

ORIGINAL ARTICLE

## LRRK2 Expression in Normal and Pathologic Human Brain and in Human Cell Lines

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### Abstract

Mutations in the leucine-rich repeat kinase 2 gene (*LRRK2*) have been recently identified in families with autosomal-dominant late-onset Parkinson disease. We report that by reverse transcriptase–polymerase chain reaction, the mRNA of *LRRK2* is expressed in soluble extracts of human brain, liver, and heart and in cultured human astrocytes, microglia, and oligodendroglia as well as in human neuroblastoma cell lines. We find by Western blotting using a polyclonal antibody of the leucine-rich repeat kinase 2 protein (Lrrk2) specific for C-terminal residues 2511–2527 that an apparent full-length protein and several of its fractions are expressed in soluble extracts of normal human brain. By immunocytochemistry, the antibody recognizes neurons, and more weakly astrocytes and microglia, in normal brain tissue. It intensely labels Lewy bodies in Parkinson disease and related neurodegenerative disorders. It also labels a subset of neurofibrillary tangles in Alzheimer disease and the Parkinsonism dementia complex of Guam (PDCG). It labels thorn-shaped astrocytes and oligodendroglial coiled bodies in PDCG; oligodendroglial inclusions in multiple system atrophy; Pick bodies in Pick disease; nuclear and cytoplasmic inclusions in Huntington disease; and intraneuronal and glial inclusions in amyotrophic lateral sclerosis. In summary, *LRRK2* is constitutively expressed in neurons and also in glial cells of human brain. It strongly associates with pathological inclusions in several neurodegenerative disorders.

**Key Words:** Alzheimer disease, Amyotrophic lateral sclerosis (ALS), Diffuse Lewy body disease, Huntington disease, Lewy body, Parkinson disease, Parkinson dementia complex of Guam, Pick disease.

### INTRODUCTION

Genetic and environmental factors both play an important role in the pathogenesis of Parkinson disease (PD) (1–6). A gene that has recently been found to be involved in PD

causation is leucine-rich repeat kinase 2 (*LRRK2*, *PARK8*) located in the chromosomal region 12q11.2–q13.1 (7–9).

The translated protein, Lrrk2, is made up of 51 exons with a total of 2527 amino acids. Although it is a large and complex protein, it has nevertheless been highly conserved through evolution, indicating important physiological functions. Homologs are found even as far back as prokaryotes. Lrrk2 belongs to the Roco protein family of the Ras/GTPase superfamily (8–10). The sequential structure includes a leucine-rich repeat zone (Lrr), a Ras of complex proteins (Roc), a C-terminal of Roc (Cor) domain, and a microtubule-associated protein kinase kinase kinase (MAPKKK) domain, with termination in beta propeller forming repeats (WD 40). Lrrk2 is thought to function, at least in part, as an active protein kinase (11) with mutations resulting in a gain of function (12).

To date, at least 19 putatively pathogenic amino acid substitutions have been identified in Lrrk2. It can be anticipated that many more will be found in the future. They occur in each of the functional domains. As examples, the mutations R1067Q, S1096C, I1122V, and S1228T occur in the LRR domain; whereas I1371V, R1441C, R1441G, and R1441H occur in the Roc domain; R1514Q, Y1699C, and M1869T occur in the Cor domain; R1941H, I2012T, G2019S, and I2020T occur in the MAPKKK domain; and T2356I and G2358R occur in the WD 40 domain. In addition, R793M and Q930R occur N-terminal to the functional domains. The most prominent of these substitutions is G2019S, which is common in French and North African families as well as Ashkenazi Jews (13). It also occurs in approximately 1% of unselected PD cases, 3% to 6% of familial cases (13, 14), and some normals. Penetrance varies. In one study, penetrance was found to range from 17% at age 50 to 85% at age 70 (15). Most postmortem studies on patients with *LRRK2* mutations have revealed the typical PD findings of nigral cell loss and extensive Lewy body distribution, but some have demonstrated Alzheimer-like neurofibrillary pathology, some multiple system atrophy-type pathology, and some progressive supranuclear palsy-like pathology.

Northern blot analyses of *LRRK2* mRNA expression have detected a 9-kb mRNA transcript in all human tissues tested, including most regions of brain with highest levels being reported in the putamen and substantia nigra (SN) (9). In rodent brain, *in situ* hybridization studies showed *LRRK2* mRNA to be widely expressed in motor and nonmotor areas

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(16, 17) with a preference for dopamine-receptive areas (17, 18) with little or no expression in the SN. Human embryonic kidney cells transfected with human recombinant Lrrk2 showed a diffuse cytoplasmic localization and an association with the outer mitochondrial membrane (9, 11).

The distribution of Lrrk2 in the brain of PD and other neurodegenerative disorders is not known. In the present study, we analyzed for the presence of *LRRK2* mRNA in human astrocytes, oligodendroglia, and microglia; in the human neuronal cell lines SH-SY5Y and LA-N-2 and in whole brain, liver, and heart. Using membrane-bound overlapping peptides covering the full Lrrk2 sequence, we identified the C-terminal binding site of a polyclonal anti-Lrrk2 antibody and performed an immunohistochemical analysis of a series of brains of patients with PD, including one member of a family with autosomal-dominant late-onset parkinsonism (family B) (19) as well as patients with diffuse Lewy body disease (DLBD), Alzheimer disease (AD), amyotrophic lateral sclerosis (ALS), Pick disease, the parkinsonism–dementia complex of Guam (PDCG), and Huntington disease. We find that the *LRRK2* gene is expressed in all cell types and organs studied; that the Lrrk2 protein occurs in the soluble state in normal brain; and that such pathologic entities as Lewy bodies, neurofibrillary tangles, and Pick bodies are recognized by a polyclonal antibody to Lrrk2.

## MATERIALS AND METHODS

### Postmortem Human Tissues

Tissue from brain, heart, and liver from 2 controls (male aged 78 and a female aged 93) were selected from our Kinsmen Laboratory Brain Bank for Western blot analysis. For immunohistochemistry, brain tissues from 5 sporadic PD cases, one familial PD case, one DLBD case, 5 AD cases (one with associated PD), one Pick disease case, 2 ALS cases, 6 PDCG cases, 2 Huntington disease, and 4 control cases, including one from Guam, were selected. In each case, the diagnosis had been confirmed by standard neuropathologic examination. The familial PD case was from family B in which a susceptibility locus on chromosome 2p13 had been identified (16). Details of the cases and a summary of the pathologic findings are given in the Table.

### Cell Lines

The human neuroblastoma SH-SY5Y cell line was a gift from Dr. R. Ross, Fordham University, New York, NY, and the LA-N-2 cells were obtained from Dr. R. C. Seeger, Children's Hospital, Los Angeles, California. The human astrocytic U-373 MG, human monocytic U937, and human monocytic THP-1 cell lines were obtained from the American Type Culture Collection (ATCC). These cells were grown in Dulbecco modified Eagle medium–nutrient mixture F12 Ham (DMEM-F12) supplemented with 10% fetal bovine serum (FBS; GIBCO BRL, Life Technologies, Burlington, ON, Canada) containing gentamicin (50 µg/mL). Incubation was carried out in a humidified 5% CO<sub>2</sub>/95% air atmosphere at 37°C. Retinoic acid-differentiated SH-SY5Y cultures were prepared as previously described (20, 21). THP-1 cells were stimulated for 4 hours and 24 hours by a combination of LPS

TABLE. Cases Studied by Immunohistochemistry

Case	Age/Sex	Diagnosis	Lrrk2					
			LB	NFT	GI	PB	INI	
1	76/F	PD	+	+	–	–	–	
2	81/M	PD	+	–	–	–	–	
3	76/F	PD	+	+	–	–	–	
4	63/M	PD	+	–	–	–	–	
5	82/M	FPD	+	+	–	–	+	
6	87/F	DLBD	+	+	–	–	–	
7	87/F	AD and PD	+	+	–	–	–	
8	78/M	AD	–	+	–	–	–	
9	82/F	AD	–	+	–	–	–	
10	62/F	AD	–	+	–	–	–	
11	84/F	AD	–	+	–	–	–	
12	73/M	Pick	–	–	–	+	–	
13	75/F	ALS	–	+	+	–	–	
14	48/M	ALS	–	–	+	–	–	
15	43/M	Huntington	–	–	–	–	+	
16	76/M	Huntington	–	+	–	–	+	
17	39/M	Control	–	–	–	–	–	
18	43/F	Control	–	–	–	–	–	
19	59/F	Control	–	–	–	–	–	
20	69/M	PDCG	+	+	+	–	+	
21	68/F	PDCG	–	+	+	–	+	
22	67/F	PDCG	+	+	+	–	+	
23	65/M	PDCG	+	+	+	–	–	
24	79/M	PDCG	+	+	+	–	–	
25	72/F	PDCG	+	+	+	–	–	
26	40/M	Guam control	+	(rare)	–	–	+	(rare)

AD, Alzheimer disease; ALS, amyotrophic lateral sclerosis; DLBD, diffuse Lewy body disease; FPD, familial Parkinson disease; GI, glial inclusion; INI, intranuclear inclusion; LB, Lewy body; NFT, neurofibrillary tangle; PB, Pick body; PD, sporadic Parkinson disease; PDCG, Parkinson dementia complex of Guam; Pick, Pick disease.

