α-Synuclein Gene Rearrangements in Dominantly Inherited Parkinsonism

Frequency, Phenotype, and Mechanisms

Pablo Ibáñez, PhD; Suzanne Lesage, PhD; Sabine Janin, BS; Ebba Lohmann, MD; Frank Durif, MD; Alain Destée, MD; Anne-Marie Bonnet, MD; Christine Brefel-Courbon, MD; Simon Heath, PhD; Diana Zelenika, PhD; Yves Agid, MD, PhD; Alexandra Dürr, MD, PhD; Alexis Brice, MD, for the French Parkinson’s Disease Genetics Study Group

Objective: Genomic multiplications of the α-synuclein gene (SNCA) cause autosomal dominant Parkinson disease (ADPD). The aim of this study was to assess the frequency and phenotype of SNCA rearrangements in a large series of families with typical or atypical AD parkinsonism.

Design: Patients were screened by the exon dosage of the SNCA gene. The genotype of patients and relatives carrying SNCA rearrangements, the size of the multiplied regions, and the centromeric and telomeric breakpoints were determined by microsatellite dosage and 250K Affymetrix Single Polymorphism Nucleotide microarrays (Affymetrix, Santa Clara, California).

Subjects: Index cases and, whenever appropriate, relatives of 286 mainly European families with ADPD were screened.

Results: Four of 264 families (1.5%) with typical ADPD carried duplications and 1 of 22 families (4.5%) with atypical AD parkinsonism carried a triplication of SNCA. Genotyping and dosage analyses showed that the multiplied regions were variable in size (0.42-5.29 megabase pairs), suggesting that SNCA multiplications occurred independently. Phenotype analyses showed that the severity of the disease correlated with the SNCA copy number, but not with the minimal number of multiplied genes (1 to 33). Haplotype analysis of polymorphic markers suggested that multiplication of the SNCA gene occurred by both interchromosomal and intrachromosomal rearrangement.

Conclusions: Our results suggest that SNCA rearrangements may be more frequent than point mutations in ADPD. Furthermore, our results indicate that the phenotype associated with SNCA multiplications correlates with the number of copies of the gene and provides the first insight into the mechanisms underlying SNCA multiplication.


PARKINSON DISEASE (PD) (OMIM 168600) is a frequent progressive neurodegenerative disorder. Symptoms are caused mainly by loss of dopaminergic neurons in the substantia nigra. However, the mechanisms by which these neurons degenerate remain unknown. The study of familial forms of the disease compatible with mendelian inheritance and accounting for less than 10% of PD cases has led to the identification of 11 genes and/or loci implicated in the pathology. Polymeropoulos et al identified the first mutation causing PD (p.A53T) in the α-synuclein gene (SNCA; OMIM 163890) in a large Italian American family with autosomal dominant parkinsonism. After intensive screening of the SNCA gene in patients with PD of different origins, only 2 additional point mutations have been identified (p.A30P and p.E46K), suggesting that point mutations in SNCA are a rare cause of PD. In contrast, different duplications and triplications of the whole SNCA gene are found in families with autosomal dominant forms of the disease. More recently, SNCA duplications were also identified in rare, apparently sporadic cases. The severity of the phenotype, ranging from typical PD to dementia with Lewy bodies (DLB); (OMIM 127750) and PD dementia, and the brain structures affected have been shown to increase with the number of copies of the gene. Most SNCA duplication carriers (3 copies of the gene) have typical late-onset parkinsonism, whereas those with triplications (4 copies of the gene) have earlier-onset and atypical parkinsonism. However, owing to the limited number of studies, the prevalence and mechanisms leading to these rearrangements have not been precisely determined.

The aim of this study was to assess the frequency of SNCA rearrangements in a large series of families with typical or atypi-
cal parkinsonism compatible with autosomal dominant inheritance and to determine the mechanism by which they occur.

