

Population Genetics of a Functional Variant of the Dopamine β -Hydroxylase Gene (DBH)

Joseph F. Cubells,^{1,2} Kazuto Kobayashi,⁴ Toshiharu Nagatsu,⁵ Kenneth K. Kidd,^{2,3} Judith R. Kidd,³ Francesc Calafell,³ Henry R. Kranzler,⁶ Hiroshi Ichinose,⁵ and Joel Gelernter^{1,2*}

¹Department of Psychiatry, VA Connecticut Health Care System, West Haven ²Department of Psychiatry, Yale University School of Medicine, New Haven, Connecticut

³Department of Genetics, Yale University School of Medicine, New Haven, Connecticut

⁴Research and Education Center for Genetic Information, Nara Institute of Science and Technology, Takayama, Ikoma, Nara, Japan

⁵Institute for Comprehensive Medical Science, Fujita Health University School of Medicine, Toyoake, Japan

⁶Department of Psychiatry, University of Connecticut School of Medicine, Farmington, Connecticut

Dopamine β -hydroxylase (E.C. 1.14.17.1; protein abbreviation: D β H) catalyzes conversion of dopamine to norepinephrine. Previous work identified two expressed alleles of the gene encoding D β H (locus symbol DBH), containing either G or T at nucleotide position 910, resulting in specification by codon 304 of alanine (DBH*304A) or serine (DBH*304S), respectively. The current study employed denaturing gradient gel electrophoresis to identify these alleles, and after developing a PCR RFLP for rapid genotyping, estimated the frequencies of the alleles in African-Americans, European-Americans, and in several geographically dispersed populations (Mbuti, Danes, Adygei, Chinese, Japanese, Surui, Maya, and Nasioi).

DBH*304A was the most common allele in all populations tested, with allele frequencies greater than 0.80 in each case. There was significant heterogeneity in allele frequency across population groups. The DBH*304S allele was most common in subjects of African descent, and least common in East Asians and individuals from indigenous populations of North and South America. The frequency of DBH*304S was significantly higher in African-Americans (0.16) than in European-Americans (0.06; $P < 0.004$). Of the four DBH*304S homozygotes

observed, all were Europeans and three of the four were Danes. Based on empirical P -values generated by computer simulation, the observed proportions of DBH*304S homozygotes did not differ significantly from Hardy-Weinberg expectations in any of the populations after Bonferroni correction for multiple comparisons. The observation of significant heterogeneity in DBH*304S allele frequency across different population samples demonstrates the importance of controlling for population stratification in future studies testing for associations between DBH*304S and clinical phenotypes. *Am. J. Med. Genet.* 74:374–379, 1997.

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INTRODUCTION

Dopamine β -hydroxylase (E.C. 1.14.17.1; protein abbreviation: D β H) catalyzes the conversion of dopamine to norepinephrine, and therefore plays an important role in noradrenergic neurotransmission. D β H activity in human serum or plasma is a stable, heritable biochemical phenotype [Weinshilboum, 1978; Vuchetich et al., 1991]. The major gene controlling serum D β H activity is linked to the ABO blood group locus on human chromosome 9q34 [Wilson et al., 1988, 1990], as is the structural gene (locus abbreviation: DBH) encoding D β H [Craig et al., 1988; Perry et al., 1991; Gelernter et al., 1991a]. DBH thus appears to be the major gene controlling serum D β H activity. Wei et al. [1997] provided further evidence for this hypothesis by demonstrating significant associations between alleles of a

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*Correspondence to: Joel Gelernter, Psychiatry 116A2, VA Connecticut, West Haven Campus, 950 Campbell Ave., West Haven, CT 06516. E-mail: gelernter-joel@cs.yale.edu

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DBH simple tandem repeat polymorphism [Porter et al., 1992] and serum D β H activity.

Serum D β H activity has been reported to be lower in patients suffering from schizophrenia [Fujita et al., 1978] or psychotic depression [Meltzer et al., 1976; M6d et al., 1986; Sapru et al., 1989] than in control subjects. Other studies, while finding no differences in D β H activity between schizophrenic patients and controls, have found low serum or cerebrospinal fluid (CSF) D β H activity to be associated with the paranoid subtype of schizophrenia [Meltzer et al., 1980; Arat6 et al., 1983], with better response to antipsychotic medications [Sternberg et al., 1983], or with differences in measures of psychosocial functioning [Sternberg et al., 1983; van Kammen et al., 1994]. DBH is therefore an important candidate gene for causing or modifying psychotic illness.

