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

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Abbreviations: *ASPM*, asp (abnormal spindle)-like, microcephaly gene; *BRCA1*, breast cancer 1 gene; cDNA, complementary DNA; *CD1C*, CD1C antigen precursor gene; *CHORDC1*, cysteine and histidine-rich domain-containing gene; *CHMP2B*, chromatin modifying protein 2B *gene; CNIH4*, cornichon homolog 4 gene; *DICER1;* dicer 1 gene; *DPY19L4*, dpy-19-like 4 gene; *DST*, Dystonin gene; *FAM188A*, Homo sapiens family with sequence similarity 188, member A gene, FDR, false discovery rate; *FUS*, fused in sarcoma gene; *HSP5*, Hermansky-Pudlak syndrome 5 gene; *LRRK2*, leucine-rich repeat kinase 2 gene; *MAPK8*, mitogen-activated protein kinase 8 isoform gene; *MAPT*, microtubule-associated protein tau gene; *MPHOSPH10*, M-phase phosphoprotein 10 gene; *NLGN1*, neuroligin 1 gene; *NDUFA4*, NADH dehydrogenase (ubiquinone) 1 alpha gene; *PPM1A*, protein phosphatase 1A gene; *PRKaR2B*, cAMP-dependent protein kinase, regulatory gene; *RAB1A*, member RAS oncogene family isoform 1 gene; RT-PCR, reverse transcription-polymerase chain reaction; *SMAD2*, Sma- and Mad-related protein 2 gene; SNCA, alpha-synuclein gene; *TARDBP*, TAR DNA binding protein gene; *TMEM100*, transmembrane protein 100 gene; *VANGL1*, vang-like 1 gene; *ZNRD1*, zinc ribbon domain containing 1 gene.

ABSTRACT

Background: 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 hypothesise that *LRRK2* mediates its pathogenic effect via alternative splicing of neurodegeneration genes.

Methods: Western blot analysis of subcellular protein fractions. Exon-array analysis of RNA from cultured neuroblastoma cells transfected with *LRRK2* expression vectors. Reverse-transcription PCR (RT-PCR) of RNA from cultured cells and post-mortem tissue.

Results: Over-expression of *LRRK2* G2019S mutant resulted in a significant 2.6 fold ($p = 0.020$) decrease in nuclear TDP-43 levels. Exon-array analyses revealed that wildtype *LRRK2* had a significant effect on 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. Over-expression of *LRRK2* resulted in altered splicing of two genes associated with PD, with an increased inclusion of exon 10 of *MAPT* (1.7 fold; $p = 0.001$) and exon 5 of the *SNCA* gene (1.6 fold; $p = 0.005$). Moreover, over-expression of *LRRK2* (G2019S), *TARDBP* (M337V) and *FUS* (R521H) mutants was associated with decreased inclusion out of the *DST* 1e precursor exons in SK-N-MC cells. Altered splicing of *SNCA* (1.9 fold; $p < 0.001$) and *DST* genes (log2 2.3 fold; $p = 0.005$) was observed in a cohort of PD compared with neurologically normal brains.

Conclusions: Our study highlights the importance of aberrant RNA metabolism as a common pathogenic pathway for idiopathic PD, mediated in part by *LRRK2* dysfunction.

INTRODUCTION

Parkinson's disease (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 characterised clinically by a combination of motor symptoms including tremor, bradykinesia and rigidity.[1] Neuropathologically, the disease is characterised 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 2527 amino acid, GTPase/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] While most patients with *LRRK2* mutations have typical Lewy body pathology, some cases have substantia nigra degeneration alone (one with TAR DNA binding protein 43 (TDP-43) immunopositive inclusions), while others have tau immunopositive inclusions consistent with frontotemporal lobar degenerations (FTLD).[9] These observations suggest that *LRRK2* serves as an upstream central integrator of multiple cell signalling 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 mRNA metabolism and alternative splicing. Firstly, 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) implicated in exon splicing and transcription regulation.[19] 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 exon inclusion of a number of genes.[13] Whilst 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 over-expressing LRRK2 wildtype or G2019S mutant protein that demonstrate the effects of *LRRK2* on alternative splicing, and the association of the G2019S mutation with altered splicing of key neurodegenerative genes.

