

sequence of 17 amino acids within H5 (432–448) as a β hairpin that extends ~ 20 Å into the membrane. The juxtaposition of β -hairpins from each subunit of the channel could form the central pore as a β barrel that spans the distance between the internal and external vestibules. β -barrel channel structures have been proposed for some less selective ion channels such as the voltage-dependent anion channel and porins^{18,19}.

Our mutations increased permeability to larger cations, Rb⁺ and NH₄⁺, without removing the ability of the channel to exclude Na⁺. These data provide evidence that different structural mechanisms are involved in selecting against cations that are larger or smaller than K⁺. These results are consistent with previous models in which multiple barriers exploit both steric hindrance and differences in ionic charge density^{2,20}. If the

channel complex is a tetramer, changing a single residue would produce a fourfold symmetrical change in the channel. Mutation of threonine to serine, for example, could increase the local pore diameter by the equivalent of two methyl groups. Thus, the flux of larger ions through the channel could be increased if the altered residue contributes to a limiting constriction. The absence of changes in K⁺ conductance in our mutants suggests that a binding site of intermediate field strength, not affected by the mutations, remains limiting for K⁺ flux but has less influence on Rb⁺ and NH₄⁺, which have lower charge densities. Further mutagenesis in H5 and in the equivalent S5–S6 linker of Na⁺ and Ca²⁺ channels may reveal the molecular bases of the energy barriers that limit permeation and determine ionic selectivity. □

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1. Bezanilla, F. & Armstrong, C. M. *J. gen. Physiol.* **60**, 558–608 (1972).
2. Hille, B. & Schwarz, W. *J. gen. Physiol.* **72**, 409–442 (1978).
3. Armstrong, C. M. *Q. Rev. Biophys.* **7**, 179–210 (1975).
4. Latorre, R. & Miller, C. *J. memb. Biol.* **71**, 11–30 (1983).
5. MacKinnon, R. & Yellen, G. *Science* **250**, 276–279 (1990).
6. Tempel, B. L., Papazian, D. M., Schwarz, T. L., Jan, Y. N. & Jan, L. Y. *Science* **237**, 770–775 (1987).
7. Schwarz, T. L., Tempel, B. L., Papazian, D. M., Jan, Y. N. & Jan, L. Y. *Nature* **331**, 137–142 (1988).
8. Timpe, L. C. *et al. Nature* **331**, 143–145 (1988).
9. Timpe, L. C., Jan, Y. N. & Jan, L. Y. *Neuron* **1**, 659–667 (1988).
10. Iverson, L. E., Tanouye, M. A., Davidson, N. & Rudy, B. *Proc. natn. Acad. Sci. U.S.A.* **85**, 5723–5727 (1988).
11. Zagotta, W. N., Hoshi, T. & Aldrich, R. W. *Proc. natn. Acad. Sci. U.S.A.* **86**, 7243–7247 (1989).
12. Guy, H. R. in *Monovalent Cations in Biological Systems* (ed Pasternak, C. A.) (CRC Press, Cleveland, Ohio, 1989).
13. Jan, L. Y. & Jan, Y. N. *Nature* **345**, 672 (1990).
14. Hille, B. *J. gen. Physiol.* **61**, 669–686 (1973).

15. Taylor, P. S. *J. Physiol., Lond.* **388**, 437–447 (1987).
16. Matteson, D. R. & Swenson, R. P. Jr. *J. gen. Physiol.* **87**, 795–816 (1986).
17. Hoshi, T., Zagotta, W. N. & Aldrich, R. W. *Science* **250**, 533–538 (1990).
18. Blachly-Dyson, E., Peng, S., Colombini, M. & Forte, M. *Science* **247**, 1233–1236 (1990).
19. Van der Ley, P., Struyvé, M. & Tommassen, J. *J. Biol. Chem.* **261**, 12222–12225 (1986).
20. Eisenman, G. & Horn, R. *J. memb. Biol.* **76**, 197–225 (1983).
21. Tempel, B. L., Jan, Y. N. & Jan, L. Y. *Nature* **332**, 837–839 (1988).
22. Stühmer, W. *et al. EMBO J.* **8**, 3235–3244 (1989).
23. Butler, A., Wei, A., Baker, K. & Salkoff, L. *Science* **243**, 943–947 (1989).
24. Frech, G. C., VanDongen, A. M. J., Schuster, G., Brown, A. M. & Joho, R. H. *Nature* **340**, 642–645 (1989).
25. Kunkel, T. A. *Proc. natn. Acad. Sci. U.S.A.* **82**, 488–492 (1985).