**METHODS**

**PATIENTS**

In this study, 345 patients with parkinsonism from 286 families (including 1st and 119th already published) compatible with autosomal inheritance were examined by a member of the French Parkinson’s Disease Genetics Study Group. A total of 210 families had at least 1 affected parent-child pair, and 76 families had at least 2 affected relatives in 2 different generations (unrelated or/and/or nephew-niece pairs). In addition, 37 of the 76 index cases had an affected sibling. Diagnostic criteria for definite PD were the presence of at least 3 of the following cardinal features: resting tremor, bradykinesia, rigidity, and good (>30%) response to levodopa therapy. In 264 of the families, exclusion criteria such as ophthalmoplegia, dementia, and dysautonomia early in the course of the disease, as well as apraxia, cerebellar, and pyramidal syndromes were absent. The clinical features of the 3 families carrying duplications (FPD-131, FPD-321, and FPD-410) have already been published. In addition, we screened 22 index cases with atypical parkinsonism. These patients had 1 or more atypical symptoms including pyramidal signs (n=8), early dementia (n=7), myoclonus (n=2), apraxia (n=2), supranuclear ophthalmoplegia (n=2), hyperventilation (n=1), axonal neuropathy (n=1), dysautonomia (n=1), no response to levodopa treatment (n=2), cerebellar ataxia (n=1), and subacute onset (n=1).

The mean (SD) age at onset of disease in index patients (140 women; 146 men) was 48.6±13.2 years (range, 8-86 years) and at examination was 57.3±13.0 years (range, 25-88 years). The 286 families were mostly of European descent (n=259), including families from France (n=230), Italy (n=11), Spain (n=4), Germany (n=3), the United Kingdom (n=3), Portugal (n=3), the Netherlands (n=1), Ireland (n=1), Greece (n=1), and Eastern Europe (n=2). There were also patients from North Africa (n=15), South (n=2) and North America (n=1), the West Indies (n=5), Turkey (n=1), Asia (n=1), and the Middle East (n=2).

Local ethics committees approved the study and written informed consent was obtained from all participants. Peripheral blood was collected from each patient; genomic DNA was extracted from leukocytes according to standard procedures and was used for subsequent molecular analyses.

**MOLECULAR ANALYSES**

**Detection of SNCA Rearrangements**

Dosage of the SNCA gene was performed by semiquantitative multiplex polymerase chain reaction (PCR), as previously described. Briefly, exons 3 and 4 of this gene were coamplified with 2 internal controls: a 236-base pair sequence of a reference gene, transthyretin (OMIM 176300), and the exon 4 of the parkin gene (OMIM 602544). Two diploid subjects were used as negative controls and a patient with a known heterozygous deletion of parkin exon 4 as positive control. The PCR products were quantified using GeneMapper 3.5 software (Applied Biosystems, Foster City, California) on an ABI 3730 genetic analyzer (Applied Biosystems). The number of copies of the gene was determined from the ratios of mutant to control alleles compared with ratios obtained from DNA of the control subjects. Ratios between 0.8 and 1.2 indicated a normal individual; between 1.3 and 1.7, a heterozygous duplication; and between 1.8 and 2.2, a triplication or homozygous duplication. Multiplied regions were further validated with intronic and flanking microsatellite markers in available members of positive families as previously reported; probands were tested for the LRRK2 G2019S mutation. All tests were performed at least twice.

**Refinement of the Multiplied Regions**

To determine the extent of the multiplied region in each positive family, we analyzed 23 existing and 13 newly developed quantitative microsatellite markers spanning approximately 8 megabase pairs (Mb) within the 4q21.3 to 4q22.3 genomic region (primer sequences available on the Genome Database human genome database Web site; http://www.gdb.org).

To pinpoint the breakpoint ends of the multiplied regions spanning SNCA, Affymetrix GeneChip Human Mapping 250K Nsp arrays genotyping and single-nucleotide polymorphism (SNP) dosage analysis were performed according to the manufacturer’s protocol (Affymetrix, Santa Clara, California). Affymetrix GCOS v.4.1 software was used to obtain raw microarray feature intensities (Raw microarray feature intensities [RAS] scores; raw data available on request). The RAS scores were processed using Affymetrix GTYPE v4.0 software to derive SNP genotypes. Copy number estimates were obtained using the program dChip. Raw copy number estimates were then smoothed using a Hidden Markov Model approach that treats copy number along the chromosome as a Markov process with a very low probability of switching between different copy number states.

The minimal size of the multiplied regions was defined by the last informative multiplied microsatellite or SNP marker and the maximal size extended up to the first informative but not multiplied marker on each boundary of the SNCA gene.

