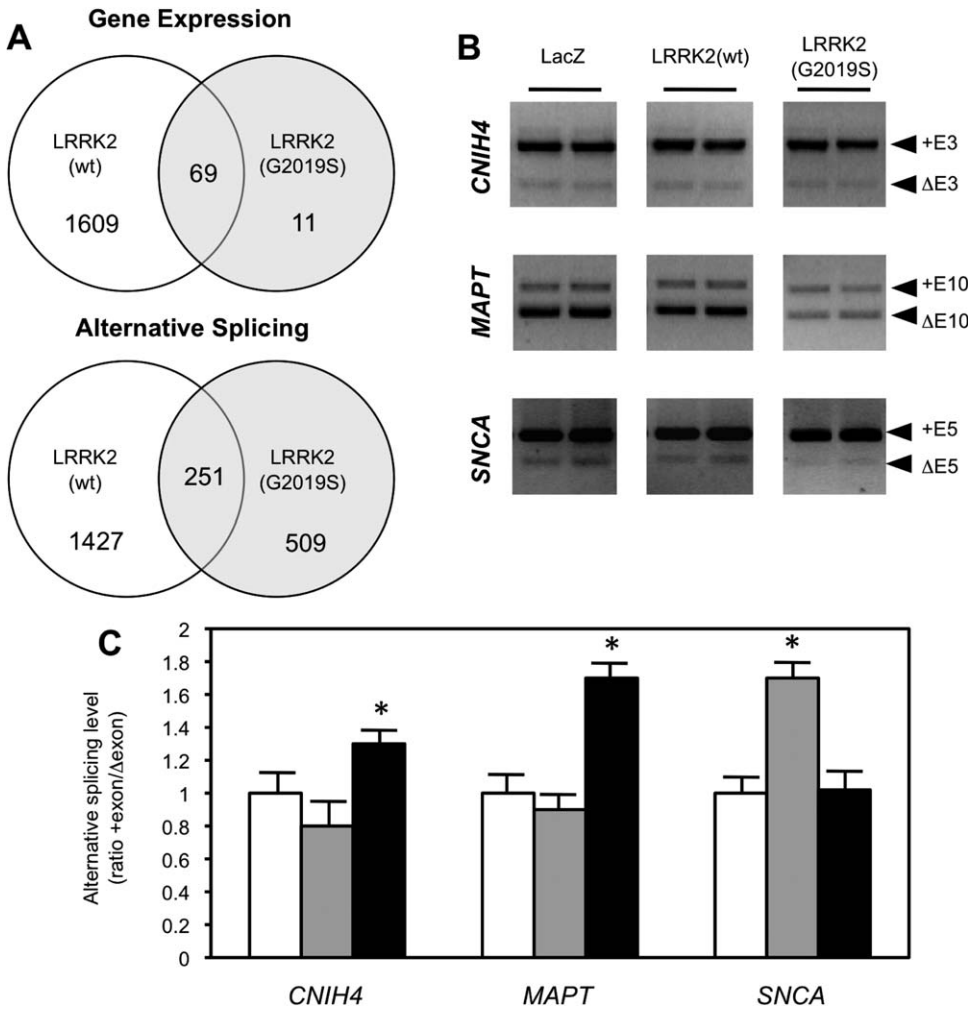

FIG. 2. Comparison between the effects of WT and G2019S mutant *LRRK2* overexpression on gene expression and alternative splicing in transfected cells. (A) Venn diagrams indicate marked overlap in the list of genes whose expressions were differentially regulated by the WT and mutant LRRK2. Little overlap was observed in the list of genes whose exons are differentially spliced in cells overexpressing the two *LRRK* cDNAs. (B) Agarose gel electrophoresis of RT-PCR products showing differential alternative splicing of endogeneous *CNIH4*, *MAPT*, and *SNCA* transcripts isolated from transfected cells. (C) Quantification of ethidium-bromide–stained RT-PCR bands for cells transfected with LacZ (white columns), LRRK2 (WT) (gray columns), and LRRK2 (G2019S) (black columns) cDNAs. Mean and SEM from five transfections. Significance of corrected $P < 0.05$ (*) is indicated.