Two allelic DBH cDNAs were isolated [Kobayashi et al., 1989] from a human pheochromocytoma library prepared from a single tumor [Kaneda et al., 1987]. In addition to several silent sequence differences scattered throughout the coding region, these allelic cDNAs differ by a single base at position 910, in exon 5. As a result, codon 304 encodes either alanine (DBH*304A, encoding D β H-ala) or serine (DBH*304S, encoding D β H-ser [Kobayashi et al., 1989]). These data suggest that at least two different expressed alleles of DBH exist in humans, but give no hint as to their relative frequencies. An alternative explanation for the presence of apparent expressed variation in the DBH cDNAs could have been a sequence change that arose only in the tumor from which the clones were isolated.

Data on the relative enzymatic activities of D β H-ala and D β H-ser have not been consistent across laboratories [Ishii et al., 1991; Li et al., 1996]. However, biochemical evidence on the heat sensitivity of D β H activity in human serum is consistent with the existence of variant D β H proteins in European-Americans [Dunnette and Weinshilboum, 1982]. In that study, approximately 5.5–8.0% of subjects exhibited thermo-labile D β H activity when the data were categorized according to a numerical cutoff in the ratio of D β H activity measured in heat-treated vs. untreated samples (H/C ratios). When H/C ratio was treated as a quantitative trait, the model that best fit the data was a 2-allele, single locus model, in which the estimated frequency of the allele conferring heat lability was approximately 0.28 [Vuchetich et al., 1991]. Given this evidence for heritable variation in the D β H protein, it is important to investigate expressed variation at the DBH locus.

To our knowledge, studies of DBH*304A and DBH*304S in human populations have not yet been undertaken. The present study confirms these sequence variants as a polymorphism in human genomic DNA, and estimates their frequencies in European- and African-Americans, as well as a variety of geographically dispersed populations.

MATERIALS AND METHODS

Subjects

The European- and African-American samples were collected from unrelated adults residing in Connecti-

cut, USA, using consent procedures approved by the Human Investigations Committees of the West Haven VA Medical Center, the Yale University School of Medicine, or the University of Connecticut Health Center. Forty-six percent (46/100) of the European-Americans and 75% (41/55) of the African-Americans were screened for absence of psychiatric and substance use diagnoses, using the Psychoactive Substance Use Disorders section of the Structured Clinical Interview for DSM-III-R (SCID [Spitzer et al., 1992]) and portions of the Computerized Diagnostic Interview Schedule (C-DIS-R [Levitan et al., 1991; Blouin et al., 1988]), or the SCID for psychiatric diagnostic assessment. Three African-American subjects and three European-American subjects did not meet criteria for any present substance use disorder, but met criteria for past abuse of alcohol or other substances. The data from these subjects were included in the analysis, given our previous demonstration that unscreened (or random population) control groups containing common phenotypes (such as alcoholism, at an estimated lifetime population prevalence of 13%) are valid for comparisons, even with affected groups, provided the affected phenotype is not extremely common in the general population [Gelernter et al., 1991b]. The remainder of the African- and European-American subjects (25% and 54%, respectively) were not screened. The Japanese samples were collected, using procedures approved by the Ethical Committee of the Fujita Health University, from healthy, unrelated ethnic Japanese adults residing throughout Japan. DNA samples from the other population groups (Mbuti, Adygei, Danes, Chinese, Nasioi, Surui, and Maya) were extracted from Epstein-Barr virus-transformed lymphocytes, as described previously [Kidd et al., 1991; Chang et al., 1996; Tishkoff et al., 1996] (further information on these samples, including acknowledgment of the numerous individuals who helped assemble the collection, can be found on the Internet at <http://info.med.yale.edu/genetics/kkidd/>).