**Bioinformatics Analyses**

Information available from the Ensembl Genome Browser (http://www.ensembl.org), the University of California Santa Cruz Genome Browser (http://genome.ucsc.edu/), the National Center for Biotechnology Information Web site (http://www.ncbi.nlm.nih.gov/), and the Genome Database Web site (http://www.gdb.org) was used to design PCR primers and to identify known and predicted genes in the multiplied regions. Repeat elements and low-copy repeats at the centromeric and telomeric boundaries of each multiplied region were searched for using the RepeatMasker (http://www.repeatmasker.org/) and BLASTN (http://www.ncbi.nlm.nih.gov/blast/) programs. For this purpose, genomic sequences of interspersed sequence repeats that had been filtered out were compared with the complete human sequence genome draft.

**Haplotype Analysis**

To identify alleles shared among and within families and to further analyze the mechanism of the rearrangement in each family, haplotypes for all available members of families with SNCA gene multiplications were constructed manually, allowing a minimum number of recombinations. We used 7 microsatellite markers (NACP-REP1 and 6 newly developed markers) located within the shared SNCA-containing region. Flanking markers NACP-REP1 and D4S3460 are located approximately 9 and 10 kilobase pairs (Kb) from the 5’ and 3’ ends of the SNCA gene, respectively. The D4S3435 and D4S3436 markers are located in introns 1 and 2, respectively, with D4S3457, D4S3458, and D4S3459 in intron 3 of the SNCA gene.
Statistical Analysis

Comparisons between patient groups were made with the χ² test or the Fisher exact test when appropriate for qualitative variables and analyses of variance for quantitative variables using SPSS Software (SPSS Inc, Chicago, Illinois).

RESULTS

DETECTION AND REFINEMENT OF THE MULTIPLIED REGION CONTAINING SNCA

Using semiquantitative multiplex PCR to detect exon rearrangements, we found SNCA duplications in 4 families and a triplication in 1, but no deletions. The presence of extra copies of the whole SNCA gene in these families was confirmed by analyzing 2 flanking and 7 intragenic SNCA microsatellite markers. The families were from France (FPD-014, FPD-131, FPD-410, FPD-437) and Italy (FPD-321). All affected members of these families whose DNA was analyzed were heterozygous carriers of an SNCA multiplication. Analyses of 36 polymorphic microsatellite markers confirmed these results and allowed estimation of the size of the rearrangements. Refinement of the multiplied regions sizes in 4 of the 5 families was performed by dosage analysis using Affymetrix 250K SNP microarrays. In the remaining family (FPD-131), the duplication was confirmed, but the boundaries could not be refined because no more genomic DNA was available for analysis. The minimal size of the multiplied regions differed among the families and ranged from 0.42 to 4.50 Mb, suggesting that the events occurred independently (Figure 1). Family FPD-014 had a triplicated region of 2.61 to 2.64 Mb that contained 4 known genes (SNCA, multimerin 1 [MMRN1] [OMIM 601456], thymosin-like 3 [TMSL3]; NM_183049], G protein–regulated inducer of neurite outgrowth 3 [GPRIN3] [OMIM 611241]), and a gene encoding the hypothetical protein LOC401145 (NP_997374.1). Members of family FPD-131 had the largest duplication (4.50-5.29 Mb), according to microsatellite dosage, which contained 33 to 34 genes (Figure 1). Several of these genes encode mitogen-activated protein (MAP) kinases or phosphatases; 3 others encode members of the HECT domain and RCC1-like domain family (HERC1) of ubiquitin ligases. Five of these genes, the MAP kinase 10 (MAPK10) (OMIM 602897), SNCA, MMRN1, polycystic kidney disease 2 (PKD2) (OMIM 173910), and dentin matrix acidic phosphoprotein (DMP1; OMIM 600980) genes have been associated with human diseases. The duplicated region in family FPD-321 was the second largest (3.47-3.58 Mb) and contained at least 23 genes. The duplicated region in family FPD-410 (approximately

Figure 1. Breakpoint analysis of 5 families with SNCA multiplications. Mb indicates megabase pairs; min, minimum; and max, maximum.
0.63-0.65 Mb) contained SNCA and MMRN1. The duplication in family FPD-437 (approximately 0.42-0.43 Mb) contained only SNCA.