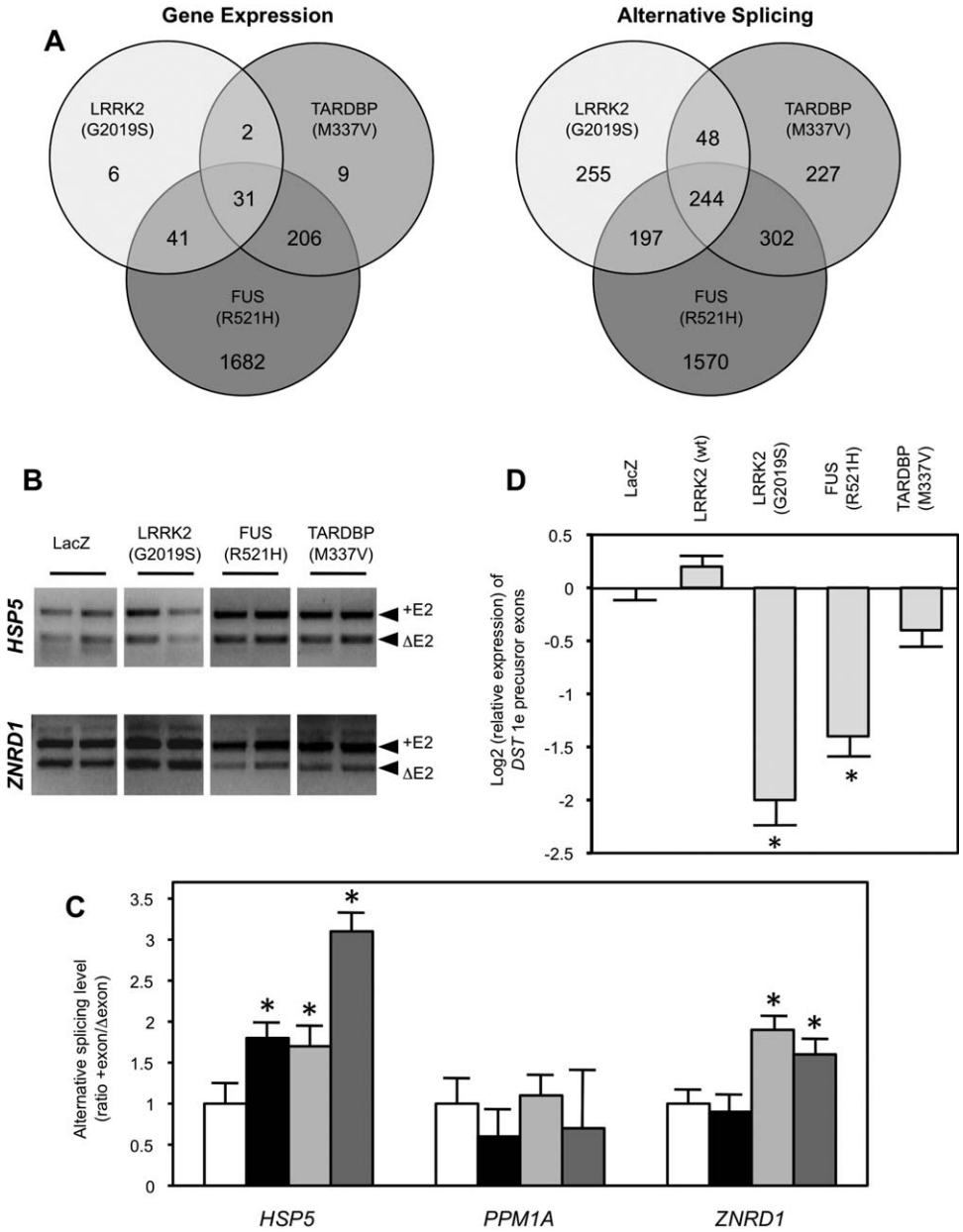

FIG. 3. Comparison between the mutant forms of three neurodegenerative genes on gene expression and alternative splicing in transfected cells. (A) Venn diagrams indicate marked overlap in genes whose expressions were differentially regulated by the mutant *LRRK2*, *TARDBP*, and *FUS*. (B) Agarose gel electrophoresis of RT-PCR products showing differential alternative splicing of endogeneous *HSP5* and *ZNRD1* transcripts isolated from transfected cells. (C) Quantification of ethidium-stained RT-PCR bands for cells transfected with LacZ (white columns), LRRK2 (G2019S) (black columns), FUS (R521H) (light gray columns), and TARDBP (M337V) (dark gray columns) cDNAs. (D) Quantification of *DST* alternatively spliced exon by quantitative real-time RT-PCR. Mean and SEM from n = 5 transfections. Significance of corrected $P < 0.05$ (*) is indicated.