DNA Preparation

DNA from African- and European-Americans was extracted from whole blood by the method of Lahiri and Nurnberger [1991], with the addition of a phenol-chloroform extraction prior to final precipitation. DNA from the Japanese samples was extracted as follows: EDTA-anticoagulated blood was mixed with lysis buffer containing 0.32 M sucrose, 10 mM Tris-HCl, pH 7.5, 5 mM MgCl₂, and 1% triton X-100, and nucleated cells were collected by centrifugation. After resuspension in 10 mM Tris-HCl (pH 8), 100 mM NaCl, 25mM EDTA, and 0.5% SDS, the pellet was then digested in proteinase K (final concentration of 75 μ g/ml) by incubation at 50°C for at least 4 hr. DNA was purified by phenol/chloroform extraction and ethanol precipitation. Procedures for extraction of DNA from the transformed lymphocytes have been described previously [Kidd et al., 1991; Chang et al., 1996].

Polymerase Chain Reaction (PCR)

Exon 5 of the DBH gene was amplified by PCR as follows: In initial studies, 25 μ l reactions contained 0.5

U of Taq polymerase (Gibco-BRL), 2.25 mM MgCl₂, 50 mM KCl, 10 mM Tris, pH 8.4, 0.5 μM of each primer, and 25 ng of template DNA. Primer sequences for DBH exon 5 were based on previously published data [Kobayashi et al., 1989; sense: 5'-ccc aca cag GCA TTT TAC TAC CC-3'; antisense: 5'-cta cCT TCT ATC ACC AGT GGG-3', where uppercase letters represent exonic sequence and lowercase intronic sequence; amplicon length: 116 bp]. Temperature cycles were: 94°C for 5 min, followed by 30 cycles of 94°C for 30 sec, 55°C for 30 sec, and 72°C for 30 sec. For DGGE, PCR was as above, except that the antisense primer contained a 39 bp GC clamp. PCR-RFLP analysis was performed on longer amplicons (200 bp), in which the primers were based entirely on flanking intronic sequence (sense: 5'-gcc ctc tca gga cac acc-3'; antisense: 5'-aca cag ctg agt cct agg g-3'). For these amplifications, 15 or 20 μl reactions contained the foregoing concentrations of reagents, except for MgCl₂ (1.5mM). Temperature cycles were: 95°C for 5 min, followed by 30 cycles of 95°C for 30 sec, 59°C for 30 sec, and 72°C for 30 sec.

Denaturing Gradient Gel Electrophoresis (DGGE) and Sequencing

DGGE [Myers et al., 1987] was performed in a Bio-rad D-Gene apparatus. Perpendicular DGGE was run at 60°C in Tris-acetate/EDTA buffer, pH 8.0, at 5.0 V/cm overnight. The gels were 1 mm thick and contained 10% 37.5:1 acrylamide/bis acrylamide, with a 20–80% gradient (100% denaturant = 7 M urea + 40% formamide). Parallel DGGE was run under the same conditions as above, except that the gradients were either 40–80% or 30–70%. Bands on perpendicular gels were identified using ethidium bromide or silver staining, while only ethidium bromide was used for parallel gels. DNA was eluted from parallel DGGE bands after staining with ethidium bromide by cutting the bands out of the gel, crushing the polyacrylamide in a 1.5 ml tube, and adding 0.4 ml saturated phenol and 0.4 ml 10 mM Tris, pH 8.0/0.5M NaCl. After overnight incubation at room temperature, 0.4 ml of chloroform was added and the aqueous phase was extracted again with fresh phenol/chloroform. After passage over a Sephadex G-50 minicolumn, the DNA was ethanol-precipitated and air-dried prior to final resuspension in 10mM Tris buffer, pH 8.0. This gel-purified DNA, representing different alleles from heterozygous individuals, was sequenced directly by automated cycle sequencing, using the above primers spanning the intron–exon boundaries, at the core sequencing facility of the Yale University Boyer Center for Molecular Medicine.

PCR-RFLP Analysis

Sequencing confirmed the presence of a G to T substitution at nucleotide position 910 [numbering as in Kobayashi et al., 1989], which results in a change in predicted amino acid sequence from alanine to serine [Kobayashi et al., 1989]. This substitution results in the loss of a restriction site for Mwo I (New England Biolabs); thus, the more common allele (DBH*304A), encoding alanine at codon 304, is cut by Mwo I to pro-

duce fragments of approximately 150 and 50 bp, while the less common serine-specifying allele (DBH*304S) remains uncut. To distinguish the two alleles, 15 μl aliquots of PCR product, in the reaction buffer described above, was added to 30 μl of the enzyme buffer recommended by the manufacturer. Digestions were incubated from 3–8 hr at 60°C. The digests were separated by electrophoresis on agarose/Synergel (0.7%/0.75%; Diversified Biotech, Boston MA) and visualized using ethidium bromide under UV transillumination. To confirm the accuracy of the PCR-RFLP, 13 subjects were typed by PCR-RFLP and DGGE. The results showed 100% concordance.