RepeatMasker software revealed a variable number of interspersed repetitive elements, including Alu elements, long interspersed nuclear elements, and long terminal repeats in both the centromeric and telomeric boundaries of the multiplied regions containing SNCA in all families except FPD-321 and FPD-014. No low-copy repeats, 10- to 400-kb DNA blocks with more than 95% identity between copies, were found in the intervals containing the breakpoints.

**HAPLOTYPE ANALYSIS**

Analysis of 7 microsatellite markers within the SNCA multicopy region showed that each family had a different set of alleles, confirming that the mutations were not inherited from a common ancestor (Figure 2). In families FPD-131, FPD-321, and FPD-410, all SNCA duplication carriers had 2 identical copies of the region (Figure 2). Similarly, the SNCA triplication carrier FPD-014-011 had 3 identical copies of the triplicated haplotype plus a haplotype of the region that he shared with his unaffected brother. In contrast, both duplication carriers from family FPD-437 shared a duplicated region containing 2 haplotypes, but the haplotype of the third copy of the region was not shared (Figure 2).

**CLINICAL CHARACTERISTICS**

All families with duplications had typical PD, whereas the family with a triplication had atypical features. Patients with duplications and triplications of the SNCA gene had similar ages at examination, ages at onset, and disease durations (Table). In the 4 families with SNCA duplications, all patients had typical PD that was similar to the phenotype of patients with typical autosomal dominant PD (ADPD) without SNCA rearrangements. Two SNCA duplication carriers were still asymptomatic at ages 56 and 54 years (FPD-321-015 and FPD-321-017; Figure 2). By 8 and 10 years of age, respectively, both were older than the age at onset of their affected sister (FPD-321-21) whose disease began at 46 years but had not reached the latest age at onset in patients with duplications in this series, which was 65 years. These observations were consistent with incomplete age-dependent penetrance.

The patients carrying SNCA triplications had atypical parkinsonism with rapidly evolving symptoms and severe cognitive impairment. Their response to levodopa treatment was limited at onset or decreased very rapidly after onset. Triplication carriers had shorter disease durations until death than duplication carriers (mean [SD] disease duration, 7.0 [2.6] years; range, 5-10 years; n = 3 vs 10.5 [7.2] years; range, 1-23 years; n = 3), assuming that patients who could not be analyzed for gene dosage carried the multiplication. Owing to the small number of cases, however, the difference was not statistically significant. Despite shorter disease durations, patients with triplications had more severe dementia (mean [SD] Mini-Mental State Examination score, 15.0 [12.8]; range, 6-24; n = 2 vs 27.8 [2.0]; range, 26-30; n = 5; P = .05), were more severely affected (mean [SD] Unified Parkinson Disease Rating Scale motor score during treatment, 44.5 [2.1]; range, 43-46; n = 2 vs 11.7 [6.1]; range, 5-17; n = 3; P = .006), and had urinary incontinence more frequently (3/3 vs 0/6; P = .01) than those with duplications.
In addition to PD caused by SNCA multiplications, a very limited number of human genetic diseases have been reported to be caused by increased gene dosage, including autism spectrum disorder (OMIM 209850, caused by duplication or deletion of contactin 4 [CNTN4] (OMIM 607280)),17 Charcot-Marie Tooth disease type 1A (OMIM 118220, caused by duplication of the peripheral myelin protein 22 [OMIM 601097] locus),18 Pelizaeus-Merzbacher disease (OMIM 312080, caused by duplication of proteo-lipid protein 1 [OMIM 300401]),19 early-onset Alzheimer disease (OMIM 104760.0020, caused by duplication of the amyloid precursor protein [OMIM 104760] locus).20 In this study, we found 4 of 264 families (1.5%) with typical ADPD that carried SNCA triplications have been associated with dementia with Lewy bodies, indicating that the increase in the number of copies of SNCA has an effect on phenotype.6-11 However, 2 recent studies reported SNCA duplications in patients with either dementia7 or variable symptoms resembling those of multiple system atrophy.6 The absence of dementia in our patients with SNCA duplications may be more frequent than point mutations in familial PD.12-22 Our results suggest that SNCA duplications might be due to shorter disease durations or to ages at onset that were earlier than those previously reported (mean [SD], 46.0 [8.7] vs 52.0 [13.3] years).6,7,9 Conversely, the age at onset in our patients with an SNCA triplication was later (48.3 [12.5] years) than that of the Iowan (36.0 [10.5] years)11 or Swedish American (31 years) patients.8,10 Comparison of patients carrying duplications and triplications of the SNCA gene showed no statistically significant differences in age at examination, age at onset, or disease duration. However, patients with triplications had shorter disease durations until death than duplication carriers. They had more severe dementia, were more severely affected, and had more frequent urinary incontinence than those with

