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Genetic Evidence for The Neuronal Nitric Oxide Synthase Gene (NOS1) as a Susceptibility Locus for Infantile Pyloric Stenosis

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Summary

The etiological role of the gene for neuronal nitric oxide synthase (NOS1) in infantile pyloric stenosis (PS) was investigated by analysis of two intragenic polymorphisms (NOS1a and NOS1b) in 27 families. There was significant overall transmission disequilibrium between PS and NOS1a ($P = .006$). Consideration of each allele independently revealed a highly significant tendency for allele 7 (210 bp) to be preferentially transmitted to the affected offspring ($P = .0006$). These observations suggest that NOS1 is a susceptibility locus for PS.

Introduction

Infantile pyloric stenosis (PS, MIM 179010) has an incidence of 1–5 per 1,000 live births in Caucasian populations, with a marked preponderance of males to females (4:1) (Davison 1946; Mclean 1956; Dodge 1975; Tam and Chan 1991). Hypertrophy of the smooth muscle of the pylorus develops a few weeks after birth, giving rise to gastric-outlet obstruction.

The pathophysiological basis of PS is unknown. A genetic contribution toward its etiology is well established. Familial aggregation of PS was first reported by Cockayne and Penrose (Cockayne 1934; Cockayne and Penrose 1943). Twin studies show a concordance rate of 25%–40% in monozygotic twins (Metrakos 1953; MacMahon and Mckeown 1955). The recurrence risk is 10% for males born after an affected child and 2% for females (Lalouel et al. 1977). By using pooled data on siblings of PS probands from several family studies and assuming a population prevalence of 0.3% for PS, the ratio of risk for first-degree relatives compared with the general population was estimated to be 18 (5.5%/0.3%) (Mitchell and Risch 1993).

The classical studies by Carter identified PS as a paradigm for the multifactorial threshold model of inheritance (Carter 1961; Carter and Evans 1969). This is supported by subsequent studies (Chakraborty 1986; Mitchell and Risch 1993). A recent reanalysis of the genetics of PS concluded that PS is determined by either a multifactorial inheritance or multiple interacting loci with no single gene accounting for more than a fivefold increase in the risk to first-degree relatives and that, at most, two or three loci of moderate effect are involved in the etiology of PS (Mitchell and Risch 1993).

Recent evidence has implicated the enzyme neuronal nitric oxide synthase (nNOS) in the etiology of PS, rendering its gene (NOS1) a candidate gene for the condition. nNOS belongs to a class of enzymes that catalyze the formation of nitric oxide (NO) from L-arginine via what is now known as the L-arginine–NO pathway. The mechanism and actions of this pathway have been extensively reviewed (Marletta 1993; Moncada and Higgs 1993; Lowenstein et al. 1994).

nNOS catalyzes the formation of NO in neurones in both the central and peripheral nervous systems. NO in parallel with vasoactive intestinal polypeptide and ATP have been shown to function as neurotransmitters of the nonadrenergic noncholinergic nerves of the enteric nervous system. These neurones are known to mediate smooth muscle relaxation (Bult et al. 1990; Li and Rand 1990; Keef et al. 1994). A defect in pyloric relaxation may be responsible for the gastric-outlet obstruction in PS (Hayes and Goldenberg 1957). In biopsy specimens of nine infants with PS, NADPH-diaphorase (NDP) staining was found to be absent in the neurones that innervate the circular muscle of the pylorus, and the nerve fibers themselves appeared abnormal and tortuous (Vanderwinden et al. 1992). NDP is an enzyme that reduces tetrazolium dyes in the presence of NADPH but not NADH. This histochemical enzymatic activity has been shown to be due to nNOS (Dawson et al. 1991; Hope et al. 1991). It was suggested that reduction of

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NO mediated relaxation resulting from dysfunction of nNOS could account for pylorospasm and smooth muscle hypertrophy in PS. Furthermore, NOS1 knock-out mice show phenotypic features of an enlarged stomach with hypertrophy of the pyloric sphincter and the circular muscle layer, resembling those of PS (Huang et al. 1993).

NOS1 has been cloned, physically located (Xu et al. 1993), and fully characterized (Hall et al. 1994). In view of the recent identification of NOS1 as a very strong candidate gene for PS, its etiological role was investigated by performing linkage analysis and the transmission disequilibrium test using two NOS1 intragenic simple-sequence length polymorphisms (NOS1a and NOS1b) in 27 families (see fig. 1). The results provide support for the hypothesis that NOS1 is a susceptibility locus for PS.