Hardy-Weinberg Equilibrium

The very small number of DBH*304S homozygotes observed in this study precluded the use of χ^2 -based tests for deviation from Hardy-Weinberg equilibrium, because such testing would have produced exaggerated *P*-values. To test for deviation from Hardy-Weinberg expectations, empirical *P*-values were obtained by a Monte Carlo permutation method. For each population, the observed numbers of each allele were shuffled among individuals at random in 10,000 iterations. The *P*-values given (Table II) are the observed proportion of iterations in which the observed number of genotypes were equal to those found in the original samples, or were more deviant from the Hardy-Weinberg expectation. If the observed data showed an excess of homozygotes, the shuffling replicates with that result and all cases with even more homozygotes were counted. If the observed data showed a deficiency of homozygotes, replicates with that result and all cases with even fewer homozygotes were counted. HWSIM, the computer program that generated these simulations, was written by one of the authors (FC). It is available by anonymous FTP from paella.med.yale.edu, directory pub/hwsim.

RESULTS

To determine initially whether polymorphisms in DBH exon 5 [Kobayashi et al., 1989] were present in the US population, samples from European- and African-American subjects were screened using DGGE. Perpendicular DGGE (not shown) showed a prominent sigmoid band, with a faint secondary band diverging from the main one, indicative of a variation in sequence. Parallel DGGE confirmed heterozygosity at DBH exon 5 in several individuals (Fig. 1). Elution and sequencing of the homoduplex bands showed that the lower band contained DNA identical in sequence to exon 5 of DBH*304A and the upper band DBH*304S.

DBH*304A and DBH*304S allele frequencies, determined by PCR-RFLP analysis in DNA samples from different human populations, are shown in Table I. Homogeneity testing strongly rejected the null hypothesis that the observed allele frequencies resulted from sampling a single population ($\chi^2 = 36.3$, 9 d.f., $P < 0.0001$). The DBH*304S allele was most common in the African-American, Mbuti, and Nasioi samples, and was not observed in the Chinese and Surui. This allele appears to be rare in East Asian-derived populations. DBH*304S was more frequently observed in African-American

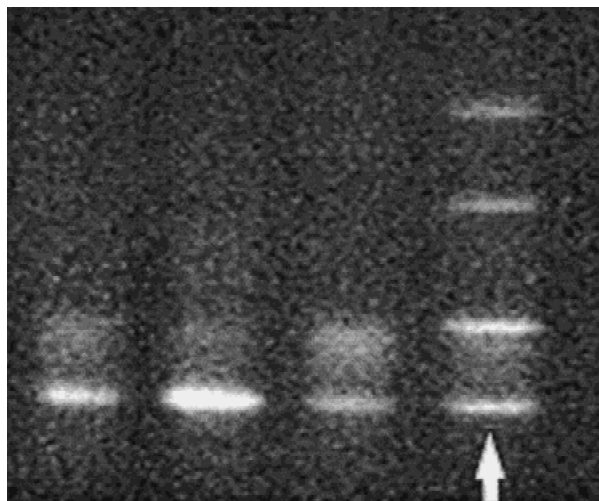


Fig. 1. Identification of DBH*304A and DBH*304S alleles by DGGE. DBH exon 5 was amplified, parallel DGGE performed, and the bands visualized, as described in Methods. A heterozygote (arrow) displays four bands. The upper two bands in the heterozygote lane represent heteroduplexes, formed by reannealing of mismatched single strands from each allele. These are always more faint than homoduplex bands because they are less stable, but their greater displacement can facilitate identification of heterozygotes [Ruano and Kidd, 1992]. Correctly matched homoduplexes, each representing a distinct allele, form the bottom pair of bands in the heterozygote lane, with the DBH*304S allele forming the upper homoduplex. The single bands are from DBH*304A homozygotes. A faint PCR artifact is present in all lanes, approximately at the level of the DBH*S band.

samples than in European-American samples ($P = 0.004$, Fisher's Exact Test). Across the population samples examined, the mean frequency of DBH*304S was 0.07, and the F_{st} was 0.05.