Materials and Methods

Expression constructs

Expression constructs with wildtype 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 Localisation

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

Lipid raft assay

Lipid raft and non-raft 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 prior to total RNA extraction. Microarray analysis using the human GeneChip® Exon 1.0 ST Arrays (Affymetrix) was performed by the Ramaciotti Centre for Gene Function Analyses (University of New South Wales, Australia).

SYBR semi-quantitative realtime RT-PCR validation of gene expression

PCR Primers for the validation of each candidate gene were designed to span exon/exon boundaries to minimise the effects of genomic DNA contamination. The complete set of primers is listed in Supplementary 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 Supplementary Table 2.

PD and neuropathologically normal control autopsy cases for comparison

Brain tissue from 10 non-demented PD cases (7 males, 3 females, age = 79 ± 2.7 years, postmortem delay 9 ± 2.9 hours) and 8 controls (4 males, 4 females, age = 86 ± 1.4 years, postmortem delay 11 ± 1.8 hours) was obtained with approval from the Sydney Brain Bank and New South Wales Tissue Resource Centre, part of the Australian Brain Bank Network of the National Health and Medical Research Council.

Statistics

Mean differences in quantitative measures were compared with the use of 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 are given for all variables. Regression analyses were used to examine the relationship between alternative splicing levels, sex, age and post-mortem delay.

RESULTS

Over-expression of wildtype 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 over-expression of the LRRK2 protein by approximately 12 fold (as determined by realtime RT-PCR) in 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 with LacZ transfected cells (FIG. 1A and B). However, no significant changes were observed for TDP-43 in the cytoplasmic fractions (FIG. 1A and B). Cells transfected with wildtype *LRRK2* construct did not result in significant changes of TDP-43 in the cytoplasmic or nuclear fractions (FIG. 1A and B) relative to the LacZ control.

The LRRK2 protein has been previously shown to localise 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 wildtype and G2019S mutant LRRK2 proteins co-localise to lipid rafts by western blotting following extraction of lipid raft and non-raft components (Supplementary materials and methods, FIG. 1C). We confirmed that both wildtype 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 via a change in lipid raft localization of the mutant protein.

Over-expression of wildtype and mutant LRRK2 resulted in significant overlap in number of differentially expressed genes

We compiled a list of genes that are differentially regulated by over-expression of either wildtype or G2019S mutant *LRRK2* cDNAs compared with LacZ control (Supplementary materials and methods, Supplementary Data 1). We observed that over-expression of the wildtype *LRRK2* affected the regulation of 20 fold more genes than mutant LRRK2, despite similar levels of transgene expression. Biological pathway analyses[28] showed that the wildtype *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 9

target genes for validation by realtime RT-PCR (Table 1). These were genes that were specific to the wildtype *LRRK2* gene list (*CHORDC1*, *CHMP2B*, *TARDBP*, *BRCA1*, *MAPK8*, *DICER1* and *TMEM100*), genes known to be involved in neurodegeneration (*TARDBP*[22] and *CHMP2B*[31]), and two genes whose expression were discordantly regulated by the wildtype and mutant *LRRK2* (*NDUFA4* and *CD1C*). We were able to significantly replicate 3 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 wildtype *LRRK2* (log2 3.2 fold, p = 0.005) on the expression of *DICER1*, a key regulator of microRNA biogenesis.[32]

Over-expression 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 over-expression of wildtype or mutant *LRRK2* (Supplementary materials and methods, Supplementary Data 2) cDNAs. We observed that wildtype *LRRK2* over-expression resulted in a 2-fold increase in the number of differentially spliced genes compared with 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 with wildtype *LRRK2* (1678: 1678) (Pearson's Chi square = 452, p < 0.0001) (FIG. 2A). Biological pathway analyses did not reveal any significant enrichment for the wildtype or mutant *LRRK2* gene lists. Two genes were chosen from the exon list for validation with an emphasis placed on a top hit (*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 allows us to measure both splice isoforms from each sample as a single reaction and to normalise for differences in sample concentration. Cells transfected with *LRRK2* G2019S mutant cDNA resulted in a significant 1.3 fold (p = 0.007) increase in inclusion of exon 3