(0.5 µg/mL) plus IFN-γ (150 U/mL) after being seeded into 10-cm tissue culture plates at a concentration of  $5 \times 10^5$  cells/mL in 15 mL of DMEM-F12 medium containing 5% FBS.

Human microglial, astrocytic, and oligodendroglial cells were isolated from surgically resected temporal lobe tissues and isolation protocols described by De Groot et al (22) were used with minor modifications (23). Before their mRNA was extracted, microglial and astrocytic cells were grown for 7 days, whereas oligodendroglial cells were grown for 1 to 7 days as previously described (23).

### RNA Isolation and cDNA Synthesis by Reverse Transcription

Total RNA was isolated from cultured cells by using the Trizol reagent (GIBCO BRL; Life Technologies) according to the manufacturer's instructions. Two micrograms of the resultant RNA extract was then used to prepare cDNA. RNA was treated with 10 U of DNase I (Invitrogen Life Technologies, Burlington, ON, Canada) for 60 minutes at 37°C in 25 µL of  $1 \times$  reverse transcription buffer (50 mM Tris-HCl, 75 mM KCl, 3 mM MgCl<sub>2</sub>) containing 40 U of RNase inhibitor (Amersham Biosciences, Baie d'Urfé, PQ, Canada)

and 1 mM dithiothreitol (DTT) followed by incubation at 85°C for 5 minutes to inactivate the enzyme. Reverse transcription was performed at 42°C for 90 minutes in 50 µL of the following mixture: 1 × reverse transcription buffer containing 2 µg of RNA, 5 mM DTT, 0.2 µg random hexamer primers (Amersham Biosciences), 1 mM deoxynucleotides (Invitrogen Life Technologies), 40 U of RNase inhibitor, and 400 U of SuperScript II reverse transcriptase (Invitrogen Life Technologies). At the end of the incubation period, the enzyme was inactivated by heating at 65°C for 10 minutes (24).

### Polymerase Chain Reaction

One microliter of the transcription reaction was amplified for *LRRK2*. The polymerase chain reaction (PCR) was carried out in a 25-µL mixture containing 1 × GeneAmp PCR buffer II, 1.25 U of AmpliTaq Gold DNA polymerase, 2 mM MgCl<sub>2</sub> (all from Applied Biosystems, Streetsville, ON, Canada), 200 µM dNTPs (Invitrogen Life Technologies), and 0.5 µM of each primer. The amplification program consisted of an initial denaturation step at 94°C, which was extended to 9 minutes to activate AmpliTaq Gold enzyme. This was followed by an annealing step at 55°C for 1 minute and an initial synthesis step at 72°C for 3 minutes. The remaining cycles were 1 minute at 94°C, 1 minute at 55°C, and 1 minute at 72°C. The number of cycles performed was 35. Two sets of primers were used. The primer sequences (5' to 3') were as follows: LRRK315F, 5'-AGCTTATTGTCTGGTAGGATCTG; LRRK315R, 5'-ATGGTGAGCCTTGTTGATC; LRRK260F, TGAATGCACCGAGGAATCTG; LRRK260R, GTGAAGTGCCTGAGGAAGCT. After amplification, PCR products were separated on a 6% polyacrylamide gel and visualized by incubation for 10 minutes in a solution containing 10 ng/mL of ethidium bromide. Polaroid photographs of the gels were taken. The predicted sizes of the PCR product were 315 and 260 bp, respectively. Restriction digest analysis of the 315 bp product by *Dde* I and that of the 260 bp product by *Mbo* I (GIBCO BRL; Life Technologies, Burlington, ON, Canada) resulted in 2 fragments corresponding to the predicted sizes of 255 and 32 bp and those of 180 and 73 bp, respectively (data not shown).

### Western Blotting

Frozen brain tissue was homogenized in 5 volumes of ice-cold TS buffer (50 mM Tris-HCl, pH 7.6, 150 mM NaCl). The homogenates were centrifuged at 100,000 × g for 1 hour at 4°C. The supernatant was taken and separated by 7.5% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). Proteins in the gel were electrotransferred onto a polyvinylidene fluoride (PVDF) membrane (Millipore Co., Bedford, MA). After blocking with 5% skim milk and 0.1% Tween 20 in TS buffer, the membrane was incubated with the rabbit polyclonal antibody to Lrrk2 (NB 300-268; Novus Biologicals, Littleton, CO). After incubation with horseradish peroxidase (HRP)-conjugated anti-rabbit IgG, immunoreactivity was visualized by the chemiluminescence method using the ECL Western blotting system (Amersham Pharmacia Biotech, Uppsala, Sweden).

### Preparation of 12-mer Lrrk2 Peptide Membranes

Full-length human Lrrk2 contains 2527 amino acids. Twelve-mer overlapping peptides were synthesized as arrays on cellulose membranes as previously described (25). A total of 1259 Lrrk2 peptides were programmed for robotic synthesis by shifting each peptide by 2 amino acids from its N-terminal to its C-terminal. The spots were arranged in rows of 12 with 34 rows per membrane. A total of 3 full membranes (panels A, B, and C) and 3 rows of a fourth membrane (panel D) were required to cover the full sequence. After preparation, the membranes were dried and stored in the cold. Used membranes were regenerated by washing twice with dimethyl formamide for 15 minutes followed by soaking at 37°C overnight in a buffer containing 8 M urea, 1% SDS, and 0.1% 2-mercaptoethanol. After washing twice with a solution of 40% methanol, 10% glacial acetic acid in H<sub>2</sub>O, v/v for 20 minutes, and then 3 times with methanol for 2 minutes, the membranes were dried in air and stored in the cold for future use.

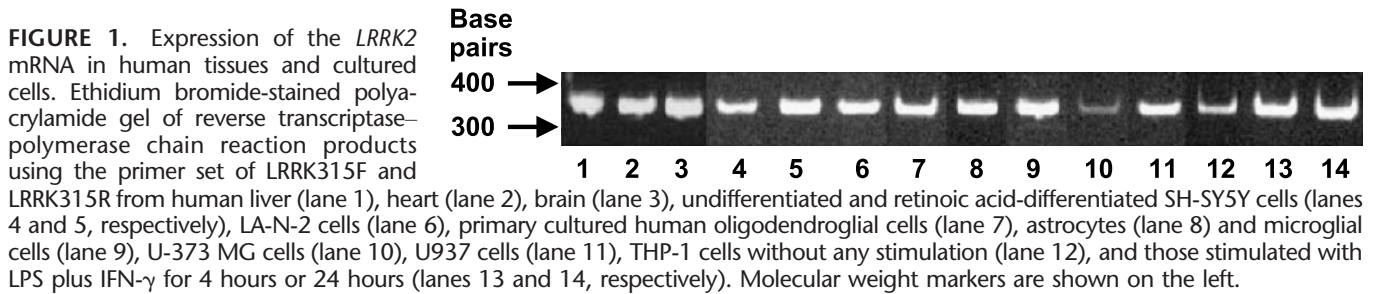
### Epitope Probing of Rabbit Anti-Human Lrrk2 Polyclonal Antibody NB 300-268

Two sets of Lrrk2 12-mer peptide array membranes were presoaked in methanol for 1 minute and washed with TBST0.2 (10M Tris-HCl, pH 7.4, 150M NaCl, 0.2% Tween-20). One set was probed with buffer (1% bovine serum albumin [BSA] in TBST0.05) and the other with antibody NB300-268 (1:1,000 dilution with 3% BSA in TBST0.05). After 2 hours of incubation at 37°C, the membranes were washed 5 times with TBST0.2, blocked with 5% BSA for 1 hour, then incubated with goat anti-rabbit IgG-HRP conjugate (1: 6,000 in 1% BSA-TBST0.05) for 1 hour at 37°C. Finally, the membranes were washed with TBST0.2, developed with ECL Western blotting detection reagents, and scanned under an imager (Bio-Rad Fluorescent Imager, Hercules, CA).

