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27. Polyclonal $Ab_{P\text{-}Thr286}$ was generated in rabbits using as antigen phosphopeptide His-Arg-Gln-Glu-Thr($PO_4$)-Val-Asp-Cys-Leu, corresponding to residues 282 to 290 of α-CaM-KII, conjugated to keyhole limpet hemocyanin. Serum was passed over a column of nonphosphopeptide coupled to agarose, and the flow-through was affinity purified on a phosphopeptide column. A similar antibody has been previously described (*25*). For protein immunoblots using $Ab_{P\text{-}Thr286}$, 1 μM microcystine was included in the blotting buffer to inhibit protein phosphatases in the Carnation milk.
28. Hippocampal slices from male Sprague-Dawley rats (4 to 6 weeks old) were prepared and maintained at 33°C and pH 7.4 in an interface chamber under continuous perfusion as previously described (*10*). The $^{32}P$-labeled slices were incubated in phosphate-free medium for 15 min and then in $^{32}PO_4$ (0.5 mCi/ml) for 45 min before induction of LTP. The LTP was induced by theta-burst stimulation, whereas control slices received low-frequency stimulation; both protocols were applied by coactivation of two independent groups of Schaffer-commissural fibers (*10*). At 5, 15, or 60 min after stimulation, the CA1 region was dissected and frozen in liquid nitrogen. Three CA1 slices (either from LTP or CON) were pooled together and homogenized at 5°C in 500 mM NaCl, 30 mM $Na_4P_2O_7$, 50 mM NaF, 50 mM tris-HCl, 200 mM EDTA (pH 7.5), 200 mM EGTA, 100 mM $Na_3VO_4$, 0.1 mM leupeptin, 1 mM phenylmethylsulfonyl fluoride, soybean trypsin inhibitor (20 μg/μl), 1 μM microcystine-LR, 0.5% Triton X-100, and 0.1% deoxycholate. Samples were sonicated and centrifuged at 10,000 rpm for 15 min, and 10 μl of supernatant was spotted onto P81 papers, which were then washed in 10% trichloroacetic acid to determine total $^{32}P$-protein. Supernatants were diluted to give equal amounts (in counts per minute) of $^{32}P$-protein, incubated with GluR1 antibody (*13*) and protein A–Sepharose at 4°C for 5 hours, and centrifuged. CaM-KII was then immunoprecipitated from the supernatant (*20*). Immunoprecipitates were run on SDS–polyacrylamide gel electrophoresis (PAGE) and exposed on a Phosphor Screen (Molecular Dynamics) for analyses. The SDS-PAGE of the GluR1 immunoprecipitates were also subjected to protein immunoblot analyses to quantitate the amount of GluR1. Protein content of homogenates was determined by protein assay (Bio-Rad).
29. $^{32}P$–AMPA-R was immunoprecipitated (GluR1 antibody) from hippocampal slices or HEK-293 cells expressing GluR1 ± CaM-KII H282R (*26*). Immunoprecipitated AMPA-R was isolated by SDS-PAGE; $^{32}P$–AMPA-R was cut from the gel, subjected to two digestions with trypsin (16 and 4 hours with 75 μg of trypsin per milliliter), oxidized, and subjected to two-dimensional peptide mapping (cathode on the right) (*20*).
30. AMPA GluR1(flip) was expressed in HEK-293 cells by the lipofectine method (Glbco protocol) with the use of 2 μg of plasmid per 35-mm dish for transfection of $5 \times 10^4$ to $7 \times 10^4$ cells. Whole cell recordings were made 16 to 20 hours after transfection. Patch pipette solution was 160 mM CsCl, 2 mM $MgCl_2$, 4 mM Na-ATP, 1 mM EGTA, and 10 mM Hepes. Series resistance of 10 to 12 megohms was 80% compensated and monitored through the recordings. Extracellular solution contained 165 mM NaCl, 2.5 mM KCl, 1 mM $MgCl_2$, 0.2 mM $CaCl_2$, and 5 mM Hepes. When added to intracellular solution, activated CaM-KII [truncated at residue 316 and autothiophosphorylated (*8*)] was at 0.4 μM. Glutamate (10 mM) was delivered to single cells by rapid application (*9*). Currents were filtered at 2 kHz and digitized at 10 kHz.
31. This work was supported by NIH (NS27037, T.R.S.), the Swiss National Research Foundation (3100-040815.94, D.M.), the International Human Frontier Science Program (RG-30/96B, D.M. and T.R.S.), and an Alfred P. Sloan and Klingenstein fellowship (L.G.). We thank D. Brickey for providing the activated CaM-KII, and Z. Wang for making the P-Thr[286] antibody.