### Table. Clinical Comparison of Patients With PD With Typical or Atypical Presentations and With SNCA Duplications or Triplications

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>SNCA Triplcation (n=3, 1 Genotyped)</th>
<th>SNCA Duplication (n=9, 7 Genotyped)</th>
<th>Atypical Parkinsonism (n=24)</th>
<th>Typical Parkinsonism (n=313)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean (SD)</td>
<td>Mean (SD)</td>
<td>P Value</td>
<td>Mean (SD)</td>
</tr>
<tr>
<td>Sex, women:men</td>
<td>1:2</td>
<td>7:2</td>
<td>.24</td>
<td>16:18</td>
</tr>
<tr>
<td>Age, y</td>
<td>55.3 (12.2)</td>
<td>42-66</td>
<td>.88</td>
<td>56.8 (15.2)</td>
</tr>
<tr>
<td>Age at onset, y</td>
<td>48.3 (12.5)</td>
<td>36-61</td>
<td>.72</td>
<td>49.9 (14.3)</td>
</tr>
<tr>
<td>Disease duration, y</td>
<td>7.0 (2.6)</td>
<td>5-10</td>
<td>.43</td>
<td>6.9 (7.8)</td>
</tr>
<tr>
<td>Age at death, y</td>
<td>58.7 (15.6)</td>
<td>42-73 (3)</td>
<td>.50</td>
<td>8.7 (6.9)</td>
</tr>
<tr>
<td>Clinical signs at onset</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Micrography, No.</td>
<td>0/3</td>
<td>1/7</td>
<td></td>
<td>10/41</td>
</tr>
<tr>
<td>Rest tremor, No.</td>
<td>2/3</td>
<td>2/7</td>
<td></td>
<td>5/18/43</td>
</tr>
<tr>
<td>Rigidity, No.</td>
<td>3/3</td>
<td>8/9</td>
<td></td>
<td>39/43</td>
</tr>
<tr>
<td>Asymmetry, No.</td>
<td>2/3</td>
<td>7/9</td>
<td></td>
<td>&gt;99/21/43</td>
</tr>
<tr>
<td>Self-estimated levodopa</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>improvement</td>
<td>36 (22)</td>
<td>17-60</td>
<td>.43</td>
<td>34 (19)</td>
</tr>
<tr>
<td>Clinical signs at examination</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bradykinesia, No.</td>
<td>3/3</td>
<td>9/9</td>
<td></td>
<td>35/44</td>
</tr>
<tr>
<td>Rigidity, No.</td>
<td>3/3</td>
<td>8/9</td>
<td></td>
<td>&gt;99/39/43</td>
</tr>
<tr>
<td>Rest tremor, No.</td>
<td>2/2</td>
<td>6/9</td>
<td></td>
<td>&gt;99/99</td>
</tr>
<tr>
<td>Urinary incontinence, No.</td>
<td>3/6</td>
<td>0/6</td>
<td></td>
<td>.01/11/40</td>
</tr>
<tr>
<td>MMSE scoreb</td>
<td>15.0 (12.8)</td>
<td>6-24 (2)</td>
<td>.05</td>
<td>25 (7.0)</td>
</tr>
<tr>
<td>UPDRS score ond</td>
<td>44.5 (2.1)</td>
<td>43-46 (2)</td>
<td>.006</td>
<td>26.2 (16.2)</td>
</tr>
<tr>
<td>Daily dose of levodopa, mg</td>
<td>51.2 (6.7)</td>
<td>47-56 (2)</td>
<td>.69</td>
<td>34.8 (14.9)</td>
</tr>
<tr>
<td>Duration of treatment, y</td>
<td>NA</td>
<td>6.3 (3.4)</td>
<td>.3-12 (5)</td>
<td>7.1 (8.9)</td>
</tr>
<tr>
<td>Dysthnia</td>
<td>NA</td>
<td>4/5</td>
<td></td>
<td>6/6</td>
</tr>
<tr>
<td>Fluctuation</td>
<td>NA</td>
<td>3/5</td>
<td></td>
<td>2/7</td>
</tr>
<tr>
<td>Dystonia</td>
<td>NA</td>
<td>2/4</td>
<td></td>
<td>6/20</td>
</tr>
</tbody>
</table>