### Interaction of α-Synuclein on Lrrk2 Peptide Array Membranes

Two sets of Lrrk2 peptide array membranes were pretreated as described previously. One set was incubated with recombinant α-synuclein (gift of Dr. Benoit Giasson), 10 µg/mL in 10 mmol phosphate-buffered saline (PBS), pH 7.4 plus proteinase inhibitor cocktail at 37°C overnight; the other with buffer as a control. After blocking with 5% BSA at 37°C for 2 hours, the membranes were incubated with the anti-α-synuclein monoclonal antibody LB509 (Synuclein Clone LB509; Zymed Lab, San Francisco, CA; 1:100, in 3% BSA-TBST0.05) at 37°C for 2 hours. Then, after washing 5 times with TBST0.2, the membranes were incubated with rabbit anti-mouse IgG-HRP conjugate (Sigma-Aldrich, Oakville, ON, Canada; 1: 4,000, in 3% BSA-TBST0.05) at 37°C for 1 hour. Finally, the membranes were washed with TBST0.2, developed with ECL Western blotting detection reagents (Amersham Biosciences, Baie d'Urfé, PQ, Canada), and scanned under the imager as described previously.





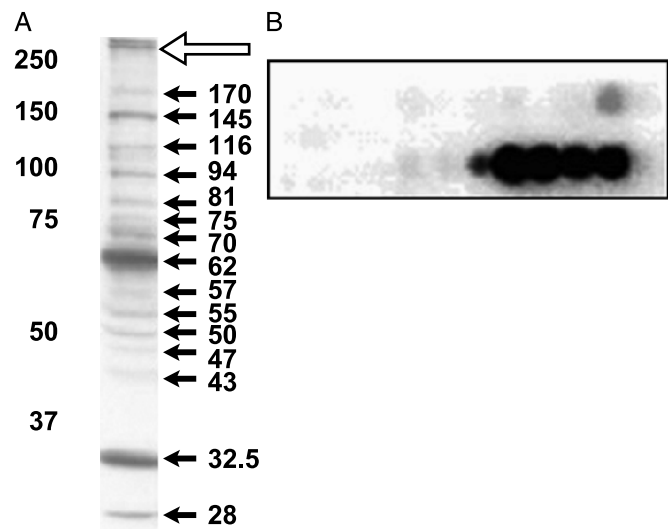
### Immunohistochemistry

Approximately 7-mm-thick transverse tissue blocks from representative levels of the cerebral hemispheres, including hippocampus, frontal, temporal, parietal, primary motor and occipital cortices, the amygdala, basal ganglia and thalamus, mesencephalon, pons, medulla oblongata, and cerebellum were analyzed. In the ALS cases, spinal cords at the cervical and thoracic levels were analyzed. These samples were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer pH 7.4 for 2 days and were maintained in 15% sucrose in 0.01 M PBS, pH 7.4, until processed.

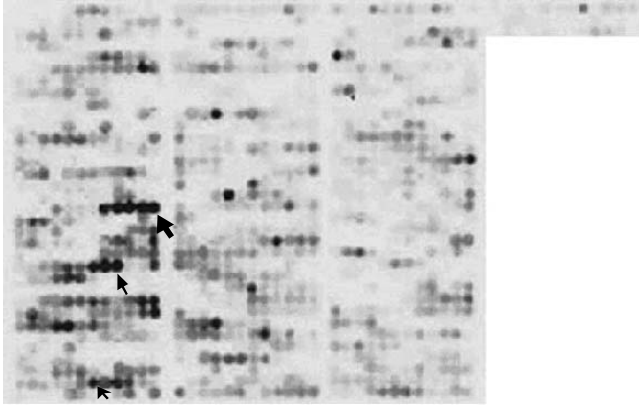
Sections 30- $\mu$ m thick were cut on a freezing microtome from all specimens and were investigated immunohistochemically as free-floating sections. A rabbit polyclonal anti-Lrrk2 antibody (NB 300-268) was used to detect Lrrk2. Two monoclonal antibodies to  $\alpha$ -synuclein (Synuclein Clone LB509, 1:1,000 and Synuclein VP-A106; Vector Labs, Burlingame, CA; 1:1,000) and a polyclonal antibody to ubiquitin (Z0458; DakoCytomation, Carpinteria, CA; 1:1,000) were used to detect Lewy bodies. To immunostain sections with AD-type changes and tau pathologies, a monoclonal anti-beta amyloid (A $\beta$ ) antibody (6F/3D, M 0872; DakoCytomation; 1:1,000) was used. The anti-tau antibodies used were: anti-tau A0024 (C-243-441; DakoCytomation; rabbit IgG, 1:1,000), which recognizes phosphorylated and nonphosphorylated tau; monoclonal anti-PHF antibody (AT8; Innogenetics, Ghent, Belgium; 1:500); and Tau-C3 (gift of Dr. L. I. Binder), which recognizes the D421 truncated site of tau (26).

For immunostaining, the avidin-biotin-peroxidase technique was used. To remove endogenous peroxidase activity, the sections were first incubated in 0.3% hydrogen peroxide for 30 minutes. The sections were next preincubated with 5% skim milk for 1 hour. They were then incubated with the Lrrk2 primary antibody diluted 1:1,000 in PBST containing 1% normal swine serum overnight at room temperature, for 48 to 72 hours at 4°C, or at room temperature overnight. After washing 3  $\times$  5 minutes with PBST, the sections were incubated for 30 minutes at room temperature with swine-anti-rabbit biotinylated secondary antibody diluted at 1:300 in PBST. The sections were washed in PBST (3  $\times$  5 minutes) and incubated with a mixture of avidin and biotinylated HRP (DakoCytomation; ABCComplex/HRP-Kit, K0355) following the recommendation of the manufacturer. The stock solution of the avidin-biotin complex was prepared by adding 10  $\mu$ L of solution A and 10  $\mu$ L of solution B to 1 mL PBST. The stock solution was used at a dilution of 1:100 to incubate sections for 1 hour at room temperature, which was followed

by a wash of 3  $\times$  5 minutes in PBST. Finally, the sections were incubated with 3, 3-diaminobenzidine (DAB), which produced a brown reaction product, or a combination of DAB and nickel ammonium sulfate that produced a dark purple reaction product. For this combined DAB-nickel ammonium sulfate reaction, the sections were incubated in 0.2% nickel ammonium sulfate (Fluka, Buchs, Switzerland, 09885) diluted in Tris buffer at pH 7.4 for 10 minutes and then were transferred to a 0.2% nickel ammonium sulfate solution that contained DAB (50 mg for 100 mL Tris). Finally, the sections were incubated in the same mixture of nickel ammonium sulfate and DAB to which 30% H<sub>2</sub>O<sub>2</sub> (10  $\mu$ L to 200 mL) was added. During this last step of incubation, positive control sections were monitored by



**FIGURE 2.** Western blotting with the Lrrk2 antibody NB 300-268 (A) and its recognition site on peptide array membranes (B). (A) Western blot of the soluble fraction of normal brain. Ten microliters of the sample was loaded. The antibody recognized a close doublet of higher molecular weight than 250 kDa (light arrow) as well as several bands from 28 to 145 kDa (dark arrows). Molecular weight markers are shown on the left. (B) Panel of peptides on Lrrk2 membrane panel D, which covers the far C-terminal region. Panels A, B, and C were blank. The large dark spots indicate the 4 12-mer peptides strongly reacting with the antibody. From left to right, they are IEVKELAEKMR, VRKELAEKMRRT, KELAEMRRTSV, and ELAEKMRRTSVE (residues 2511-2527). Note that the final peptide was shifted by only one amino acid compared with 2 amino acid shifts for the other 12-mers.