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# Mutation in the α-Synuclein Gene Identified in Families with Parkinson's Disease

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Parkinson's disease (PD) is a common neurodegenerative disorder with a lifetime incidence of approximately 2 percent. A pattern of familial aggregation has been documented for the disorder, and it was recently reported that a PD susceptibility gene in a large Italian kindred is located on the long arm of human chromosome 4. A mutation was identified in the α-synuclein gene, which codes for a presynaptic protein thought to be involved in neuronal plasticity, in the Italian kindred and in three unrelated families of Greek origin with autosomal dominant inheritance for the PD phenotype. This finding of a specific molecular alteration associated with PD will facilitate the detailed understanding of the pathophysiology of the disorder.

Parkinson's disease (PD) was first described by James Parkinson in 1817 (*1*). The clinical manifestations of this neurodegenerative disorder include resting tremor, muscular rigidity, bradykinesia, and postural instability. A relatively specific pathological feature accompanying the neuronal degeneration is an intracytoplasmic inclusion body, known as the Lewy body, which is found in many regions, including the substantia nigra, locus ceruleus, nucleus basalis, hypothalamus, cerebral cortex, cranial nerve motor nuclei, and the central and peripheral divisions of the autonomic nervous system (*1*).

In many cases a heritable factor predisposes to the development of the clinical syndrome (*2*). We have recently shown that genetic markers on human chromosome 4q21–q23 segregate with the PD phenotype in a large family of Italian descent (*3*). The clinical picture of the PD phenotype in the Italian kindred has been well documented to be typical for PD, including Lewy bodies, with the exception of a relatively earlier age of onset of illness at 46 ± 13 years. In this family the penetrance of the gene (the proportion of people with the genotype who actually manifest the disease) has been estimated to be 85%, suggesting that a single gene defect will be sufficient to determine the PD phenotype.

Alpha-synuclein, a presynaptic nerve terminal protein, was originally identified as the precursor protein for the non–β amyloid component of Alzheimer's disease amyloid plaques NAC (*4*). The human α synuclein gene was previously mapped in the 4q21–q22 region (*5*). Genotype analysis in the Italian PD kindred with additional genetic markers showed recombination events. One recombination was observed for genetic marker D4S2371 at the centromeric end of the PD interval and one recombination was inferred for marker

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D4S2986 at the telomeric end of the interval. These recombinations redefined the location of the PD gene to an interval of approximately 6 cM between markers D4S2371 and D4S2986 (6). A minimal physical contig of yeast artificial chromosome (YAC) clones was constructed to span the interval from marker D4S2371 to marker D4S2986 (6). Using this contig, we established that the α-synuclein gene is located within the D4S2371–D4S2986 interval, just telomeric to marker D4S2371. Thus, α-synuclein represented an excellent candidate gene for PD. Sequence analysis of the fourth exon of the α-synuclein gene (7) revealed a single base pair change at position 209 from G to A (G209A) relative to the published sequence of the gene (GenBank ID L08850), which results in an Ala to Thr substitution at position 53 (Ala53Thr) and the creation of a novel Tsp45 I restriction site (Fig. 1). Mutation analysis for the G209A change in the Italian kindred showed complete segregation with the PD phenotype with the exception of individual 30, who is affected but not carrying this mutation (Fig. 2A). This individual apparently inherited a different PD mutation from his father because we have shown that he shares a genetic haplotype with his unaffected maternal uncle, individual 3, for genetic markers in the PD linkage region.