of the cornichon homolog 4 (*CNIH4)* gene (FIG.2B and C). No significant effects were observed for the wildtype *LRRK2* cDNA (FIG.2B and 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 in of exon 10 of *MAPT*, but had no effect on the inclusion of exon 5 of the *SNCA* gene (FIG. 2B and C). In contrast, cells which over-expressed wildtype *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 and 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 gene expression and RNA metabolism.[12] Our western blot data suggests that *LRRK2* might act to control subcellular levels of TDP-43 (FIG. 1), and thus may act in part via the same pathogenic pathway on gene expression and RNA metabolism. In order to identify common effects of the *LRRK2* (G2019S), *TARDBP* (M337) and *FUS* (R521H) mutants, we compiled a list of differentially expressed genes (Supplementary Data 1) and spliced exons (Supplementary Data 2) in SK-N-MC cells over-expressing these mutant cDNAs. Thirty-one genes appear to be consistently affected by the over-expression 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, VANGL1*) were chosen from this list for validation by realtime RT-PCR with an emphasis on genes with neuronal functions (*NLGN1*[25], *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 (Supplementary Data 2), 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*, *ZNRD1*) were chosen for validation based on top hits (*HSP5*, *PPM1A* and *ZNRD1*) and a gene involved in neurodegeneration (*DST*).[28] Over-expression of all three mutant genes in SK-N-MC cells resulted in 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 and C). A similar effect was seen for the zinc ribbon domain containing 1 (*ZNRD1*) gene, in which increased inclusion of exon 2 was observed for cells over-expressing the FUS(R521H) (1.9 fold; $p = 0.001$) and TARDBP(M337V) (1.6 fold; $p = 0.010$) mutants (FIG. 3B and C) compared to LacZ control.

Several Affymetrix exon probes for Dystonin (*DST)* gene were identified as being significantly affected by over-expression of the three mutant genes (Supplementary Data 2), 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). Over-expression of all three mutants, but not wildtype LRRK2, decreased the inclusion of the *DST* 1e precursor specific exons compared with lacZ control, with significance reached for LRRK2(G2019S) (log2 2 fold; $p = 0.001$) and FUS(R521H) (log2 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 post-mortem brain tissue of neuropathologically defined PD cases (Supplementary materials and methods, 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 inclusion of exon 5 of the *SNCA* gene, compared with neurologically normal brains (FIG. 4A). There was also a significant log2 2.3 fold ($p = 0.005$) decrease in inclusion of the *DST* 1e precursor specific exons in

PD compared with neurologically normal brains (FIG. 4B). No significant changes in alternative splicing of *MAPT* were observed in brain tissue (FIG. 4A). Sex, age and post mortem 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 frontotemporal dementia 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 cell systems we demonstrate that the over-expression of mutant *LRRK2* caused a similar change in *MAPT* splicing to frontotemporal dementia. 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 due more to gene expression[32] than alternative splicing. Our cell systems also revealed that overexpression of wildtype LRRK2 increased the alternatively spliced, aggregate-forming *SNCA* Δexon5 isoform, providing a mechanism linking LRRK2 to α-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]

Proteins that interact with the basal splicing protein complex[11] affect alternative splicing of genes and are regulated by growth factor–dependent kinase phosphorylation.[19] Such proteins include the hnRNPs FUS and TDP-43. Subcellular fractionation of cells over-expressing *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, but may act indirectly by phosphorylating hnRNPs.

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 and D) may give rise to neurodegeneration (FIG. 3A and 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 inclusion of the exon encoding the *DST* 1e precursor splice isoform in PD brain tissue. It still remains to be elucidated the exact biological role of the 1e precursor isoform and how alterations in the levels of this splice isoform may contribute to PD. The main limitation of this study is the use of over-expression of the wildtype 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 importantly 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 as 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] Finally, while we have demonstrated that cellular *LRRK2* overexpression changes the isoform expression of *MAPT*, *SNCA* and *DST* genes, only the latter two genes were observed to have altered splicing in post-mortem PD brains. This suggests to us the importance of aberrant RNA metabolism as a common pathogenic pathway in idiopathic PD that may in part be regulated by *LRRK2* dysfunction.