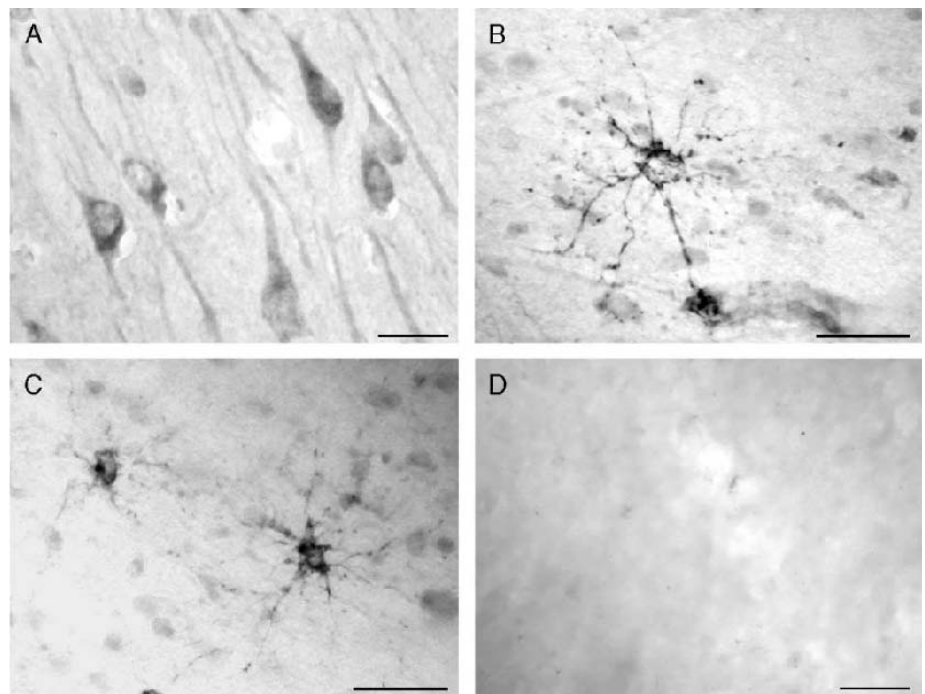


**FIGURE 3.** Panels A, B, C, and D of Lrrk2 membranes. Recombinant  $\alpha$ -synuclein binds to at least 3 regions of adjacent 12-mer peptides on panel A of the membrane (arrows). They span regions from residues 423 to 442, 541 to 556, and 781 to 798. Their sequences are STLLEQNVNFR-KILLSKGIH, DIHKLVLAALNRFIGN, and KGDSQIISLLRLALDV, respectively. The control membrane, which was exposed only to the synuclein antibody, was blank (data not shown).

microscopic analysis for efficiency of the immunoreaction. The sections were rinsed in distilled water, dehydrated in graded ethanol, passed  $2 \times 5$  minutes in xylene, and were mounted in Entellan (VWR Canlab, Mississauga, ON, Canada, UN 1866). Control sections, in which the primary antibody was replaced by normal serum, were immunostained in parallel with the Lrrk2-immunostained sections. Sections were pretreated with 80% formic acid for 15 minutes before immunostaining for  $\alpha$ -synuclein.

Double immunofluorescence staining to detect overlapping expression of Lrrk2 and  $\alpha$ -synuclein in Lewy bodies

was also performed on SN sections of patients with PD in whom the presence of Lewy bodies had previously been confirmed by ubiquitin and  $\alpha$ -synuclein staining. The SN sections were simultaneously incubated with a mixture of polyclonal anti-Lrrk2 (1:300) and monoclonal anti- $\alpha$ -synuclein (clone LB509, 1:200) antibodies overnight at room temperature. The sections were rinsed in PBST ( $3 \times 5$  minutes) and then incubated for 1 hour at room temperature with FITC-conjugated swine anti-rabbit antibody (DakoCytomation; F0205, 1:30) to yield a green fluorescence for Lrrk2. The sections were washed in PBST ( $3 \times 5$  minutes). Some were incubated with 1% normal rabbit serum for 1 hour at room temperature to quench any crossreaction between the secondary antibodies. Other sections were incubated directly with TRITC-labeled rabbit anti-mouse antibody (DakoCytomation; R0270, 1:30) to yield a red fluorescence for  $\alpha$ -synuclein. No differences were observed between these 2 treatments. SN sections were similarly incubated with the Lrrk2 polyclonal antibody and monoclonal antibodies either to Tau-C3, glial fibrillary acidic protein (GFAP, clone F2; DakoCytomation) to detect astrocytes or HLA-DR (CR3/43; DakoCytomation; R0270) to detect microglia. The primary antibodies were identified using the same combination of fluorescent secondary antibodies. The sections were coverslipped with Fluoromount-G (Southern Biotechnology Associates, Birmingham, AL) and examined with a Carl Zeiss Axi-overt-200 fluorescence microscope. The capture of the green Lrrk2 fluorescence and the red fluorescence of  $\alpha$ -synuclein, tau, GFAP, or HLA-DR as well as the merged images were performed using the Northern Elite program. Staining of cell nuclei with Hoechst dye 3258 (1  $\mu$ g/mL) produced a white fluorescence in ultraviolet light that changed to a blue color in the merged fluorescent images.



**FIGURE 4.** Neurons, astrocytes, and microglia show positive Lrrk2 immunostaining in control brain. **(A)** Lrrk2 immunoreactivity in the cytoplasm of pyramidal neurons in the hippocampus of a young control patient. **(B)** Some astrocytes in the white matter also showed positive Lrrk2 staining. **(C)** Some Lrrk2-positive cells were morphologically compatible with microglia. **(D)** Nearby section of the hippocampus showing negative immunostaining with normal serum alone. Scale bars = **(A)** 70  $\mu$ m; **(B–D)** 50  $\mu$ m.



To analyze the coexpression of Lrrk2 and tau in hippocampal sections of patients with AD and Pick disease, double immunostaining with the polyclonal anti-Lrrk2 antibody (1:300) and the monoclonal AT8 anti-tau antibody (1:200) was carried out.

### Immunoabsorption Assay

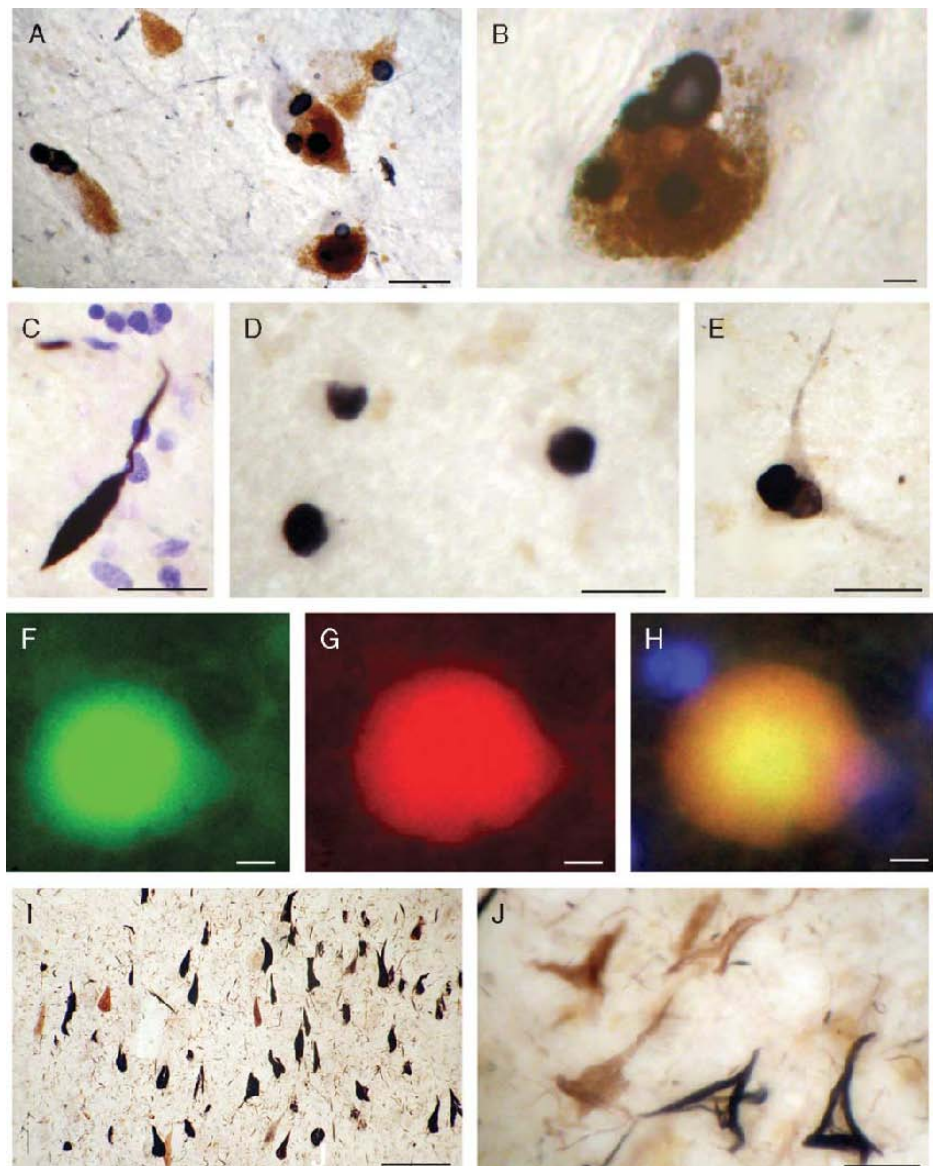
An immunoabsorption assay was done using the synthetic peptide that had been used as the antigen in generating the Lrrk2 antibody. This peptide spanned residues 2500–2527 of Lrrk2 (Novus Biologicals, Inc.; NB-300-268). Five micrograms per milliliter Lrrk2 268 peptide in PBS was mixed with 1  $\mu\text{g}/\text{mL}$  anti Lrrk2 antibody in continuous rotation at 4°C for 4 hours. In parallel, 1  $\mu\text{g}/\text{mL}$  anti-Lrrk2 268 antibody was incubated in PBS under the same conditions. Parallel immunostaining of adjacent sections of the hippocampus of patients with DLBD, AD, and Pick disease was carried out

with the immunoabsorbed and nonimmunoabsorbed anti-Lrrk2 antibodies as described previously.