Subjects and Methods

Families

Multiplex families were ascertained through collaboration with Prof. M. Pembrey and Ms. R. Coffey (London), Prof. J. Dodge (Belfast), Mr. P. Tam (Oxford), Dr. R. Bell (Banbury, United Kingdom), and Dr. A. Day (Cheltenham, United Kingdom). Twenty-seven families were studied (fig. 1), 21 of which have at least three affected individuals. There is no consanguinity in any of our families, and the majority (25/27) is of Caucasian origin. Affected individuals have not been specifically evaluated for the presence of underlying syndromes or additional malformations, but no such abnormalities were reported by the referring physicians or recorded in the case records. No cytogenetic analysis has been performed. Diagnosis of PS was made according to standard criteria and confirmed at laparotomy in all affected individuals. Blood samples for DNA analysis were obtained from a total of 229 individuals, of whom 87 were affected (28 females and 59 males).

Marker Typing

Genomic DNA was extracted from white cells by using standard methods (Miller et al. 1988). Individuals were typed with two NOS1 intragenic polymorphisms: NOS1a (courtesy of Dr. P. Marsden) and NOS1b (courtesy of Dr. R. Twells). Primer sequences of NOS1a are 5′-CCTGCGTGGCTACTACATTC-3′ and 5′-AGACGTCGCAACCCTCATTA-3′, with an annealing temperature of 52°C. Primer sequences of NOS1b are 5′-CTGGGGGCAATGGTGTGT-3′ and 5′-GAGTAAAATTAAGGGTCAGC-3′, with an annealing temperature of 51°C. Genomic DNA was amplified using PCR. PCR was performed in 96-well microtitre plates (Hybaid). Each well contained 50–100 ng of genomic DNA; 1.5 mM $MgCl_2$; 1 × reaction buffer (Bioline); 200 μM each of dGTP, dATP, and dTTP; 20 μM of dCTP; 0.05 μl ^{32}P-dCTP (3,000 Ci/mmol); 50 ng of each primer; and 0.2 U of BIOTAG polymerase (Bioline), in a total volume of 20 μl. Thirty cycles were performed in a thermocycler (Hybaid Omnigene™). Alleles were separated by 6% denaturing acrylamide gel electrophoresis and detected by autoradiography. Details of the polymorphisms have been published elsewhere (Hall et al. 1994; Twells et al. 1995). Allele frequencies and heterozygosity of the two polymorphisms were estimated in a random control population of unrelated British individuals (120 chromosomes for NOS1a; 220 chromosomes for NOS1b). The allele sizes and frequencies are listed in table 1. NOS1a has nine alleles and an observed heterozygosity of .86. NOS1b has nine alleles and an observed heterozygosity of .76.

Linkage Analysis

Linkage was performed with the LINKAGE (version 5.1) package of computer programs under two models of inheritance (Lathrop et al. 1984). The data were analyzed by using only affected subjects, i.e., apparently normal individuals are classified as unknown with respect to the PS trait. Sex-specific penetrance values of .60 for males and .15 for females were used for both the autosomal dominant (AD) model and the autosomal recessive (AR) model. A phenocopy probability of .002 was used, to allow for occasional sporadic cases or misdiagnosis. The disease allele frequency was set equal to .002 for the AD model and .05 for the AR model, to produce the correct population prevalence of PS. Lod scores were calculated between PS and NOS1 by using the MLINK program assuming 0 recombination between the two NOS1 intragenic polymorphisms. The analysis was carried out allowing for locus heterogeneity, and the A-test procedure was used to produce lod2 statistics maximized over the proportion of linked families (α) (Risch 1989). In order to test the main hypothesis that NOS1 itself modified susceptibility, the lod scores at 0 recombination with the markers were studied, although lod scores were also computed at other recombination fractions.

Tests for Linkage Disequilibrium

Transmission disequilibrium testing was carried out according to the method described by Spielman et al. (1994) as extended to incorporate multiple marker alleles (Sham and Curtis 1995). Only genotypes measured directly were included in this analysis. Although it would have been possible to deduce some additional genotypes, doing this can sometimes introduce subtle bias into the analysis (Curtis and Sham 1995). The genotype-wise analysis incorporates a "saturated" model to examine each kind of heterozygous parental genotype separately to see if there is deviation from the expected 50:50 trans-