significant enrichment for the WT or mutant *LRRK2* gene lists. Two genes were chosen from the exon list for validation, with an emphasis placed on a top hit (e.g., *CNIH4)* and gene involved in the regulation of cell death (*FAM188A*).[23] Genes were validated by RT-PCR, followed by gel electrophoresis, to visualize products corresponding to the inclusion or exclusion of the candidate exon. This allowed us to measure both splice isoforms from each sample as a single reaction and to normalize for differences in sample concentration. Cells transfected with *LRRK2* G2019S mutant cDNA resulted in a significant (1.3-fold; $P = 0.007$) increase in the inclusion of exon 3 of the cornichon homolog 4 (*CNIH4)* gene (Fig. 2B,C). No significant effects were observed for the WT *LRRK2* cDNA (Fig. 2B,C) or *FAM188A* gene (data not shown).

We then proceeded to examine previously validated alternatively spliced exons from two major PD genes: the microtubule-associated protein tau (*MAPT*) and alpha-synuclein (*SNCA*) genes.[24] Similar to the *CNIH4* gene, cells transfected with *LRRK2* G2019S mutant cDNA resulted in a significant (1.7-fold; $P = 0.001$) increase in splicing of exon 10 of *MAPT*, but had no effect on the inclusion of exon 5 of the *SNCA*

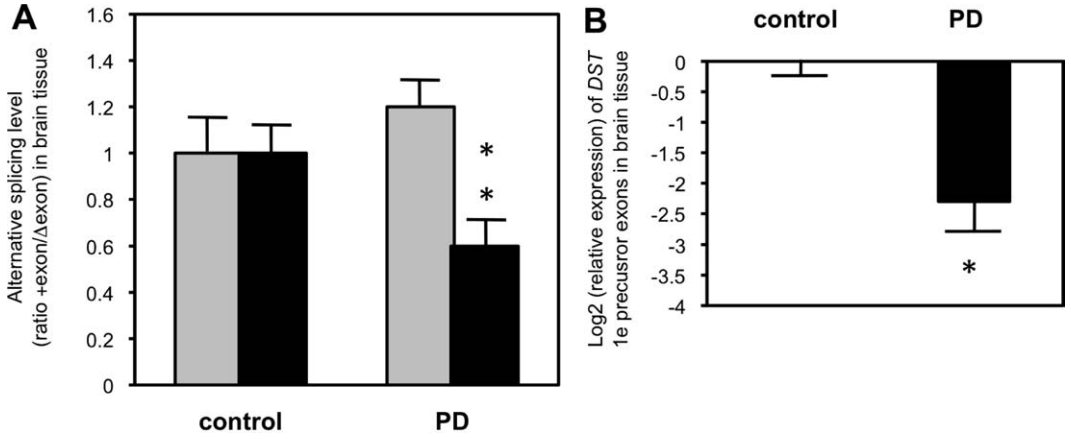

FIG. 4. Examination of alternative splicing in affected brain samples from patients with PD, compared to neurologically healthy controls. (A) Quantification of ethidium-stained bands for *MAPT* (gray columns) and *SNCA* (black columns) RT-PCR products. (B) Quantification of *DST* alternatively spliced exon by quantitative real-time RT-PCR. Mean and SEM from n = 10 PD and n = 8 control samples. Significance of corrected $P < 0.05$ (*) and $P < 0.001$ (**) are indicated.

gene (Fig. 2B,C). In contrast, cells that overexpressed WT *LRRK2* significantly increased (1.6-fold; $P = 0.005$) inclusion of exon 5 of *SNCA*, but had no effect on the alternative splicing of *MAPT* (Fig. 2B,C).

Dysregulated Alternative Splicing Is a Common Effect in PD Brains

TARDBP and *FUS* genes are two major neurodegenerative genes that code for hnRNPs involved in the gene expression and RNA metabolism.[12] Our western blotting data suggest that *LRRK2* might act to control the subcellular levels of TDP-43 (Fig. 1) and thus may act, in part, through the same pathogenic pathway on gene expression and RNA metabolism. To identify common effects of the *LRRK2* (G2019S), *TARDBP* (M337), and *FUS* (R521H) mutants, we compiled a list of differentially expressed genes (Supporting Data 1 in Supporting Materials) and spliced exons (Supporting Data 2 in Supporting Materials) in SK-N-MC cells overexpressing these mutant cDNAs. Thirty-one genes appear to be consistently affected by the overexpression of mutant *LRRK2*, *TARDBP*, or *FUS* (Fig. 3A). Biological pathway analysis did not reveal any significant enrichment of pathways from this gene list. Eight genes (*ASPM*, *DPY19L4*, *MPHOSPH10*, *NLGN1*, *PRKaR2B*, *RAB1A*, *SMAD2*, and *VANGL1*) were chosen from this list for validation by real-time RT-PCR, with an emphasis on genes with neuronal functions (*NLGN1*[25] and *VANGL1*[26]) or known interactions with LRRK2 protein (*RAB1A*[27]). None of the changes in gene expression were successfully replicated with samples from all three mutant transfection groups.