## RESULTS

### LRRK2 mRNA Expression

All tissues and cell lines tested by reverse transcriptase–polymerase chain reaction demonstrated expression of *LRRK2*. This is illustrated in Figure 1, which shows the expression of *LRRK2* mRNA in human liver (lane 1), heart (lane 2), brain (lane 3), undifferentiated and retinoic acid-differentiated SH-SY5Y cells (lanes 4 and 5, respectively), LA-N-2 cells (lane 6), primary cultured human oligodendroglial cells (lane 7), human astrocytes (lane 8), human microglial cells (lane 9), as well as U-373 MG cells (lane 10), U937 cells (lane 11), and THP-1 cells unstimulated and stimulated with LPS plus IFN- $\gamma$  (lanes 12–14).



**FIGURE 5.** Lewy bodies show a positive immunoreaction to Lrrk2. **(A, B)** Lrrk2-immunoreactive Lewy bodies in degenerating dopaminergic neurons of the substantia nigra in a patient with Parkinson disease. **(C)** A Lewy neurite immunolabeled with the anti-Lrrk2 antibody. **(D, E)** Lrrk2-positive cortical Lewy bodies in pyramidal neurons of the temporal **(D)** and frontal cortex **(E)** of a patient with diffuse Lewy body disease. **(F–H)** Colocalization of Lrrk2 and alpha-synuclein. The images were taken from a section that was doubly stained by immunofluorescence using the polyclonal antibody to Lrrk2, exhibiting green FITC fluorescence, and a monoclonal antibody to phosphorylated  $\alpha$ -synuclein exhibiting a red TRITC fluorescence. Merging of the captured images shows the overlap of Lrrk2 and  $\alpha$ -synuclein staining in Lewy bodies. **(I, J)** Lrrk2-immunoreactive neurofibrillary tangles in Alzheimer disease (AD). **(I)** Section of the hippocampus of a patient with sporadic AD was doubly immunostained with anti-Lrrk2 polyclonal antibody (black) and anti-tau monoclonal antibody Tau C3. **(J)** Higher power of the same section. A number of neurofibrillary tangles show the black Lrrk2 immunoreaction. Some other neurons show only brown tau immunostaining. Scale bars = **(A, C–E)** 50  $\mu\text{m}$ ; **(B)** 10  $\mu\text{m}$ ; **(F–H)** 7  $\mu\text{m}$ ; **(I)** 200  $\mu\text{m}$ ; **(J)** 70  $\mu\text{m}$ .

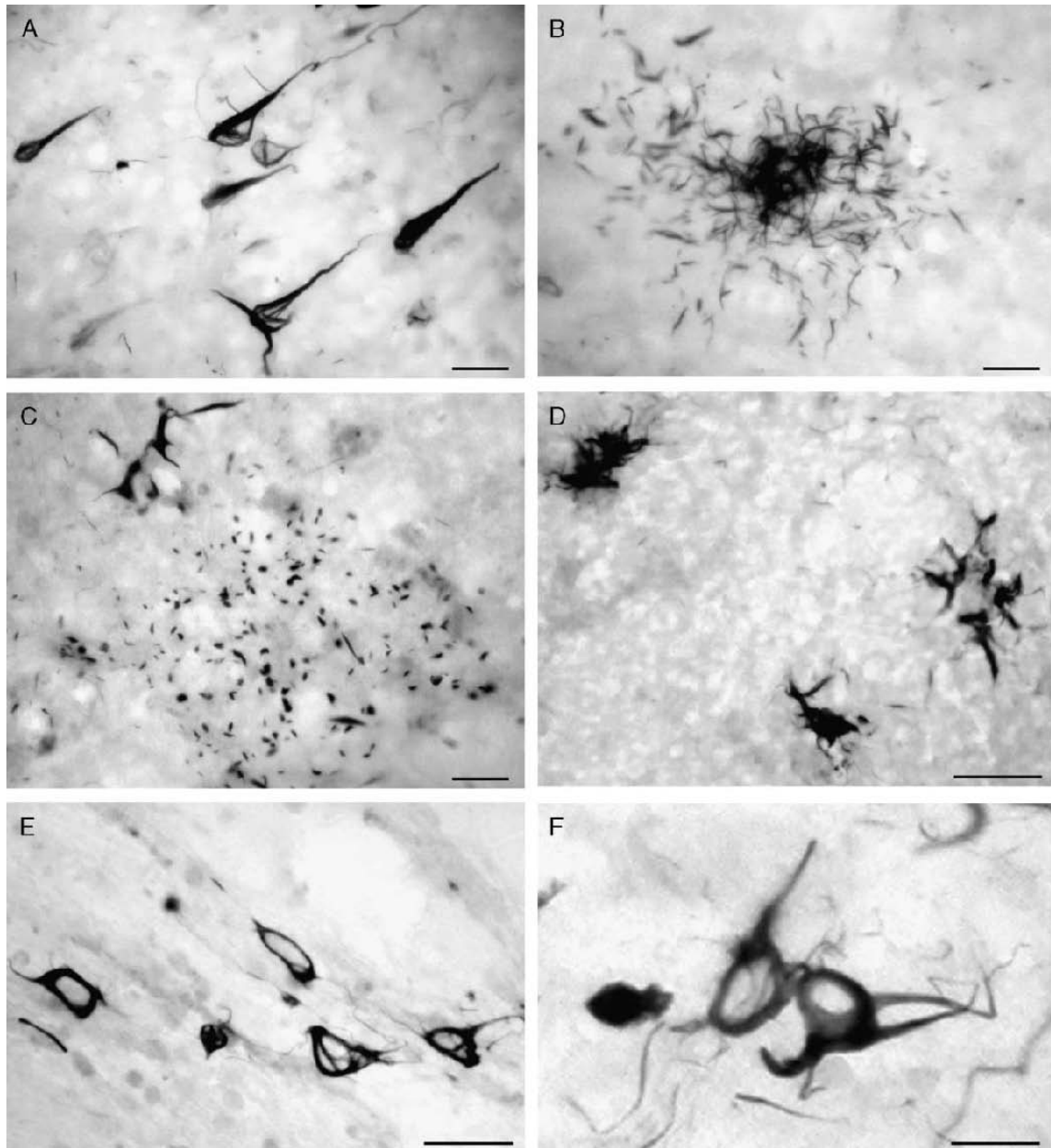
### Lrrk2 Protein Expression

Figure 2 A shows a typical Western blot of human brain supernatant. The antibody to Lrrk2 stained a close doublet of higher molecular weight than 250 kDa, which roughly corresponds to the predicted value of full-length Lrrk2 with possible posttranslational modifications. Several other positive bands from 28 to 145 kDa may represent C-terminal fragments of Lrrk2. Strong bands were observed at approximately 62 and 32.5 kDa, suggesting major cleavages in the

MAPKKK and WD 40 domains. This indicates that significant amounts of Lrrk2 C-terminal fragments are produced in brain, which, if retained in an insoluble state, would be recognized immunohistochemically.

### Epitope Recognition

Figure 2B shows the results of probing Lrrk2 peptide membranes with the Lrrk2 polyclonal antibody NB 300-268. The figure shows that the region of the molecule recognized



**FIGURE 6.** In Parkinson dementia complex of Guam (PDCG), the neuronal and glial pathologic lesions are Lrrk2-immunopositive. **(A)** Neurofibrillary tangles in the middle temporal gyrus in a patient with PDCG. **(B)** An Lrrk2-positive astrocytic plaque is seen in Brodmann area 9 of the frontal cortex of a patient with PDCG. “Hazy” granules **(C)** and thorn-shaped astrocytes **(D)** both show a positive Lrrk2 immunoreaction in the middle temporal gyrus of the same PDCG case. **(E, F)** Lrrk2-positive oligodendroglial coiled bodies in the internal capsule **(E)** and in the white matter of the primary motor cortex **(F)** in PDCG. Scale bars = 50  $\mu$ m.



by the antibody is included in the sequence IEVKKE-LAEKMRRTSVE spanning residues 2511 to 2527 of the far C-terminal region of Lrrk2.