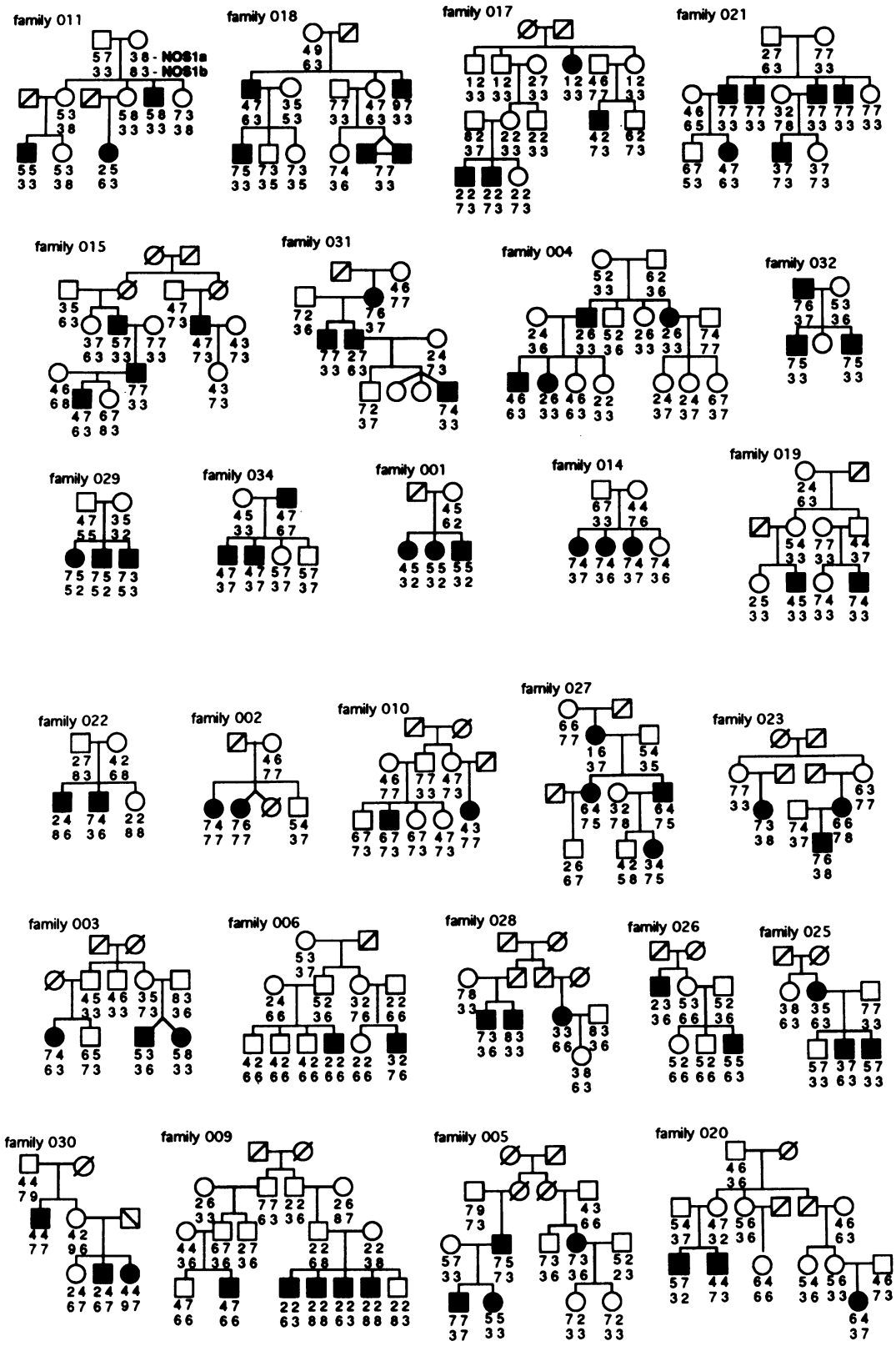

Figure 1 PS pedigrees, showing haplotypes of two intragenic polymorphisms at NOS1. Order of loci is as shown in family 011.