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Segregation of a missense mutation in the amyloid precursor protein gene with familial Alzheimer's disease

Alison Goate*, Marie-Christine Chartier-Harlin*, Mike Mullan*, Jeremy Brown*, Fiona Crawford*, Liana Fidani*, Luis Giuffra†, Andrew Haynes‡, Nick Irving*, Louise James‡, Rebecca Mant||, Philippa Newton*, Karen Rooke*, Penelope Roques*, Chris Talbot*, Margaret Pericak-Vance§, Allen Roses§, Robert Williamson*, Martin Rossor*, Mike Owen|| & John Hardy*¶

* Alzheimer's Disease Research Group, Departments of Biochemistry and Neurology, St Mary's Hospital Medical School, London W2 1PG, UK

† Department of Human Genetics, Yale University Medical School, 333 Cedar Street, New Haven, Connecticut 06150, USA

§ Duke University Medical Center, Durham, North Carolina NC 27710, USA

|| Departments of Psychological Medicine and Medical Genetics, University of Wales College of Medicine, Cardiff CF4 4XN, UK

A LOCUS segregating with familial Alzheimer's disease (AD) has been mapped to chromosome 21 (ref. 1), close to the amyloid precursor protein (APP) gene^{2–5}. Recombinants between the APP gene and the AD locus have been reported^{6–8} which seemed to exclude it as the site of the mutation causing familial AD. But recent genetic analysis of a large number of AD families has demonstrated that the disease is heterogeneous⁹. Families with late-onset AD do not show linkage to chromosome 21 markers^{9,10}. Some families with early-onset AD show linkage to chromosome 21 markers, but some do not^{8,9,11}. This has led to the suggestion that there is non-allelic genetic heterogeneity even within early

onset familial AD^{8,9}. To avoid the problems that heterogeneity poses for genetic analysis, we have examined the cosegregation of AD and markers along the long arm of chromosome 21 in a single family with AD confirmed by autopsy. Here we demonstrate that in this kindred, which shows linkage to chromosome 21 markers, there is a point mutation in the APP gene. This mutation causes an amino-acid substitution (Val → Ile) close to the carboxy terminus of the β -amyloid peptide. Screening other cases of familial AD revealed a second unrelated family in which this variant occurs. This suggests that some cases of AD could be caused by mutations in the APP gene.

Segregation of polymorphic DNA markers spanning 55% of the physical and genetic length of the long arm of chromosome 21 was analysed in family F23 in which early-onset AD has been confirmed after autopsy (Fig. 1). These markers, represented by the loci (from centromere to telomere) D21S16, D21S13, D21S1, APP, D21S17, D21S156 and D21S167 (refs 12–16), cover the genetic distance in which the AD locus has been proposed

TABLE 1 Two-point linkage analyses between Alzheimer's disease and polymorphic DNA markers on the long arm of chromosome 21