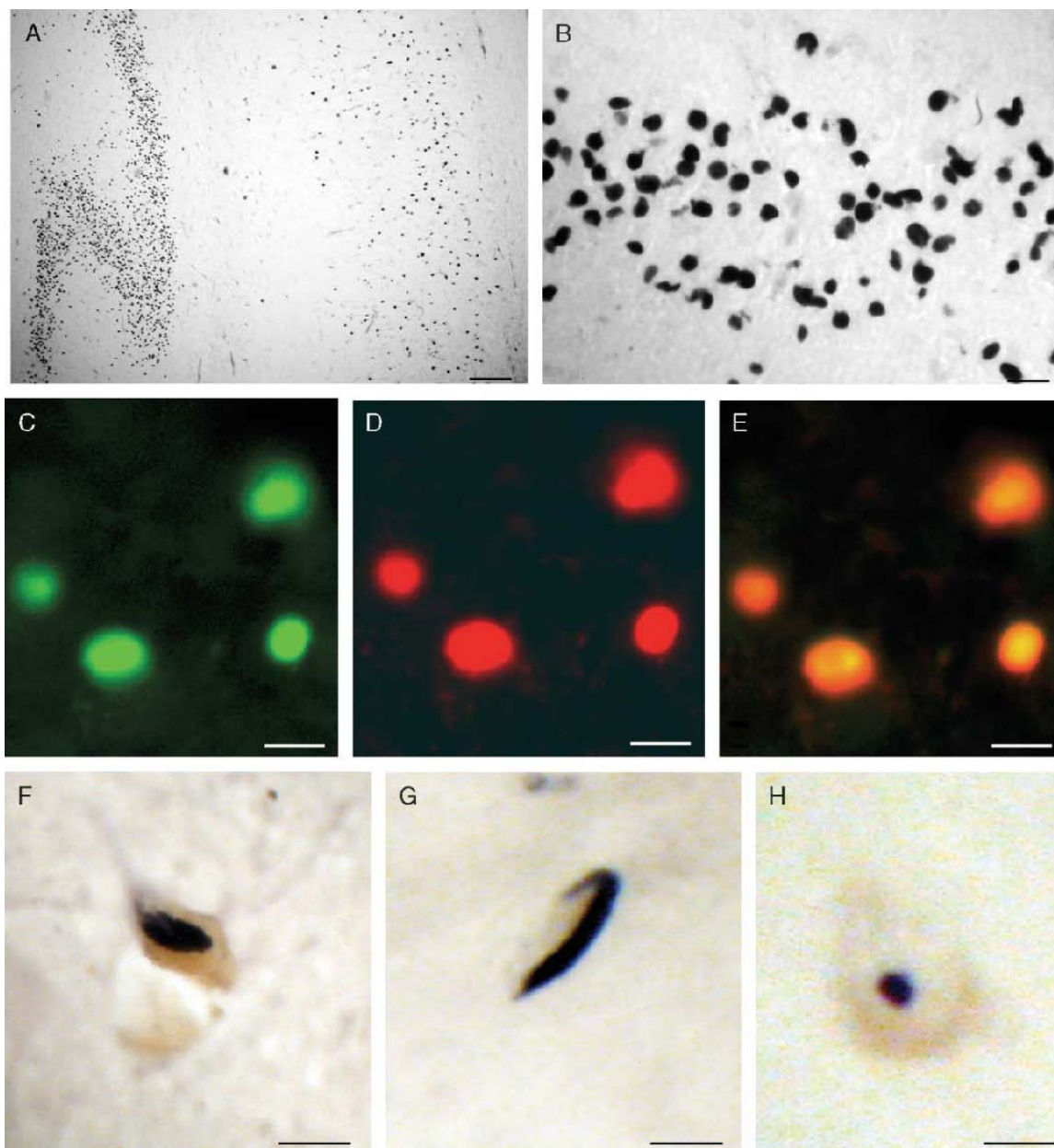
### Interaction With $\alpha$ -Synuclein

Figure 3 shows panels A, B, C, and D of the Lrrk2 peptide membranes. It shows that recombinant  $\alpha$ -synuclein binds to at least 3 regions in panel A (arrows) encompassing

residues 423 to 442, 541 to 556, and 781 to 798. Their sequences are, respectively, STLLQNVNFRKILLSKGIH, DIHKLVLAAALNRFIGN, and KGDSQIISLLLRLALDV.

### Immunohistochemistry

Figure 4A illustrates positive cytoplasmic Lrrk2 immunostaining in pyramidal neurons of the hippocampus in a patient without neurologic symptoms and without



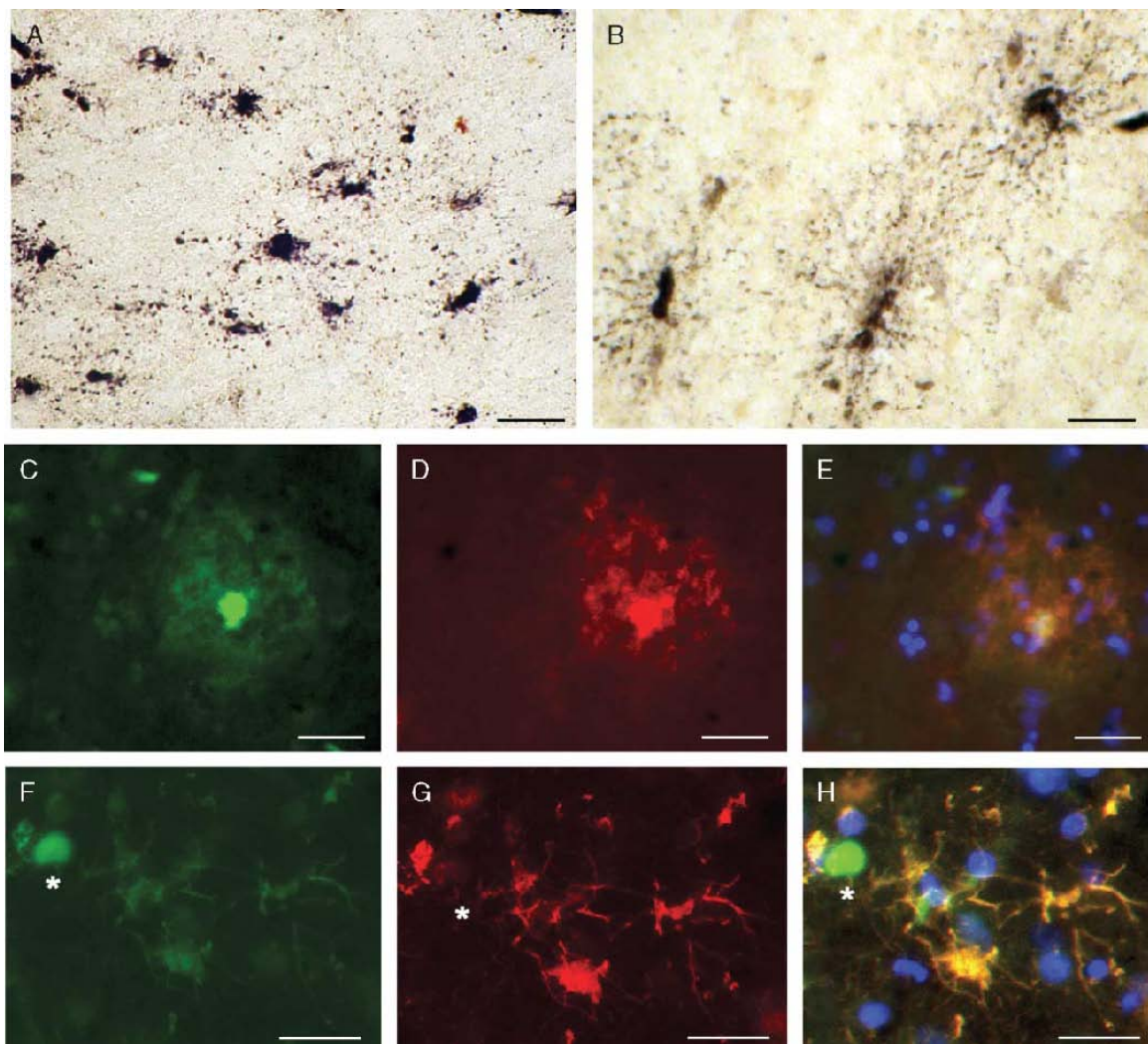
**FIGURE 7.** Lrrk2 in Pick disease, amyotrophic lateral sclerosis (ALS), and Huntington disease. **(A, B)** Strong Lrrk2 immunoreactivity of Pick bodies in neurons of the dentate gyrus and in pyramidal neurons of the hippocampus. **(C–E)** Double immunofluorescence staining of Pick bodies (hippocampus of the same case as in **[A]** and **[B]**) with the anti-Lrrk2 antibody (**[C]**, exhibiting green fluorescence) and with the monoclonal AT8 antibody that recognizes hyperphosphorylated tau (**[D]**) exhibiting red fluorescence. **(E)** The overlap of Lrrk2 and phosphorylated tau appears orange in the merged image. **(F, G)** Lrrk2-positive intraneuronal **(F)** and glial **(G)** inclusions in the anterior horn of the spinal cord in a case of ALS. **(H)** Lrrk2-immunoreactive intranuclear inclusion in the frontal cortex of a patient with Huntington disease. Scale bars: **(A)** 200  $\mu\text{m}$ ; **(B)** 80  $\mu\text{m}$ ; **(C–F)** 50  $\mu\text{m}$ ; **(G, H)** 25  $\mu\text{m}$ .