Table 1

Allele Sizes and Frequencies of NOS1a and NOS1b

Allele	Size (bp)	Frequency
	NOS1a	
1	182	.009
2	188	.200
3	190	.028
4	198	.182
5	206	.173
6	208	.090
7	210	.282
8	212	.027
9	216	.009
	NOS1b	
1	404	.009
2	407	.082
3	410	.463
4	413	.005
5	416	.009
6	419	.227
7	422	.141
8	425	.050
9	428	.014

mission of alleles to affected offspring. These deviations are combined into an overall χ^2 statistic with number of df equal to the number of distinct parental genotypes. Additionally, a more parsimonious allele-wise analysis was used, which applies logistic regression to examine whether certain alleles are more likely than others to be transmitted to affected offspring, across a number of different parental genotypes. In order to carry out this analysis, it is assumed that each allele has a parameter B associated with it such that, if a parent has genotype ij, the ratio of the probabilities of transmitting allele i or j to an affected offspring is given by $\ln(p_i/p_j) = B_i - B_j$. The value of one of these parameters is arbitrarily fixed to 0, and values for the other parameters are chosen to maximize the likelihood of the observed data. This maximized likelihood, $L1$, is compared with the likelihood under the null hypothesis, $L0$, which is obtained by constraining all parameters to be equal and hence there being a 50:50 probability to transmit either allele from any parental genotype. If the marker locus has m alleles, $2\ln(L1 - L0)$ can then be taken as a χ^2 statistic with $m - 1$ df in order to provide a statistical test for transmission disequilibrium. By parameterizing the analysis in this way, the maximization can be carried out by any standard software designed to perform logistic regression analysis, as described in full elsewhere (Sham and Curtis 1995). In the present case, the analyses were carried out using ETDT, a DOS program specially written to analyze transmission disequilibrium test (TDT) data for multiallelic markers (available from ftp.gene.ucl.ac.uk/pub/packages/dcurtis). The advantage of the allele-wise analysis is that it is more parsimonious than the genotype-wise method, which has up to $m(m - 1)/2$ df, and that it avoids the problems of multiple-testing that arise if each allele is considered individually against the rest, as would be required by the TDT in its original formulation. In addition, considering each allele individually may miss true linkage disequilibrium if two alleles are associated with the disease, since each will tend to mask the other. In the present application, analyses of individual alleles of the polymorphisms were only carried out after obtaining some initial evidence for overall linkage disequilibrium between the disease and NOS1 by using the genotype-wise and allele-wise analyses, in order to clarify which particular alleles appeared to be associated with the disease. For those pedigrees in which no recombinations were observed, the cosegregating allele was noted. These allele and haplotype frequencies were compared to the frequencies in the control population by using Fisher's exact test.

Results

Linkage Analysis

The alleles of each individual for the two NOS1 intragenic polymorphisms are shown in figure 1, arranged according to haplotypes insofar as these could be deduced. The total lod score summed over all pedigrees was <-2 for ≥ 20 cM around the NOS1 locus. Thirteen families (011, 018, 017, 021, 015, 031, 004, 032, 029, 034, 001, 014, and 019) did not demonstrate any recombination between PS and NOS1 and had positive lod scores, the highest being 0.87 with family 011. The maximum lod score incorporating heterogeneity (lod2) is 1.26 at $\theta = .0$ and $\alpha = .35$. Lod scores obtained with the recessive model were more negative and did not provide any suggestion that some families might be linked.

Tests for Linkage Disequilibrium

The data for the multiallele TDT are shown in table 2. There was significant evidence for overall transmission disequilibrium between the disease locus and NOS1a when both the genotype-wise test ($\chi^2 = 53.9$; 21 df; $P = .0001$) and the allele-wise test ($\chi^2 = 21.6$; 8 df; $P = .006$) were used. Consideration of each allele independently revealed a highly significant tendency for allele 7 (210 bp) to be preferentially transmitted to affected offspring (see table 3). This occurred 29 of 37 times ($\chi^2 = 11.9$; 1 df; $P = .0006$). This result remains significant at $P = .005$ after a Bonferroni correction has been applied to allow for testing of nine alleles. There was no significant transmission disequilibrium of this allele to unaffected offspring (12 of

Table 2

Data for Multiallele TDT of PS with NOS1 Polymorphisms

	Allele Not Transmitted									Total No. of Transmissions
	1	2	3	4	5	6	7	8	9	
	NOS1a									
Allele transmitted:										
1	...	...	...	...	...	...	...	...	...	0
2	1	(11)	...	2	3	...	2	2	...	10
3	...	3	...	1	2	...	...	1	...	7
4	...	4	...	(5)	6	6	2	...	1	19
5	...	1	10	1	...	...	3	1	...	16
6	2	4	...	3	1	(2)	...	...	...	10
7	...	7	...	9	3	8	(14)	1	1	29
8	...	...	2	...	...	...	1	...	...	3
9	...	...	...	1	...	...	...	...	...	1
Total no. of nontransmissions	3	19	12	17	15	14	8	5	2	
	NOS1b									
Allele transmitted:										
1	...	...	...	...	...	...	...	...	...	0
2	...	...	3	...	...	3	...	...	...	6
3	...	2	(34)	...	...	17	11	4	...	34
4	...	...	...	...	...	...	...	...	...	0
5	...	...	2	...	(3)	...	1	...	...	3
6	...	...	7	...	1	(4)	1	5	1	15
7	...	...	7	...	...	5	(5)	2	...	14
8	...	...	3	...	...	2	1	...	...	6
9	...	...	...	...	...	1	...	...	...	1
Total no. of nontransmissions	0	2	22	0	1	28	14	11	1	