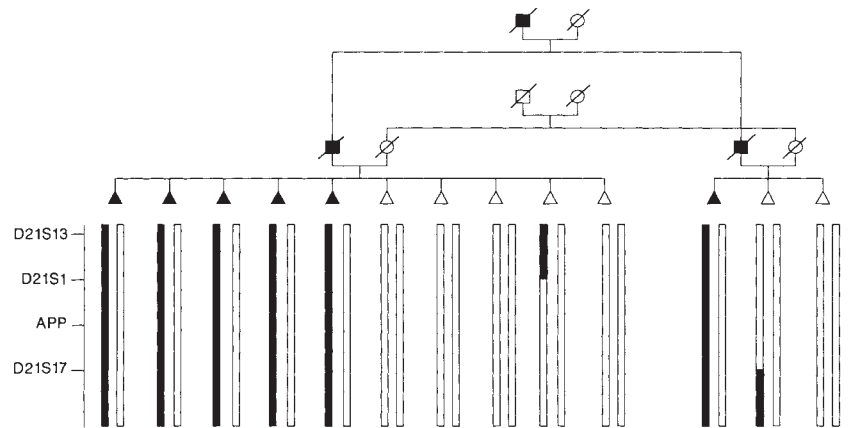
Locus	Recombination fraction (θ)				
	0.00	0.01	0.05	0.10	0.20
D21S16	1.36	1.35	1.28	1.18	0.92
D21S13	2.21	2.17	2.06	1.89	1.44
D21S1	2.65	2.61	2.45	2.22	1.67
APP(EcoRI)	2.93	2.88	2.82	2.36	1.72
D21S17	0.45	0.51	0.61	0.63	0.54
D21S156	−6.31	−4.29	−2.73	−1.76	−0.77
D21S167	−8.02	−3.44	−1.88	−1.17	−0.49
APP(BclI)	3.37	3.31	3.07	2.76	2.09

Allele frequencies used for the loci D21S16, D21S13, and D21S1 have been calculated from unrelated individuals in a British population (M.M. *et al.*, manuscript in preparation). Allele frequencies used for the other polymorphisms are as previously reported^{12–16}. The allele frequency used for the BclI polymorphism was 0.01, which was the highest frequency predicted from non-detection of the polymorphism in 200 normal chromosomes (99% confidence, $[1 - (1 - \text{confidence interval})^{1/200}]$). The following loci were not informative within the pedigree: D21S80, D21S52, D21S11, D21S8, D21S111 and D21S82.

‡ Present addresses: Department of Respiratory Medicine, St Mary's Hospital Medical School, London W2 1PG, UK (A.H.); ICI Biotechnology Division, Alderley Park, Macclesfield, Cheshire, UK (L.J.).

¶ To whom correspondence should be addressed.

FIG. 1 Pedigree in which early-onset AD is apparently inherited as an autosomal dominant disorder. The average age of onset in this family is 57 ± 5 yr. Black symbols denote affected individuals and oblique lines indicate individuals who are deceased. Females are denoted by circles and males by squares. Triangles are used in the present generation to preserve anonymity. In generation II the spouses of the two affected brothers were sisters. Samples were available from the 13 individuals whose haplotypes are illustrated, from a further 19 children and spouses of these individuals and from 7 more distantly related unaffected individuals. Beneath the pedigree are ideograms of the two chromosomes 21 in each individual of the third generation at four loci on the long arm of the chromosome. The linkage data suggest that the chromosomes filled in black were inherited from the affected fathers.



to occur, with the exception of the pericentromere. Two-point linkage analyses (MLINK; ref. 17) were carried out between each of the polymorphic loci and AD (Table 1).