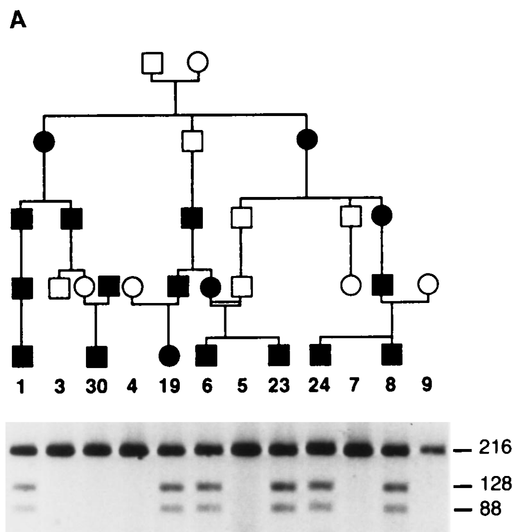
The frequency of this variation was studied in two general population samples, one consisting of 120 chromosomes of the parents of the CEPH (Centre d'Etude du Polymorphisme Humain) reference families, and the other consisting of 194 chromosomes of unrelated individuals from the blood bank in Salerno, Italy, a city near the town from which the family originated. Of these 314 chromosomes, none was found to carry the G209A mutation. Fifty-two patients of Italian descent with sporadic PD were also screened for the mutation, along with five individuals who had been used to identify previously unpublished Greek families (6).

The Ala53Thr change was found to be present in three of the Greek kindreds, and it segregated with the PD phenotype (Fig. 2B). In those three Greek kindreds the age of onset for the disease is relatively early, ranging from the mid-30s to the mid-50s. The Ala53Thr substitution in four independent PD families and its absence from 314 control chromosomes provides the strongest genetic evidence that this mutation in the α-synuclein gene is associated with the PD phenotype in these families.

We have also demonstrated by amplification by the polymerase chain reaction (PCR) of reverse-transcribed mRNA (RT PCR) that the mutant allele is transcribed in the lymphoblast cell line of an affected individual from the Italian kindred (Fig. 3) (8). These data indicate that the mutant allele is transcribed and, although no protein expression data are yet available, it is reasonable to assume that the mutant protein is indeed expressed.

The Ala53Thr substitution is localized in a region of the protein whose secondary structure predicts an α helical formation, bounded by β sheets. Substitution of the alanine with threonine is predicted to disrupt the α helix and extend the β sheet structure. Beta pleated sheets are thought to be involved in the self-aggregation of proteins, which could lead to the formation of amyloid-like structures (9). This was already tested in the case of NAC35, the 35–amino acid peptide derived from α-synuclein that was first isolated from plaques found in patients with Alzheimer's disease (4, 9). NAC35 is located in the middle of the α-synuclein molecule and extends from amino acid position 61 to 95. Residue 53, which is mutated in PD, is outside the NAC35 peptide found in amyloid plaques. However, the true size of the peptide involved in the plaques is not known as the protease used to isolate the peptide cuts at lysine 60 of the α-synuclein protein. In cross-linking experiments with β amyloid, it was demonstrated (9) that

**Fig. 1.** DNA sequence of the PCR product used for mutation detection. Oligonucleotide primers are shown by arrows and the numerals 3 and 13. Intron sequence is shown in lower case and exon sequence in upper case. Amino acid translation of the exon is shown below the DNA sequence. The circled base represents the G209A change in the mutant allele. The resulting amino acid Ala53Thr change is represented by the circled amino acid.



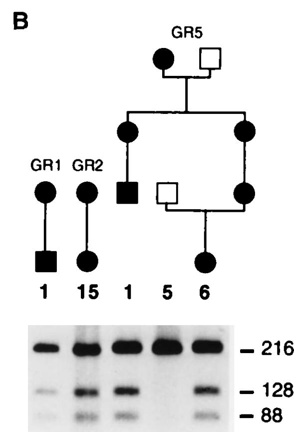




**Fig. 2.** Mutation analysis of the G209A change is shown in a subpedigree of the Italian kindred (**A**) and the three (GR1, GR2, GR5) Greek PD kindreds (**B**). Filled symbols represent affected individuals. Numerical identifiers denote the individuals immediately above. Tsp45 I digestion of PCR products (5) is shown at the bottom of the figure, and fragment sizes are indicated on the right in base pairs.