Abbreviations: MMSE, Mini-Mental State Examination; NA, not available; PD, Parkinson disease; UPDRS, Unified Parkinson Disease Rating Scale.

a The maximum score for the MMSE is 30 points.

b Indicates the number of patients with available data for the corresponding feature.

c The maximum score for the UPDRS is 180. On and off indicate that the scale was rated with or without treatment with levodopa, respectively.
duplications, underlining the dosage effect of SNCA multiplications.

The size of the rearrangements estimated by dosage of microsatellite markers was confirmed and refined by Affymetrix 250K SNP microarrays in all but one family who could not be analyzed because no more genomic DNA was available. Interestingly, our study shows that the size of the duplicated region and the number of genes it contains has no effect on phenotype. There are no additional signs or greater severity in family FPD-131 with at least 33 more duplicated genes than in families in which SNCA alone (FPD-437) or SNCA and MMRN1 (FPD-410) were duplicated. Nishioka et al7 speculated that MMRN1 overexpression may contribute to the dementia phenotype because one demented patient carried a genomic duplication containing both SNCA and MMRN1 in their entirety, whereas the duplicated region in the patient without dementia contained all of SNCA but only part of MMRN1. Our results do not confirm this hypothesis because the patients carrying multiplications of both SNCA and MMRN1 in our series did not have dementia. In addition, the MMRN1 gene is not expressed in the brain.

Penetrance of SNCA multiplications is age dependent and might also be reduced, as suggested by previous studies. However, the genetic basis of this age-at-onset variability is not known. It is probably not related to the size of the multiplication because the 2 unaffected carriers belong to the family with the second largest duplication. We have also excluded the LRRK2 G2019S mutation, which was absent in all of the families with SNCA multiplication, as a potential modifier.

The mutations found in the 5 families have different sizes, and the haplotypes in the rearranged region are not the same, indicating that all 5 mutations occurred independently. Furthermore, our haplotype analysis suggests that different mechanisms are involved in the generation of SNCA multiplications. For 3 duplications and the triplication, a single sequence was multiplied and transmitted in each family. This suggests an unequal intrachromosomal rearrangement after recombination between sister chromatids. In the 2 patients of family FPD-437, however, the microsatellite alleles on the 2 copies of the duplicated region were different, suggesting that the duplication resulted from a nonallelic homologous recombination between 2 homologous chromosomes. This mechanism is frequent in genomic disorders and is usually the consequence of nonallelic homologous recombination between low-copy repeats that flank unique genomic segments. However, in our study, bioinformatics analyses of all of the multiplied regions containing SNCA revealed no large flanking low-copy repeats, but rather numerous repetitive elements interspersed at the breakpoints. Interspersed repetitive elements at breakpoints have been reported elsewhere and their presence has been postulated to be a frequent cause of deletions and duplications by nonallelic homologous recombination. Interestingly, the most comprehensive study of genome-wide human segmental duplications found an enrichment of Alu repeats near or within breakpoints.

Our study indicates that the phenotype associated with SNCA multiplications correlates with the number of copies of the SNCA gene, but not with the number of copies of other genes in the multiplied region, and provides the first insight into the mechanisms underlying SNCA multiplication. Because disorders resulting from genome rearrangements are thought to occur with equal frequency in all populations, SNCA multiplications could account for approximately 2% of all ADPD cases.

Accepted for Publication: May 19, 2008.