DBH*304S homozygotes were absent in all but two of the groups (Danes and Adygei; see Table II). Simulations, to test whether the observed frequencies of each genotype at the DBH*304 site conformed to Hardy-Weinberg expectations, were run for each population, as described in Methods. The Danes were the only population that appeared to deviate from Hardy-Weinberg equilibrium, with a probability less than 0.05. However, the observed $P = 0.013$ does not reach significance after application of the Bonferroni correction for ten tests ($\alpha = 0.005$).

To address the possibility that the apparent DBH*304S homozygotes in the Danish sample represented amplification of only a single allele, because of mispriming artifacts in the presence of an unknown sequence variation, the genotypes in these samples were confirmed using both sets of primers (described above). Sequencing, in both directions, of the Danish homozygote samples verified that codon 304 indeed encoded serine, and that no other exon 5 sequence differences were apparent.

DISCUSSION

The present study demonstrates the presence of the DBH*304A and DBH*304S alleles in most of a variety of human populations representing all major geographic regions. The DBH*304S allele was absent in the Chinese and Surui samples. However, although

rare, it was observed in other East Asian (Japanese) and Native American (Maya) samples. It is therefore likely that we did not detect DBH*304S in the Chinese and Surui samples because of the small sample sizes and rarity of the allele in populations from East Asia and America. While the relative frequencies of the two alleles vary among groups, DBH*304A is always the more common allele, with frequencies greater than 0.80 in every group examined. The presence of DBH*304S in Africans (Mbuti) and its significantly higher frequency in African-Americans than in European-Americans suggests the mutation giving rise to allelic variation in DBH codon 304 occurred prior to the migration of humans from Africa [Tishkoff et al., 1996].

Only two population samples, both from Europe, contained DBH*304S homozygotes. While the sample of Danes did not show significant deviation from Hardy-Weinberg equilibrium, we cannot, with absolute confidence, rule out such a deviation given the small sample size, and the P -value of 0.013 that resulted from our simulation. Population stratification, wherein we may have sampled a subpopulation from Europe that carries a higher frequency of the DBH*304S allele, cannot be ruled out. Alternatively, a third allele with a deletion large enough to disrupt the amplification of DBH exon 5 would not be detected by our current assay, and could account for a potential deviation from Hardy-Weinberg expectations generated from a two-allele model. Both possibilities require further study. The observation of significant heterogeneity in DBH*304S allele frequency across different population samples emphasizes the importance of precisely specifying populations, and controlling for population stratification, in future studies testing for association between DBH*304S and clinical phenotypes.

The effect of expressed variation at DBH codon 304 on D β H protein function is not yet clear. Ishii et al. [1991] expressed the two variants in COS cells, and estimated homospecific activities (activity per unit of enzyme protein) of D β H in cellular homogenates, using a specific antiserum to human D β H to estimate D β H protein levels by Western blot. They found more than a 10-fold difference in homospecific activity between the two forms, with DBH*304S encoding the lower activity form. More recently, Li et al. [1996] expressed the two variants in *Drosophila* Schneider 2 cells, and then assayed the activity of the purified D β H proteins. They found no difference in homospecific D β H activity between the variant proteins. Our preliminary efforts to associate genotype at DBH codon 304 with plasma D β H activity have thus far shown no difference in the mean D β H activities exhibited by DBH*304A homozygotes and DBH*304 S/A heterozygotes (J.F. Cubells, J. Gelernter, G.M. Anderson, and L.H. Price, unpublished observations). While these preliminary results seem most consistent with the results of Li et al. [1996], analysis of the biochemical phenotypes of DBH*304S homozygotes, as well as estimation of plasma D β H homospecific activity in subjects of known genotype, will be necessary to resolve the issue.

Human serum D β H activity varies widely in humans (approximately 2,000-fold in European-Americans [Weinshilboum, 1978]). It thus appears unlikely that

TABLE I. Frequencies of DBH Exon 5 Alleles Observed in African-Americans, European-Americans, and Several Geographically Isolated Populations Worldwide

Global region	Group	2N	DBH*304S	DBH*304A	f(DBH*304S)	S.E.
African	African-American ^a	110	18	92	0.16*	0.04
	Mbuti	72	9	63	0.13	0.04
Europe	European-American ^a	200	12	188	0.06	0.02
	Danes	70	7	63	0.10	0.04
	Adygei	84	5	79	0.06	0.03
East Asia	Chinese	80	0	80	0.00	0.00
	Japanese	96	1	95	0.01	0.01
America	Surui	46	0	46	0.00	0.00
	Maya	66	2	64	0.03	0.02
Australo-Melanesia	Nasioi	40	6	34	0.15	0.06

2N indicates the number of chromosomes sampled in each group. S.E. indicates the binomial standard error.