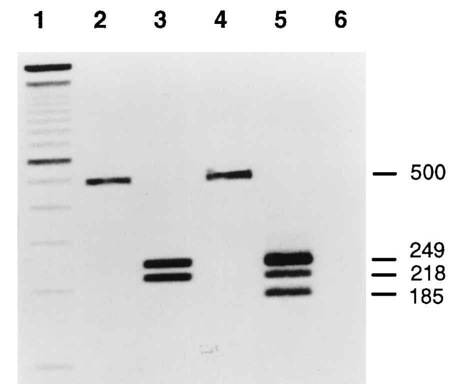



**Fig. 3.** Mutation analysis of the G209A change in RT PCR products. Lane 1: 100-bp ladder, lanes 2 and 3, normal control; lanes 4 and 5, PD patient; lane 6, negative control without RT enzyme. Sizes are indicated on the right in base pairs. Lanes 2 and 4 show uncut DNA and lanes 3 and 5 show DNA cut with Tsp45 I.

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residues 1 to 56 and 57 to 97 specifically bind amyloid and that a synthetic peptide consisting of residues 32 to 57 performed similarly.

Three members of the synuclein family have been characterized in the rat, with *SYN1* exhibiting 95% similarity to the human α-synuclein protein (*10*). *SYN1* of the rat is expressed in many regions of the brain, with high levels found in the olfactory bulb and tract, the hippocampus, dentate gyrus, habenula, amygdala, and piriform cortex, and intermediate levels in the granular layer of the cerebellum, substantia nigra, caudate-putamen, and dorsal raphe (*10*). This pattern of expression coincides with the distribution of the Lewy bodies found in brains of patients with Parkinson's disease. Decreases in olfaction often accompany the syndromic features of Parkinson's disease, and it is proposed that in many cases hyposmia (decreased sense of smell) is an early sign of the illness (*11*).

In the zebra finch the homolog to α-synuclein, synelfin, is thought to be involved in the process of song learning, suggesting a possible role for synuclein in memory and learning (*12*). In contrast to humans, rats have a threonine at the same position in their homologs to the human α-synuclein gene (Fig. 4). Similarly, the zebra finch synelfin carries a threonine, whereas both *Bos taurus* and *Torpedo californica* (*13*) do not. There are no reports that suggest the presence of Lewy bodies in the brains of the rat or the zebra finch or a phenotype resembling that of PD. Lack of any phenotype could be explained by a combination of factors, such as the relatively short life-span of rodents, the need for interaction with other cellular components not present in the rat, absence of a critical environmental trigger in the rodents, or a requirement for heterozygous status for the production of a phenotype.

Studies of early onset AD have previously documented that missense mutations can cause an adult onset neurodegenerative disorder. Of the 31 mutations described so far in the loci for presenilin 1 and 2, 30 were missense and 1 was a splice variant (*14*). Missense mutations in the prion protein have also been implicated in the amyloid production seen in Gerstmann-Straüssler-Scheinker and Creutzfeld-Jakob diseases, both forms of spongiform encephalopathy (*15*). Studies in these neurodegenerative disorders have pointed to the importance of the physical chemical properties of mutant cellular proteins in initiating and propagating neuronal lesions leading to disease. Similar studies in the synuclein protein family may provide valuable insights into the etiology and pathogenesis of PD.

Although the mutation identified in the α-synuclein gene is unlikely to account for the majority of sporadic and familial cases of PD, it may account for a significant proportion of those early-onset families with PD characterized by a highly penetrant, autosomal dominant inheritance. Even if the mutation we have described is directly related to only a small fraction of the total number of PD patients, it provides a clue that should lead to the understanding of the underlying pathways resulting in the symptoms of PD.