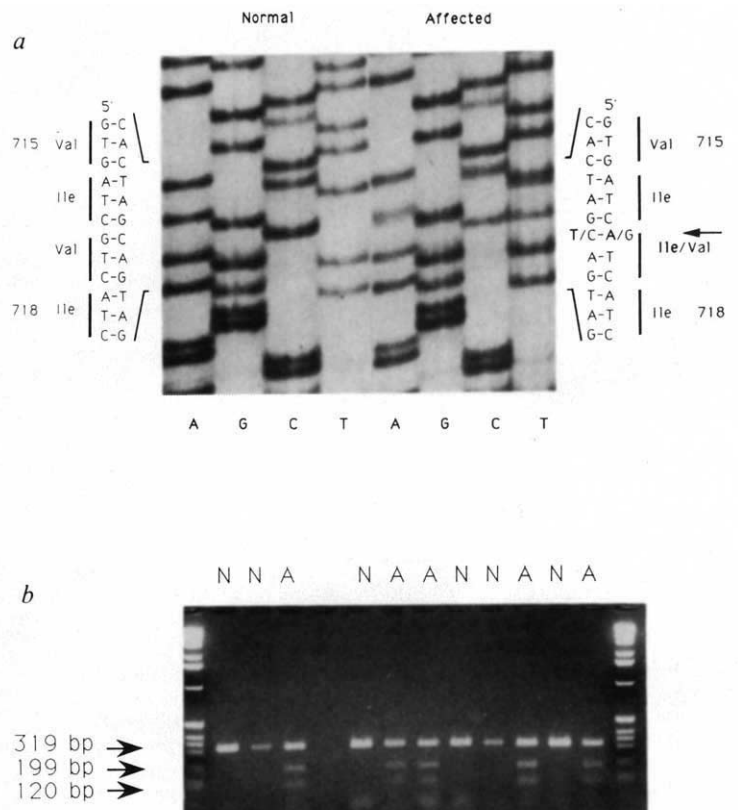
Two individuals with recombinant chromosomes provide evidence for the position of the AD locus (Fig. 1). The unaffected individual in the second sibship who shares the same allele at D21S17 as the affected individuals is 15 years over the mean age of onset of the disease in this family. This recombinant excludes the disease locus from being telomeric to this marker. The individual with a recombinant chromosome in the first sibship is unaffected but is only two years above the mean age of onset within the family (giving odds of two to one against developing the disease based upon the family's risk curve). This person shares the same alleles as the affected individuals between D21S13 and D21S1/S11, but not at APP. If this person were to develop AD in future, then the disease locus must be centromeric of APP and the APP gene is excluded as the disease locus; but

if the person remains healthy, then the AD locus must be telomeric of D21S1/S11 and APP is not excluded.

Because we could not exclude the APP gene as the site of the mutation leading to AD in this family, we analysed the APP gene in an affected family member by PCR direct sequencing using intronic primers^{18,19}. Exon 17 was sequenced first because it encodes part of the β -amyloid peptide and is the site of the mutation leading to hereditary cerebral haemorrhage with amyloidosis-Dutch type (HCHWA-D)^{20,21}.

Sequencing of exon 17 revealed a C to T transition at base pair 2,149, causing a valine to isoleucine change at amino acid 717 (transcript APP₇₇₀; ref. 19) (Fig. 2a). This valine residue is conserved in rodents. The base substitution may have involved deamination of a methylated cytosine situated 5' to guanine, a fairly common mutation in human DNA²². The substitution creates a *Bcl*I restriction site which allows detection of the corresponding polymorphism within the PCR product (Fig. 2b).

FIG. 2 a, Autoradiograph of a sequencing gel from part of exon 17 of the APP gene in a normal and an affected individual showing a single base-pair change in the affected individual. This C to T transition leads to an amino-acid substitution of a valine by an isoleucine at codon 717. PCR was carried out using the following intronic primers in order to amplify exon 17 of the APP gene: (1) 5'-CCTCATCCAAATGTCGCCGTCATT-3', and (2) 5'-GCCTAATTCTCTCATAGTCTTAATCCCAC-3'. PCR conditions were 94 °C for 10 min to denature, then 35 cycles of 60 °C for 1 min, 72 °C for 3 min, 94 °C for 1.5 min; and a single cycle of 72 °C for 10 min. The reaction volume of 25 μ l contained 50 pmol of primers, dNTPs at 200 μ M, and MgCl₂ at 1.5 mM. A second PCR was performed which contained 50 pmol primer (1) and 0.5 pmol primer (2). The PCR product was purified on a Centricon 100 microconcentrator (Amicon) and used directly for sequencing with the Sequenase kit (version 2.0) (USB) following the manufacturer's protocol. Direct sequencing of exon 15 has revealed that this exon sequence is normal in family F23. Single-strand conformational analysis has failed to detect any sequence alterations in exons 7, 15 or 16 (our unpublished data). b, *Bcl*I digests of the exon 17 PCR product from unaffected (N) and affected (A) individuals in an early-onset AD family showing cosegregation of the restriction site and the disease. *Bcl*I digests were carried out at 50 °C for 2–4 h then electrophoresed in 3% agarose and stained with ethidium bromide. Size markers (indicated in base pairs) are shown in the outside tracks of the gel.