Of the exons identified as significantly differentially spliced (Supporting Data 2 in Supporting Materials), 244 exons were common to all three mutant gene lists (Fig. 3A). This represented an enrichment of exons encoded by cytoplasmic proteins ($P = 0.010$).[20] Four exons from the list (*DST*, *HSP5*, *PPM1A*, and *ZNRD1*) were chosen for validation based on top hits (*HSP5*, *PPM1A*, and *ZNRD1*) and a gene involved in neurodegeneration (dystonin; *DST*).[28] Overexpression of all three mutant genes in SK-N-MC cells resulted in an increased inclusion of exon 2 of the Hermansky-Pudlak syndrome 5 (*HSP5*) gene (1.7- to 3-fold; $P = 0.024$ to $P = 0.005$), compared to the LacZ control (Fig. 3B,C). A similar effect was observed for the zinc ribbon domain-containing 1 (*ZNRD1*) gene, in which increased inclusion of exon 2 was observed for cells overexpressing the FUS (R521H) (1.9-fold; $P = 0.001$) and TARDBP (M337V) (1.6-fold; $P = 0.010$) mutants (Fig. 3B,C), compared to LacZ control.

Several Affymetrix exon probes for the *DST* gene were identified as being significantly affected by the overexpression of the three mutant genes (Supporting Data 2 in Supporting Materials), consistent with the observation that *DST* undergoes complex alternative splicing to generate multiple protein isoforms.[29] The probe, 2958477, corresponds to the inclusion of exons coding for the *DST* 1e precursor (RefSeq NM_001723). Overexpression of all three mutants, but not WT LRRK2, decreased the inclusion of the *DST* 1e precursor-specific exons, compared to lacZ control, with significance reached for LRRK2 (G2019S) ($\log_2$ 2-fold; $P = 0.001$) and FUS (R521H) ($\log_2$ 1.3-fold; $P = 0.005$) mutants (Fig. 3D).

We proceeded to determine whether the aberrant splicing of *DST*, *MAPT*, and *SNCA* in our cellular model was also observed in postmortem brain tissue of neuropathologically defined PD cases (Supporting Materials; Fig. 4). We demonstrated that alternative splicing of *SNCA* was significantly altered in PD cases, with a 1.9-fold ($P < 0.001$) decrease in the inclusion of exon 5 of the *SNCA* gene, compared to neurologically healthy brains (Fig. 4A). There was also a significant ($\log_2$ 2.3-fold; $P = 0.005$) decrease in the inclusion of the *DST* 1e precursor-specific exons in PD, compared to neurologically healthy brains (Fig. 4B). No significant

changes in alternative splicing of *MAPT* were observed in brain tissue (Fig. 4A). Sex, age, and postmortem delay were not significant predictors of alternative splicing in *MAPT*, *SNCA*, or *DST* (data not shown; $P > 0.05$).

Discussion

The *LRRK2* gene has emerged as one of the most prevalent genetic causes of PD.[7] This is the first study to demonstrate that the *LRRK2* gene may have a role in alternative splicing of at least two major PD genes: *MAPT* and *SNCA*. Dysfunction in the alternative splicing of *MAPT* has been shown in genetic forms of FTLD to alter the relative ratios of key splice isoforms crucial for axonal integrity.[30] Similarly, a splice isoform of the *SNCA* gene lacking exon 5 (Δexon5) had been shown to inhibit proteasomal function and preferentially form aggregates *in vitro*.[31] In our cellular model, we demonstrated that the overexpression of mutant *LRRK2* caused a similar change in *MAPT* splicing to FTLD. The lack of aberrant splicing of the *MAPT* gene in our brain cohort (Fig. 4A) was expected (the cases did not have frontotemporal dementia), and suggests that the gene association between PD and *MAPT* is more the result of gene expression[32] than alternative splicing. Our cellular model also revealed that overexpression of WT *LRRK2* increased the alternatively spliced, aggregate-forming *SNCA* Δexon5 isoform, providing a mechanism linking LRRK2 to alpha-synuclein neuropathology. The significant increase in the *SNCA* Δexon5 isoform in our PD brain tissue (Fig. 4A) is consistent with this mechanism and confirms previous studies.[31]