NOTE.—The parental genotype is defined by the allele in the corresponding row and column. The number in each cell indicates the number of parents, with the corresponding genotype who transmitted the allele defined by the row and did not transmit the allele defined by the column to affected offspring. For example, with NOS1a, the number "4" in the second cell of row 6 indicates four transmissions of allele 6 and four nontransmissions of allele 2 from parents with genotype 6/2 to affected offspring. Transmissions and nontransmissions from homozygous parents (enclosed in parentheses) are not included in the analysis.

20 transmissions; P = NS). NOS1b did not show significant evidence of transmission disequilibrium with the disease locus when either the genotype-wise (χ^2 = 19.8; 12 df; P = .07) or allele-wise (χ^2 = 9.89; 6 df; P = NS) method of analysis was used.

When transmission disequilibrium was examined in the 13 families showing positive lod scores, the transmission disequilibrium with NOS1a became even more pronounced, in that allele 7 was preferentially transmitted 21 of 24 times. When attention was confined to these families, allele 3 of NOS1b was preferentially transmitted 22 of 38 times. If one considers all the pedigrees with positive lod scores at NOS1, then, of 13 such pedigrees, 8 demonstrate cosegregation with allele 7 of NOS1a ($P < .01$) and 10 demonstrate cosegregation with allele 3 of NOS1b (P = NS). The software package GENEPOP (Raymond and Rousset, in press) was used to evaluate the distribution of these alleles among the controls. The distribution of these alleles in the control sample was not significantly different from that expected under Hardy-Weinberg equilibrium, and there was no evidence that the alleles of the two polymorphisms were in linkage disequilibrium with each other. Therefore, the expected frequency of the 7-3 haplotype can be calculated as the product of the individual allele frequencies, yielding a value of 0.13. Of the 13 possibly linked families, 6 (018, 021, 015, 031, 032, and 014) cosegregate with the haplotype 7-3, which is significant at $P < .01$ (uncorrected for multiple haplotypes).

Discussion

Genetic methods including tests of linkage and linkage disequilibrium represent powerful approaches for evalu-

Table 3

Results of TDT Analysis of PS with Individual Alleles of NOS1 Polymorphisms

	Allele								
	1	2	3	4	5	6	7	8	9
	NOS1a								
Total no. of transmissions	0	10	7	19	16	10	29	3	1
Total no. of nontransmissions	3	19	12	17	15	14	8	5	2
χ^2		2.8	1.3	0.1	0.0	0.7	11.9**		
	NOS1b								
Total no. of transmissions	0	6	34	0	3	15	14	6	1
Total no. of nontransmissions	0	2	22	0	1	28	14	11	1
χ^2			2.6			3.9*	0.0	1.54	

* $P < .05$ (NS with Bonferroni correction).
** $P = .0006$ (.005 with Bonferroni correction).

ating the role of candidate genes in human diseases that display Mendelian inheritance. Their application to diseases with so-called complex inheritance, such as PS, involves a number of factors that serve to complicate the methodology and make the interpretation of results more difficult. These factors include uncertainty about the mode of inheritance, the paucity of large multiplex pedigrees, and the possibility that there may be locus heterogeneity among small nuclear pedigrees and even within large multiplex pedigrees. All of these combine to reduce the power of this approach for complex diseases.

As discussed above, PS is a paradigm for such complex diseases, and Carter's classic studies of its inheritance gave rise to the concept of multifactorial inheritance. However, inheritance of the disease trait is not inconsistent with segregation of a single major locus of very low and sex-specific penetrance (Kidd and Spence 1976; Fried et al. 1981), and certainly one cannot exclude the possibility that this mode of inheritance may be operative in at least some families. NOS1 is a highly plausible candidate gene for PS, on account of its biological role in the control of gastrointestinal tract smooth muscle and has been strongly implicated by available evidence in man and mouse. The present observations provide genetic support for an etiological role for NOS1 in PS.