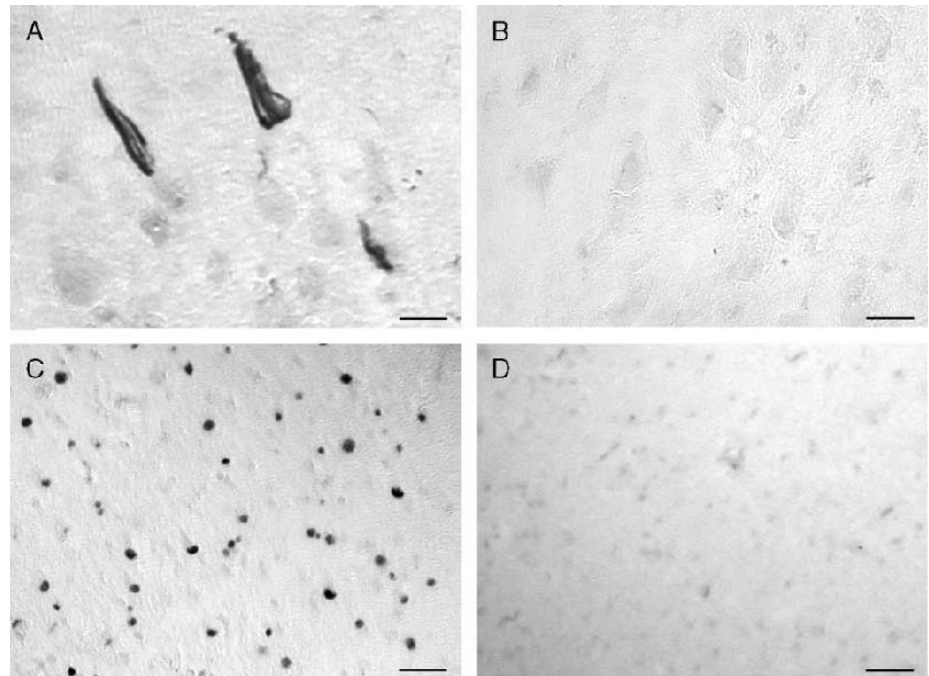
identifiable brain pathology. Such positive neuronal staining was observed in all other brain areas examined, including the temporal, parietal, primary motor, and occipital cortices. Dopaminergic neurons of the SN also showed similar staining. Some Lrrk2-positive glial cells compatible with astrocytes (Fig. 4B) and reactive microglia (Fig. 4C) were also observed. No immunoreactivity was observed with control serum (Fig. 4D).

Figure 5 illustrates immunostaining of pathologic tissue in PD and DLBD. The anti-Lrrk2 antibody identified Lewy bodies in the SN in all sporadic PD cases and also in the familial PD case from family B (Fig. 5A, B). A few Lrrk2-positive Lewy neurites were observed (Fig. 5C). Lrrk2-immunoreactive Lewy bodies were also observed in the locus ceruleus and raphe nuclei in the pons and in the

tegmentum of the medulla oblongata. A similar strong Lrrk2 immunoreactivity of Lewy bodies was observed in the SN, brainstem, parahippocampal, and neocortical areas in DLBD. It was also observed in the AD case in which the AD-type cortical changes were combined with Lewy body pathology. Lrrk2-immunoreactive Lewy bodies in the middle temporal gyrus in a patient with DLBD are illustrated in Figure 5D. An example of Lewy bodies in a pyramidal neuron in the precentral cortex with slightly stained processes from the same DLBD case is shown in Figure 5E. When the SN of a patient with PD known to exhibit Lewy body pathology was doubly immunostained for Lrrk2 and  $\alpha$ -synuclein by immunofluorescence technique, the green Lrrk2 fluorescence (Fig. 5F) and red  $\alpha$ -synuclein fluorescence (Fig. 5G) of Lewy bodies overlapped and on merged



**FIGURE 8.** Lrrk2-positive reactive microglia and reactive astrocytes. **(A, B)** Lrrk2-reactive glial cells in the temporal white matter in a case with diffuse Lewy body disease (DLBD). **(C–E)** An adjacent section to **(A)** was double immunostained for Lrrk2 [**C**], green fluorescence) and C3/43, which recognizes activated microglia [**D**], red fluorescence). The colocalization of Lrrk2 and C3/43 in the same cell on the merged captured image **(E)** indicates that the cell is an Lrrk2-positive activated microglia. **(F–H)** Temporal lobe section in the same DLBD case was double immunostained with anti-Lrrk2 antibody [**F**], green fluorescence) and anti-GFAP [**G**], red fluorescence). In the merged image **(H)**, the colocalization of Lrrk2 and GFAP is visible in orange color that contrasts with the green color of the Lewy body that is negative for GFAP [**G**], asterisk). Scale bars = 50  $\mu$ m.



**FIGURE 9.** Immunoabsorption of the Lrrk2 antibody with the immunizing synthetic peptide abolished Lrrk2 immunostaining. **(A–D)** Sections of the hippocampus of a patient with Alzheimer disease and a Pick disease immunostained with anti-Lrrk2 antibody showing neurofibrillary tangles **(A)** and Pick bodies **(C)**. On adjacent sections where the immunostaining was done with the immunoabsorbed anti-Lrrk2 antibody, Lrrk2 immunoreactivity of tangles **(B)** and Pick bodies **(D)** was abolished. Scale bars = **(A)** 50  $\mu\text{m}$ ; **(B)** 60  $\mu\text{m}$ ; **(C, D)** 200  $\mu\text{m}$ .

fluorescent images yielded a yellow–orange fluorescence with a typical central core (Fig. 5H).

Figures 5I and J show Lrrk2 compared with tau immunoreactivity in the hippocampus of a patient with AD. On doubly immunostained sections, in which Lrrk2 immunoreactivity was revealed in black and that of Tau C3-positive neurofibrillary tangles in brown, a subset only of neurofibrillary tangles exhibited a black Lrrk2 immunoreaction, whereas the remaining tangles showed only tau immunostaining.

Figure 6 illustrates Lrrk2-immunoreactive pathology in PDCG. The anti-Lrrk2 antibody labeled neurofibrillary tangles (Fig. 6A) and Lewy bodies (not shown here), which were observed in 4 of 6 PDCG cases in the brainstem and amygdala. In one case, Lewy bodies were also present in the neocortex. In addition, glial pathology, including astrocytic plaques (Fig. 6B), “hazy” granules (Fig. 6C), thorn-shaped astrocytes (Fig. 6D), and oligodendroglial coiled bodies (Fig. 6E, F) all showed strong Lrrk2 immunostaining.

Figure 7 illustrates Lrrk2 immunoreactivity in some other pathologies. Pick bodies show strong immunoreaction with anti-Lrrk2 antibody where it colocalizes with phosphorylated tau (Fig. 7A–E). Lrrk2-positive intraneuronal inclusions were observed in motor neurons of the anterior horn of the spinal cord (Fig. 7F) and glial inclusions in the degenerating corticospinal tracts (Fig. 7G) in the ALS cases. Intranuclear (Fig. 7H) and some intracytoplasmic Lrrk2-positive inclusions were also observed in the brain of the 2 patients with Huntington disease.

Figure 8 illustrates Lrrk2-positive reactive microglia and reactive astrocytes in the temporal white matter of a case with DLBD. Adjacent sections were used for double immunofluorescence staining. On the section doubly immunostained for Lrrk2 (green fluorescence, Fig. 8C) and CR3/43, which recognizes activated microglia (red fluorescence, Fig. 8D), the

orange merged image (Fig. 8E) indicates that the cell is an activated microglia. Similarly, a section was double immunostained with anti-Lrrk2 antibody (green fluorescence, Fig. 8F) and anti-GFAP (red fluorescence, Fig. 8G). The orange merged image (Fig. 8H) demonstrates the colocalization of Lrrk2 and GFAP in the same astrocytes. Some Lrrk2-positive reactive microglia and a few astrocytes were also seen, particularly in the white matter in some PD, AD, DLBD, and PDCG cases.