hnRNP proteins, such as TDP-43 and FUS, can interact with the basal splicing protein complex[11] and affect alternative splicing of genes. These proteins can be regulated by growth-factor–dependent kinase phosphorylation.[19] Subcellular fractionation of cells overexpressing *LRRK2* demonstrated that the LRRK2 protein was predominantly found in cytoplasmic and lipid raft fractions (Fig. 1C), indicating that LRRK2 does not act directly on the basal splicing protein complex within the nucleus, but may act indirectly by phosphorylating hnRNPs. It is also unclear whether the G2019S mutant has an altered enzymatic activity or changed ability to bind to these factors, compared to the WT molecule.

Dystonin belongs to the Plakins family of proteins, which function to link cellular cytoskeletal elements with each other and connect them to junctional complexes.[28] Our exon-array data suggest that altered splicing of the *DST* locus represents a common mechanism by which mutations in *TARDBP*, *FUS*, and *LRRK2* genes (Fig. 3A,D) may give rise to neurodegeneration (Fig. 3A,D). Consistent with this observation was that TDP-43 was shown to bind directly to *DST* transcripts, and depletion of the TDP-43 molecule resulted in increased splicing out of a *DST* exon.[33] We also demonstrate that there was a significant decrease in the inclusion of the exon encoding the *DST* 1e precursor splice isoform in PD brain tissue. It still remains to be elucidated what the exact biological role of the 1e precursor isoform is and how alterations in the levels of this splice isoform may contribute to PD.

The main limitation of this study was the use of overexpression of the WT and mutant proteins in transfected neuroblastoma cells as an experimental model, which does not represent the normal level of *LRRK2* expression in the brain. Thus, conclusive evidence regarding the effect of *LRRK2* on alternative splicing and, more important, whether the G2019S mutation represents a further gain of function in terms of its ability to affect alternative splicing of key neurodegenerative genes (Fig. 2A) will come from analyses of brain tissue of *LRRK2* G2019S mutation carriers. Of further interest is whether the effect on alternative splicing is specific to the G2019S mutation, because mutations have been shown to alter the function of various domains of the LRRK2 protein and are associated with different clinical symptoms and neuropathologies.[9]

Conclusion

In conclusion, we have demonstrated that *LRRK2* overexpression in a cellular model alters the isoform expression of key neurodegenerative genes, such as *MAPT*, *SNCA*, and *DST*. The latter two genes were also observed to have altered splicing in postmortem PD brains. This suggests that aberrant RNA metabolism is an important contributor to idiopathic PD. ■

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References

1. Bertram L, Tanzi RE. The genetic epidemiology of neurodegenerative disease. J Clin Invest 2005;115:1449–1457.
2. Arima K, Uéda K, Sunohara N, et al. Immunoelectron-microscopic demonstration of NACP/alpha-synuclein-epitopes on the filamentous component of Lewy bodies in Parkinson's disease and in dementia with Lewy bodies. Brain Res 1998;808:93–100.
3. Marín I, van Egmond WN, van Haastert PJ. The Roco protein family: a functional perspective. FASEB J 2008;22:3103–3110.
4. Dihanich S, Manzoni C. LRRK2: a problem lurking in vesicle trafficking? J Neurosci 2011;31:9787–9788.
5. Hatano T, Kubo S, Imai S, et al. Leucine-rich repeat kinase 2 associates with lipid rafts. Hum Mol Genet 2007;16:678–690.
6. Gehrke S, Imai Y, Sokol N, Lu B. Pathogenic LRRK2 negatively regulates microRNA-mediated translational repression. Nature 2010;466:637–641.
7. Cookson MR. The role of leucine-rich repeat kinase 2 (LRRK2) in Parkinson's disease. Nat Rev Neurosci 2010;11:791–797.