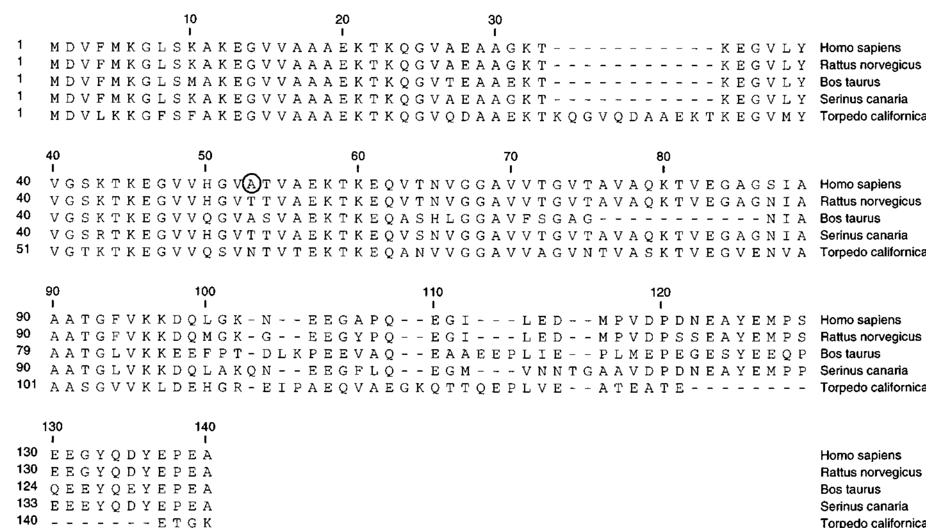


**Fig. 4.** Sequence alignments of α-synuclein homologues in different species. Accession numbers for the sequences used were as follows: *Homo sapiens* Swiss-Prot P37840, *Rattus norvegicus* Swiss-Prot P37377, *Bos taurus* Swiss-Prot P33567, *Serinus canaria* GenBank L33860, and *Torpedo californica* Swiss-Prot P37379. Numbering on top of the alignments is according to the human sequence. Amino acid 53, which is the site of the Ala53Thr change, is circled.

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7. DNA samples were collected after informed consent. For mutation analysis genomic DNA was amplified with primer 3 (5′-GCTAATCAGCAATTTAAGGCTAG-3′) and primer 13 (5′-GATATGTTCTTAGATGCTCAG-3′) of the DNA sequence (GenBank ID U46898) under standard PCR conditions. Sequence analysis was performed with the Perkin-Elmer dye terminator cycle sequencing kit on an ABI 373 fluorescent sequencer (ABI, Foster City, CA). Restriction digestion was done after the PCR with Tsp45 I according to the manufacturer's protocol (New England Biolabs, Beverly, MA). The digested PCR products were separated by electrophoresis on a 6% Visigel (Stratagene, La Jolla, CA) and visualized by ethidium bromide staining. Pedigree structures in Fig. 2 have been slightly modified to protect patient confidentiality.
8. Total RNA was extracted from the lymphoblastoid cell line of an affected individual, and first-strand synthesis was performed by oligo (dT) priming (Gibco-BRL, Gaithersburg, MD). Primer 1F: (5′-ACGACAGTGTGGTGTAAAGG-3′) and primer 13R 5′-AACATCTGTCAGCAGATCTC-3′ corresponding to nucleotides 21 to 40 and 520 to 501 of the published sequence were used to amplify a product of 500 bp containing the mutation nucleotide 209. PCR products were subjected to restriction digestion by Tsp45 I (5). The mutation at nucleotide 209 creates a novel Tsp45 I site (Fig. 1), so that the normal allele can be cut into four fragments of 249, 218, 24, and 9 bp, whereas the mutant allele will have five fragments of 249, 185, 33, 24, and 9 bp (Fig. 3). Size standards used were the 100-bp ladder as provided by Gibco-BRL (Gaithersburg, MD).
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16. Abbreviations for the amino acid residues are: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Try.


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