Author Affiliations: Institut National de la Santé et de la Recherche Médicale (INSERM), Unité Mixte de Recherche en Santé (UMR) S679 Neurologie & Thérapeutique Expérimentale, F-75013, Paris, France (Drs Ibáñez, Lesage, Lohmann, Bonnet, Agid, Durr, and Brice and Ms Janin); Université Pierre et Marie Curie (UPMC) Univ Paris 06, UMR S679, F-75005, Paris, France (Drs Ibáñez, Lesage, Lohmann, Bonnet, Agid, Durr, and Brice and Ms Janin); Wellcome Trust Centre for Gene Regulation and Expression, University of Dundee, Dundee, Scotland (Dr Ibáñez); Equipe Mixte INSERM (EMI–INSERM) 9904, Service de Neurologie, Hôpital Gabriel Montpied, Clermont-Ferrand, France (Dr Durif); Equipe d'Accueil (EA) 2683, Service de Neurologie, Hôpital R. Salengro, Centre Hospitalier Régional Universitaire de Lille, Lille, France (Dr Destée); Assistance Publique–Hôpitaux de Paris (AP-HP), Pitié-Salpêtrière Hospital, Department of Nervous System Disorders, F-75013, Paris, France (Drs Bonnet and Agid); Centre d’Investigation Clinique, Service de Pharmacologie Médicale et Clinique, Faculté de Médecine, Centre Hospitalier Universitaire Toulouse, Toulouse, France (Dr Brefel-Courbon); Commissariat à l’Energie Atomique, Institut de Génomique, Centre National de Génotypage, Evry, France (Drs Heath and Zelenika); and AP-HP, Pitié-Salpêtrière Hospital, Department of Genetics and Cytogenetics, F-75013, Paris, France (Drs Durr and Brice).

French Parkinson’s Disease Genetics Study Group Investigators: Yyves Agid, MD; Anne-Marie Bonnet, MD; Micheal Borg, MD; Alexis Brice, MD; Emmanuel Broussolle, MD, PhD; Phillippe Damier, MD, PhD; Alain Destée, MD; Alexandra Durr, MD, PhD; Frank Durif, MD; Ebba Lohmann, MD; Maria Martinez, PhD; Christianne Penet; Pierre Pollak, MD; Olivier Rascol, MD, PhD; Francois Tison, MD, PhD; Christine Tranchant, MD; Marc Vérin, MD, PhD; Francois Viallet, MD; Marie Vidailhet, MD.

Correspondence: Alexis Brice, MD, INSERM UMR 679 (former U289), Hôpital de la Salpêtrière, 47 Boulevard de l’Hôpital, 75651 Paris Cedex 13, France (alexis.brice@upmc.fr).

Author Contributions: Drs Brice, Ibáñez, Lesage, Lohmann, and Durr had full access to all of the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. Study concept and design: Ibáñez, Durr, and Brice. Acquisition of data: Ibáñez, Lohmann, Durr, Destée, Bonnet, Brefel-Courbon, and Agid. Analysis and interpretation of data: Ibáñez, Lesage, Janin, Durif, Heath, Zelenika, and Brice. Drafting of the manuscript: Ibáñez, Lesage, Janin, Lohmann, Bonnet, Zelenika, Agid, Durr, and Brice. Critical revision of the manuscript for important intellectual content: Ibáñez, Lesage, Durif, Destée, Brefel-Courbon, Heath, Agid, Durr, and Brice. Statistical analysis: Heath and

Financial Disclosure: None reported.

Funding/Support: This study was supported by the French Ministry of Research and Technology and the Verum Foundation (Dr Ibáñez); European Union contract LSHM-CT-2003-503330/APOPIS from the European Commission; and the Association France-Parkinson.

Additional Contributions: We are grateful to the patients and their families. We thank Merle Ruberg, PhD, for critical reading of the manuscript and the DNA and Cell Bank of IFR70 for sample preparation.

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15. Zelenika. Obtaining funding: Ibáñez, Durr, and Brice. Administrative, technical, and material support: Ibáñez, Lesage, Janin, Lohmann, Brefel-Courbon, Durr, Zelenika, Agid, and Brice. Study supervision: Lesage, Durif, Destee, Durr, and Brice. Financial Disclosure: None reported. Funding/Support: This study was supported by the French Ministry of Research and Technology and the Verum Foundation (Dr Ibáñez); European Union contract LSHM-CT-2003-503330/APOPIS from the European Commission; and the Association France-Parkinson. Additional Contributions: We are grateful to the patients and their families. We thank Merle Ruberg, PhD, for critical reading of the manuscript and the DNA and Cell Bank of IFR70 for sample preparation.

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