### Immunoabsorption Assay

To verify the specificity of the Lrrk2 antibody, an immunoabsorption test was carried out using the synthetic peptide that was used for rabbit immunization (residues 2500–2527; Novus Biologicals, Inc.; NB-300-268). Figure 9 illustrates parallel immunostaining of adjacent sections with the Lrrk2 antibody demonstrating positive staining of neurofibrillary tangles (Fig. 9A) and Pick bodies (Fig. 9C) that were abolished with the immunoabsorbed Lrrk2 antibody (Fig. 9B and D, respectively).

### DISCUSSION

The present results indicate that the *LRRK2* gene and the Lrrk2 protein are constitutively expressed in all central nervous system cell types. *LRRK2* has previously been reported to be expressed in human lung (9), heart, and liver (8). We found Lrrk2 to be expressed in the soluble fraction of human brain, liver, and heart indicating that the gene is translated and the product must have physiological functions in widespread tissues of the body. This is consistent with the report of Giasson et al (27) who, in addition to human brain, detected the protein in multiple mouse organs.

However, it is not understood why it is the brain, and especially the SN, which is primarily affected by mutations that alter the functional state of the protein. We found that



the anti-Lrrk2 antibody we used, which recognizes the far C-terminal sequence in the molecule, colocalized with Lewy bodies in the SN of sporadic and familial PD cases. It also identified cortical Lewy bodies in cases with DLBD. Double immunofluorescence staining clearly showed the overlap of Lrrk2 and  $\alpha$ -synuclein immunoreactions in Lewy bodies in the SN of PD cases. In PDCG, which is known to be associated with parkinsonian features, Lrrk2 labeled not only a subset of neurofibrillary tangles, but in 4 of the 6 cases identified Lewy bodies in the brainstem and in one case cortical Lewy bodies.

The astrocytic and oligodendroglial tau aggregations, which are typical of this disorder, all showed a distinctive Lrrk2-positive immunoreaction. These included astrocytic plaques, thorn-shaped astrocytes, and coiled bodies of oligodendrocytes. Such glial pathologic changes are known to occur in other tauopathies such as progressive supranuclear palsy and corticobasal degeneration. Coiled bodies are also known to occur in ALS and multiple system atrophy. It is intriguing that Pick bodies, which are tau-positive but  $\alpha$ -synuclein negative, are strongly immunoreactive for Lrrk2.

These immunohistochemical results differ from the report of Giasson et al, who used an antibody raised against residues 1246–1265 of Lrrk2 (24). Their antibody demonstrated cytoplasmic staining of normal neurons, but failed to detect  $\alpha$ -synuclein inclusions, neurofibrillary tangles, and glial inclusions. It is possible that the midregion sequence against which this antibody was raised is not as exposed in pathologic lesions as the far C-terminal sequence. It may also be that the antibody used in our experiments detects C-terminal fragments that are retained in an insoluble state and possibly bound to the pathologic deposits. The fact that  $\alpha$ -synuclein strongly binds to Lrrk2 sequences on Lrrk2 peptide membranes is evidence in favor of an *in vivo* interaction between Lrrk2 and  $\alpha$ -synuclein.

The fact that Lrrk2 appears to be present in neuronal and glial inclusions in several neurodegenerative disorders may indicate that a common link may exist in the pathogenesis of these disorders. It must be emphasized, however, that these are preliminary observations on a limited number of cases using a single antibody. The findings will need to be confirmed using multiple antibodies on a much larger series of cases. The finding that *LRRK2* mutations were also found in a few cases with AD also reinforces this possibility. This is in harmony with previous findings showing that in familial PD cases with *LRRK2* mutations, heterogenic pathologic changes were observed at autopsy. Further histologic, biochemical, and molecular analyses studies may reveal that a common thread exists, possibly in protein phosphorylations carried out by its MAPKKK domain.

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#### REFERENCES

1. Calne DB, Langston JW. Aetiology of Parkinson's disease. *Lancet* 1983; 2:1457–59

2. Calne S, Schoenberg B, Martin W, et al. Familial Parkinson's disease: Possible role of environmental factors. *Can J Neurol Sci* 1987;14:303–5
3. Feany MB. New genetic insights into Parkinson's disease. *N Engl J Med* 2004; 351:1937–40
4. Kondo K, Kurland LT, Schull WJ, et al. Parkinson's disease. Genetic analysis and evidence of a multifactorial etiology. *Mayo Clin Proc* 1973;48:465–75
5. Vaughan JR, Davis MB, Wood NW. Genetics of parkinsonism: A review. *Ann Hum Genet* 2001;65:111–26
6. Warner TT, Schapira AH. Genetic and environmental factors in the cause of Parkinson's disease. *Ann Neurol* 2003;53:16–23
7. Funayama M, Hasegawa K, Ohta E, et al. An LRRK2 mutation as a cause for the parkinsonism in the original PARK8 family. *Ann Neurol* 2005;57:918–21
8. Paisan-Ruiz C, Jain S, Evans EW, et al. Cloning of the gene containing mutations that cause PARK8-linked Parkinson's disease. *Neuron* 2004; 44:595–600
9. Zimprich A, Biskup S, Leitner P, et al. Mutations in LRRK2 cause autosomal-dominant parkinsonism with pleomorphic pathology. *Neuron* 2004;44:601–7
10. Bosgraaf L, Van Haastert PJ. Roc, a Ras/GTPase domain in complex proteins. *Biochim Biophys Acta* 2003;1643:5–10
11. Gloeckner CJ, Kinkl N, Schumacher A, et al. The Parkinson disease causing LRRK2 mutation I2020T is associated with increased kinase activity. *Hum Mol Genet* 2006;15:223–32
12. West AB, Moore DJ, Biskup S, et al. Parkinson's disease-associated mutations in leucine-rich repeat kinase 2 augment kinase activity. *Proc Natl Acad Sci U S A* 2005;102:16842–47
13. Ozelius LJ, Senthil G, Saunders-Pullman R, et al. LRRK2 G2019S as a cause of Parkinson's disease in Ashkenazi Jews. *N Engl J Med* 2006; 354:424–25
14. Lesage S, Leutenegger AL, Ibanez P, et al. LRRK2 haplotype analysis in European and North African families with Parkinson disease: A common founder for the G2019S mutation dating from the 13th century [Letter]. *Am J Hum Genet* 2005;77:330–32
15. Kachergus J, Mata IF, Hulihan M, et al. Identification of a novel LRRK2 mutation linked to autosomal dominant Parkinsonism: Evidence of a common founder across European populations. *Am J Hum Genet* 2005;76:672–80
16. Simon-Sanchez J, Herranz-Perez V, Lucha-Bordonau F, Perez-Tur J. LRRK2 is expressed in areas affected by Parkinson's disease in the adult mouse brain. *Eur J Neurosci* 2006;3:659–66
17. Melrose H, Lincoln S, Tyndall G, et al. Anatomical localization of leucine-rich repeat kinase 2 in mouse brain. *Neuroscience* 2006;139:791–94
18. Galter D, Westerland M, Carmine A, et al. LRRK2 expression linked to dopamine-innervated areas. *Ann Neurol* 2006;59:714–19
19. Gasser T, Muller-Myhsok B, Wszolek ZK, et al. A susceptibility locus for Parkinson's disease maps to chromosome 2p13. *Nat Genet* 1998;18: 262–65
20. Klegeris A, Schwab C, Bissonnette CJ, et al. Induction of complement C9 messenger RNAs in human neuronal cells by inflammatory stimuli: Relevance to neurodegenerative disorders. *Exp Gerontol* 2001;36: 1179–88
21. Pahlman S, Ruusala AI, Abrahamsson L, et al. Retinoic acid-induced differentiation of cultured human neuroblastoma cells: A comparison with phorbol ester-induced differentiation. *Cell Differ* 1984;14:135–44
22. De Groot CJA, Montagne L, Janssen I, et al. Isolation and characterization of adult microglial cells and oligodendrocytes derived from postmortem human brain tissue. *Brain Res Brain Res Protoc* 2000;5:85–94
23. Hosokawa M, Klegeris A, Maguire J, et al. Expression of complement messenger RNAs and proteins by human oligodendroglial cells. *Glia* 2003;42:417–23
24. Hosokawa M, Klegeris A, McGeer PL. Human oligodendroglial cells express low levels of C1 inhibitor and membrane cofactor protein mRNAs. *J Neuroinflamm* 2004;1:17
25. Guo JP, Petric M, Campbell W, et al. SARS corona virus peptides recognized by antibodies in the sera of convalescent cases. *Virology* 2004;324:251–56
26. Horowitz PM, Patterson KR, Guillozet-Bongaarts AL, et al. Early N-terminal changes and caspase-6 cleavage of tau in Alzheimer's disease. *J Neurosci* 2004;24:7895–902
27. Giasson BI, Covy JP, Bonini NM, et al. Biochemical and pathological characterization of Lrrk2. *Ann Neurol* 2006;59:315–22