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Authors' contributions

JBJK conceived this entire study, with GMH organising the human tissue study (tissue collection and diagnosis, ethics and tissue request). GMH is CI on the research programs that longitudinally follow brain donors with Parkinson's disease and she is also director of the Sydney Brain Bank. DE, SK, and SG performed experiments. JBJK, DE and SK participated in the management, analysis, interpretation of data and drafting of manuscript. All authors have critically revised the manuscript for important intellectual content and have seen and approved the final version.

Full Financial Disclosures of all Authors for the Past Year

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Partnerships - None	Contracts - None
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2) David Elliott

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Table 1: Validation by realtime RT-PCR of candidate genes associated with *LRRK2* over-expression

		LRRK2 (wt)		*LRRK2* (G2019S)	
Gene	Biological Process #	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)*	1.6 (0.529)	1.4 (0.218)	3.9 (0.220)
CHMP2B	cellular membrane organization	1.4 (0.023)*	2.7 (0.335)	1.2 (0.251)	2.5 (0.465)
TARDBP	Transcription regulation, mRNA processing	1.3 (0.014)*	2.9 (0.040)*	1.1 (0.168)	3.0 (0.073)
BRCA1	Cell cycle, DNA repair	1.3 (0.015)*	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)*	2.0 (0.324)	1.1 (0.241)	0.5 (0.888)
TMEM100	integral to membrane	1.2 (0.042)*	3.8 (0.005)*	1.0 (0.792)	2.9 (0.073)
DICER	RNA-mediated gene silencing	1.2 (0.006)*	3.2 (0.005)*	1.1 (0.047)*	2.2 (0.073)
NDUFA4	mitochondrial electron transport, NADH to ubiquinone	1.2 (0.115)	2.6 (0.064)	- 1.4 (0.038)*	2.1 (0.090)
CD1C	antigen processing and presentation	- 1.1 (0.115)	0.4 (0.675)	1.1 (0.048)*	0.2 (0.888)

terms derived from gene ontology databases.[20]

* significance at $p < 0.05$.

Figure Legends

FIG. 1: Modulation of *LRRK2* expression by transfection of expression constructs with the full-length wildtype or G2019S mutant *LRRK2* cDNA led to altered TDP-43 levels. A) Western blot analysis shows endogenous TDP-43 levels in the cytoplasmic (cyto-) and nuclear (nuc-) fractions of transfected cells over-expressing *LRRK2* compared with LacZ. The house keeping β-actin protein was used to normalise 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 standard error of mean from 5 transfections. Significance of $p < 0.05$ is indicated (*). C) Western blot analysis of lipids from raft and non-raft fractions show that both the wildtype and G2019S LRRK2 protein did not differ markedly in their distribution between raft and non-raft fractions. Gαi-2 (raft specific) and calnexin (non-raft specific) were used to normalise protein levels.

FIG. 2: Comparison between the effects of wildtype and G2019S mutant *LRRK2* over-expression 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 wildtype and mutant LRRK2. Little overlap was observed in the list of genes whose exons are differentially spliced in cells over-expressing 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(wildtype) (grey columns) and LRRK2(G2019S) (black columns) cDNAs. Mean and standard error of mean from 5 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 grey columns) and TARDBP(M337V) (dark grey columns) cDNAs. D) Quantification of *DST* alternatively spliced exon by quantitative realtime RT-PCR. Mean and standard error of mean from n = 5 transfections. Significance of corrected $p < 0.05$ (*) is indicated.

FIG. 4: Examination of alternative splicing in affected brain samples from patients with Parkinson's disease compared with neurologically normal controls. A) Quantification of ethidium stained bands for *MAPT* (grey columns) and *SNCA* (black columns) RT-PCR products B) Quantification of *DST* alternatively spliced exon by quantitative realtime RT-PCR. Mean and standard error of mean from n = 10 PD and n = 8 control samples. Significance of corrected $p < 0.05$ (*) and $p < 0.001$ (**) are indicated.