Although the mode of inheritance of PS is unknown, in the presence of a very strong candidate gene with intragenic polymorphisms and a family resource of mainly multigeneration families, we decided initially to use the lod score method of analysis. Because family studies and segregation analyses have failed to identify the exact inheritance, the parameters were chosen to represent plausible models. They include a low and sex-specific penetrance partly to reflect the marked difference in the sex ratio. In any event, because an affecteds-only analysis was performed, the penetrance values have little influence on the results. No other models were evaluated. By studying multiplex families, it is hoped that there is enrichment for cases with a major genetic contribution to etiology. These multiplex families, however, are atypical of PS and represent only a minority of cases.

Lod score analysis assuming locus homogeneity produces strongly negative total lod scores at NOS1 for both dominant and recessive transmission models, implying that this locus is not responsible for PS in at least some families. Allowing for locus heterogeneity, a maximum lod score of 1.26 at NOS1 is obtained, with the estimated proportion of linked families being 0.35. This is insufficient on its own to prove linkage, but it has not excluded the possibility that mutations in NOS1 may confer susceptibility to PS in a minority of families. The lod score method does, however, rely on specifying a mode of transmission that is approximately correct, and both false-positive and false-negative results can be obtained if the parameters are very wrong (Clerget-Darpoux et al. 1986; Lander and Schork 1995). Therefore, it is not possible with our observations to falsify the hypothesis that NOS1 could have a minor effect on susceptibility in all families, though we find no evidence to support this.

In the present case, the TDT provides significant sup-

port for linkage and linkage disequilibrium. In contrast to the lod score method, this test requires no assumptions concerning mode of transmission. Additionally, it is only expected to be positive if both linkage and linkage disequilibrium are present. It also differs from case-control association studies in that population stratifications will not produce false-positive results. The multiallele TDT for NOS1a is highly significant, and allele 7 of NOS1a is preferentially transmitted to cases 29 of 37 times (corrected $P = .005$) but only 12 of 20 times to unaffected offspring (P = NS). The results from the TDT cannot be considered to be independent of the results of linkage analysis, because, if a group of pedigrees is known to demonstrate linkage, the meioses within each pedigree will not constitute independent transmissions. Nevertheless, the positive TDT does suggest the presence of linkage disequilibrium, as well as linkage, implicating a region within ~1 cM of NOS1. To avoid the problems of nonindependence of transmissions within linked pedigrees, we examined which alleles were cosegregating in each of the 13 possibly linked pedigrees and observed a significant excess of allele 7 of NOS1a and of the 7-3 haplotype.

Our observations provide support for the hypothesis that a locus at or close to NOS1 modifies susceptibility to PS. This locus appears to be in linkage disequilibrium with allele 7 of NOS1a and possibly also with allele 3 of NOS1b. Although the linkage and linkage disequilibrium observed at NOS1 might reflect the influence of polymorphism at an adjacent locus in linkage disequilibrium with the NOS1 alleles, the considerable biological plausibility of NOS1's status as a candidate gene makes this possibility somewhat unlikely. Instead, it seems more likely that a mutation within NOS1 itself, or in a controlling region, is responsible for the increased susceptibility to PS in the linked families. It is even possible that the allelic variation at NOS1a may itself be pathological—its location in the 5′ flanking region of exon 1 immediately upstream of the start site of transcription between a canonical TATA element and the inverted CAAT boxes could allow variation in repeat size to contribute to nNOS mRNA transcript generation, stability, or processing (Hall et al. 1994). This would imply that allele 7 itself conferred increased risk, but it seems hard to reconcile this theory with the fact that the allele is common, that the disease is rare, and that subjects homozygous for this allele do not appear to have an appreciably higher risk than heterozygotes.

For reasons discussed earlier, no single method of analysis in a complex disease can be optimal in all circumstances. Though the lod score incorporating heterogeneity with the dominant model was only mildly positive and therefore equivocal, the TDT analysis was strongly positive. We have refrained from carrying out any nonparametric methods of linkage analysis. Applying additional analyses would have further complicated the interpretation of the results because of the issue of multiple testing.

Several strategies are available for confirmation of the hypothesis that NOS1 is a susceptibility locus for PS. Tests for transmission disequilibrium could be extended to sporadic cases, but it is of course possible that a different etiological mechanism may underlie these. Direct examination of the NOS1 gene in linked families for sequence variations with functional consequences and significant association with the PS trait probably represents the most efficient approach and is currently being undertaken.

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