Linkage analysis shows that the polymorphism cosegregates with AD (Table 1).

Screening by PCR of 100 unrelated, normal individuals from the same population as the AD pedigree, and 14 cases (9 families) of familial late-onset disease, failed to demonstrate this substitution. Screening of 18 cases (16 families) of early-onset familial disease revealed the *BclI* restriction site in two affected individuals from a second family (family 372) in which linkage between AD and chromosome 21 markers shows small positive lod scores (log of likelihood ratio; D21S16, $z = 0.6$, $\theta = 0$; D21S1, $z = 0.9$, $\theta = 0$)¹⁰.

Affected individuals from both families with the mutation were also genotyped for the rare *EcoRI* polymorphism detected by the 3' end of the APP complementary DNA¹²; this probe detects a polymorphic site in the intron between exons 17 and 18. For family F23, the disease (and the mutation) cosegregates with the rare allele, which is present in only 5% of the normal population. In contrast, affected individuals in family 372 do not share this allele, but are homozygous for the common allele. This shows that the two pedigrees are very unlikely to be related to one another, as the markers are separated by less than 20 kilobases.

The genetic data show that the disease locus is linked to the missense mutation in F23 ($z = 3.37$, $\theta = 0$), but does not prove causation. However, failure to detect this polymorphism in 200 normal chromosomes supports the contention that it is a pathogenic mutation (the frequency of this polymorphism in the general population has an upper limit of 0.01, with 99%

confidence). As we have not yet sequenced the entire APP gene in an affected individual it remains possible that the APP gene is the disease locus but that the variant observed is not the causative mutation.

If this variant is pathogenic, previous reports of recombinants between AD and the APP gene have to be explained⁶⁻⁸. The demonstration of heterogeneity in familial AD⁹ suggests that many apparent recombinants will have resulted from analysis of families without a mutation on chromosome 21. Reports of recombination in families showing evidence of chromosome 21 linkage may have been in error for several reasons, including misdiagnosis, mistyping, non-paternity and the occurrence of phenocopies. Furthermore, the APP gene is large enough for intragenic recombination to occur¹⁹. Alternatively, there may be two loci on chromosome 21 capable of causing AD, APP and a more proximal locus. If the mutation we describe is pathogenic, then the failure to detect the resulting *BclI* site in all cases of familial early-onset AD proves that this disease must be genetically heterogeneous. This could be a result of heterogeneity in mutations occurring at the APP locus, or non-allelic heterogeneity, or both.

The Val → Ile substitution occurs within the transmembrane domain two residues from the carboxy terminus of the β -amyloid peptide (Fig. 3). Computer analysis predicts that the substitution would make the transmembrane domain more hydrophobic and so might anchor the protein more firmly in the membrane²³⁻²⁹, which could influence the stability of the deposited peptide.

A clear prediction of the hypothesis that mutations in the APP gene can predispose to AD is that examination of the APP gene in other families should lead to the identification of other mutations. The occurrence of AD pathology in trisomy 21 suggests that these mutations need not be in the coding region but may also be in controlling elements, leading to overexpression of APP. Whatever the aetiology of individual cases of AD, the results reported here suggest that β -amyloid peptide deposition is the central event in the pathogenesis of the disorder³⁰. □