8. Correia Guedes L, Ferreira JJ, Rosa MM, Coelho M, Bonifati V, Sampaio C. Worldwide frequency of G2019S LRRK2 mutation in Parkinson's disease: a systematic review. Parkinsonism Relat Disord 2010;16:237–242.
9. Wider C, Dickson DW, Wszolek ZK. Leucine-rich repeat kinase 2 gene-associated disease: redefining genotype-phenotype correlation. Neurodegener Dis 2010;7:175–179.
10. Berwick DC, Harvey K. LRRK2 signaling pathways: the key to unlocking neurodegeneration? Trends Cell Biol 2011;2:257–265.
11. Black DL. Mechanisms of alternative pre-messenger RNA splicing. Annu Rev Biochem 2003;72:291–336.
12. Lagier-Tourenne C, Polymenidou M, Cleveland DW. TDP-43 and FUS/TLS: emerging roles in RNA processing and neurodegeneration. Hum Mol Genet 2010;19:R46–R64.
13. Maracchioni A, Totaro A, Angelini DF, et al. Mitochondrial damage modulates alternative splicing in neuronal cells: implications for neurodegeneration. J Neurochem 2007;100:142–153.
14. Qing H, Wong W, McGeer EG, McGeer PL. Lrrk2 phosphorylates alpha synuclein at serine 129: Parkinson disease implications. Biochem Biophys Res Commun 2009;387:149–152.
15. Sreedharan J, Blair IP, Tripathi VB, et al. TDP-43 mutations in familial and sporadic amyotrophic lateral sclerosis. Science 2008; 319:1668–1672.
16. Vance C, Rogelj B, Hortobágyi T, et al. Mutations in FUS, an RNA processing protein, cause familial amyotrophic lateral sclerosis type 6. Science 2009;323:1208–1211.
17. Luty AA, Kwok JB, Dobson-Stone C, et al. Sigma nonopioid intracellular receptor 1 mutations cause frontotemporal lobar degeneration-motor neuron disease. Ann Neurol 2010;68:639–649.
18. Adam RM, Yang W, Di Vizio D, Mukhopadhyay NK, Steen H. Rapid preparation of nuclei-depleted detergent-resistant membrane fractions suitable for proteomics analysis. BMC Cell Biol 2008;9: 30.
19. Benjamini Y, Hochberg Y. Controlling the false discovery rate: a practical and powerful approach to multiple testing. J Roy Statist Soc Ser B 1995;57:289–300.
20. Antonov AV. BioProfiling.de: analytical web portal for high-throughput cell biology. Nucleic Acids Res 2011;39 (Web Server issue):W323–W327.
21. Urwin H, Ghazi-Noori S, Collinge J, Isaacs A. The role of CHMP2B in frontotemporal dementia. Biochem Soc Trans 2009; 37:208–212.
22. Perron MP, Provost P. Protein components of the microRNA pathway and human diseases. Methods Mol Biol 2009;487:369–385.
23. Liu B, Liu Y, Chen J, et al. CARP is a novel caspase recruitment domain containing pro-apoptotic protein. Biochem Biophys Res Commun 2002;293:1396–1404.
24. Edwards TL, Scott WK, Almonte C, et al. Genome-wide association study confirms SNPs in SNCA and the MAPT region as common risk factors for Parkinson disease. Ann Hum Genet 2010;74: 97–109.
25. Lee H, Dean C, Isacoff E. Alternative splicing of neuroligin regulates the rate of presynaptic differentiation. J Neurosci 2010;30: 11435–11446.
26. Iliescu A, Gravel M, Horth C, Kibar Z, Gros P. Loss of membrane targeting of Vangl proteins causes neural tube defects. Biochemistry 2011;50:795–804.
27. Kicka S, Shen Z, Annesley SJ, Fisher PR, Lee S, Briggs S, Firtel RA. The LRRK2-related Roco kinase Roco2 is regulated by Rab1A and controls the actin cytoskeleton. Mol Biol Cell 2011;22: 2198–2211.
28. Sonnenberg A, Liem RK. Plakins in development and disease. Exp Cell Res 2007;313:2189–2203.
29. Leung CL, Zheng M, Prater SM, Liem RK. The BPAG1 locus: alternative splicing produces multiple isoforms with distinct cytoskeletal linker domains, including predominant isoforms in neurons and muscles. J Cell Biol 2001;154:691–697.
30. D'Souza I, Schellenberg GD. Regulation of tau isoform expression and dementia. Biochim Biophys Acta 2005;1739:104–115.
31. Beyer K. Alpha-synuclein structure, posttranslational modification, and alternative splicing as aggregation enhancers. Acta Neuropathol 2006;112:237–251.
32. Kwok JB, Teber ET, Loy C et al. Tau haplotypes regulate transcription and are associated with Parkinson's disease. Ann Neurol 2004;55:329–334.
33. Polymenidou M, Lagier-Tourenne C, Hutt KR, et al. Long pre-mRNA depletion and RNA missplicing contribute to neuronal vulnerability from loss of TDP-43. Nat Neurosci 2011;14:459–468.