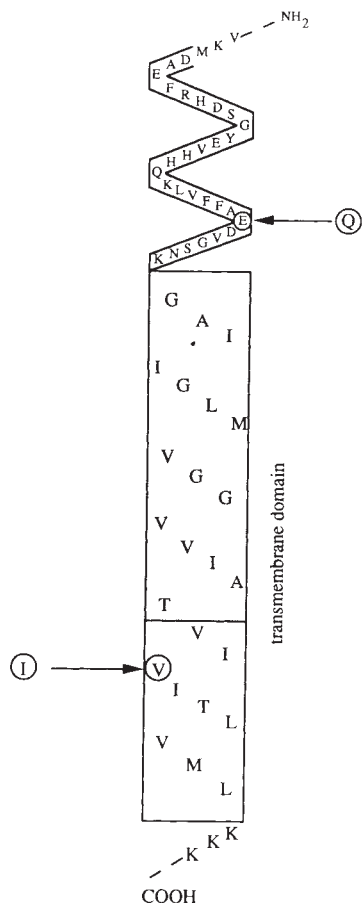


FIG. 3 Part of the amino-acid sequence (single-letter code) encoded by exons 16 and 17 of the APP gene showing the mutation described here (Val to Ile) within the transmembrane domain and the mutation causing HCHWA-D (Glu to Gln) in the extracellular domain²¹. The shaded region of the transmembrane domain and the boxed amino acids of the extracellular domain represent the sequence of the deposited β -amyloid peptide. Adapted from ref. 2.

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1. St George-Hyslop, P. *et al. Science* **235**, 885-890 (1987).
2. Kang, J. *et al. Nature* **325**, 733-736 (1987).
3. Goldgaber, D., Lerman, M. I., McBride, O. W., Sappioti, U. & Gajdusek, D. C. *Science* **235**, 877-880 (1987).
4. Robakis, N. K., Ramakrishna, N., Wolfe, G. & Wisniewski, H. M. *Proc. natn. Acad. Sci. U.S.A.* **84**, 4190-4194 (1987).
5. Tanzi, R. *et al. Science* **235**, 880-884 (1987).
6. Van Broeckhoven, C. *et al. Nature* **329**, 153-155 (1987).
7. Tanzi, R. *et al. Nature* **329**, 156-157 (1987).
8. Schellenberg, G. *et al. Science* **241**, 1507-1510 (1988).
9. St George-Hyslop, P. *et al. Nature* **347**, 194-197 (1990).
10. Pericak-Vance, M. *et al. Expl. Neurol.* **102**, 271-279 (1988).
11. Goate, A. *et al. Lancet* **1**, 352-355 (1989).
12. Tanzi, R. *et al. Genomics* **3**, 129-136 (1988).
13. Warren, A. *et al. Genomics* **4**, 579-591 (1989).
14. Lewis, G. *et al. Genomics* **8**, 400-402 (1990).
15. Guo, Z., Sharma, V., Patterson, D. & Litt, M. *Nucleic Acids Res.* **18**, 4967 (1990).
16. Kidd, K. *et al. Cytogenet. Cell Genet.* **51**, 622-947 (1989).
17. Lathrop, G. M., Lalouel, J. M., Julier, C. & Ott, J. *Proc. natn. Acad. Sci. U.S.A.* **81**, 3443-3446 (1984).
18. Gyllenstein, U. in *PCR Technology* (ed. Erlich, H. A.) 45-60 (Stockton, London, 1989).
19. Yoshikai, S., Sasaki, H., Doh-ura, K., Furnya, H. & Sakaki, Y. *Gene* **87**, 257-263 (1990).
20. Van Broeckhoven, C. *et al. Science* **248**, 1120-1123 (1990).
21. Levy, E. *et al. Science* **248**, 1124-1126 (1990).
22. Barker, D., Schafer, M. & White, R. *Cell* **36**, 131-138 (1984).
23. Argos, P., Rao, J. K. M. & Hargrave, P. A. *Eur. J. Biochem.* **128**, 565-575 (1982).
24. Garnier, J., Osguthorpe, D. J. & Robson, B. *J. molec. Biol.* **120**, 97-120 (1978).
25. Gaboriaud, C., Bissery, V., Benchetrit, T. & Morron, J. P. *FEBS Lett.* **224**, 149-155 (1987).
26. Kyte, J. & Doolittle, R. F. *J. molec. Biol.* **157**, 105-132 (1982).
27. Levin, J. M., Robson, B. & Garnier, J. *FEBS Lett.* **205**, 303-308 (1986).
28. Lim, V. *J. molec. Biol.* **88**, 857-872 (1974).
29. Gilrat, J., Garnier, J. & Robson, B. *J. molec. Biol.* **198**, 425-443 (1987).
30. Glenner, G. G. & Wong, C. W. *Biochem. biophys. Res. Commun.* **120**, 1131-1135 (1984).

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