*Significantly greater than 0.06 (European-Americans), $P = 0.004$, Fisher's exact test.

^aThe global region listed represents the predominant origin of this probably somewhat admixed group.

DBH*304A and DBH*304S, or any other single biallelic DBH polymorphism, can account for more than a small proportion of the population variance in human serum D β H activity. Weinshilbom et al. [1975] showed that approximately 5% of subjects in a large sample of European-Americans exhibited very low serum D β H activity (defined as ≤ 50 on a scale of approximately 0–2,000). They proposed that such individuals were homozygous for an allele, "DBH^L," specifying very low D β H activity. This proposed allele occurred at an apparent frequency of approximately 20%. The frequencies of the expressed DBH alleles studied here do not match those estimated by Weinshilbom for DBH^L. Neither do they correspond to those estimated in the two-allele system proposed to account for heritable variation in D β H thermal stability in European-Americans (0.28 and 0.72 for heat-labile and heat-stable phenotypes, respectively [Vuchetich et al., 1991]). Further work is necessary to evaluate the contribution of allelic variation in the DBH structural gene to heritable variation in the levels, activity, and thermal lability of D β H in serum, CSF, and, ultimately, noradrenergic neurons.

Numerous laboratories have measured D β H activities or D β H protein levels in the serum or CSF of medi-

cally ill [Kopin et al., 1976; O'Connor et al., 1994] or psychiatrically disordered patients [e.g., Fujita et al., 1978; Meltzer et al., 1976, 1980; M6d et al., 1986; Sternberg et al., 1982, 1983; van Kammen et al., 1994]. From these studies, several intriguing correlations between D β H levels and important features of disease have emerged. However, a persistent problem with these and other studies has been the lack of a precise definition of the physiological meaning of serum and CSF levels of D β H, whether measured as D β H activity or amount of D β H protein. This want of clarity arises from uncertainty about the relationship between serum or CSF D β H levels and neuronal D β H function. Linkage studies employing serological and biochemical markers have provided significant evidence for linkage between a major gene controlling serum D β H activity and the ABO blood group locus, residing at 9q34 [Goldin et al., 1982; Wilson et al., 1988, 1990]. Chromosomal in situ hybridization [Craig et al., 1988] and linkage analysis employing molecular markers [Perry et al., 1991; Gelernter et al., 1991a] show that DBH resides at 9q34, in tight linkage with ABO. These converging lines of evidence suggest that DBH is the major gene controlling the serum D β H phenotype. Thus, the most important meaning of serum and CSF D β H activities

TABLE II. Tests for Deviation From Hardy-Weinberg Equilibrium on the Observed Frequencies of DBH*304S Homozygotes, DBH*304 S/A Heterozygotes, and DBH*304*A Homozygotes, in African-Americans, European-Americans, and Several Geographically Isolated Populations Worldwide

Global region	Group	DBH*304S homozygotes	DBH*304 S/A heterozygotes	DBH*304A homozygotes	P
Africa	African-American*	0	18	37	0.094
	Mbuti	0	9	27	0.347
Europe	European-American*	0	12	88	0.429
	Danes	3	1	31	0.013
	Adygei	1	3	38	0.373
East Asia	Chinese	0	0	43	—
	Japanese	0	1	47	1.000
America	Surui	0	0	23	—
	Maya	0	2	31	0.841
Australo-Melanesia	Nasioi	0	6	14	0.416

Empirical P -values were determined by computer simulation, as described in Methods. Only the Danes exhibited deviation from Hardy-Weinberg equilibrium at $P < 0.05$. However, this value is greater than the Bonferroni-corrected $\alpha = 0.005$, required for statistical significance after ten tests.

*The global region listed represents the predominant origin of this probably somewhat admixed group.

may be as indicators of allelic variation in DBH. The DBH gene is present as a single copy per haploid genome [Kobayashi et al., 1989]. It is therefore certain that expressed allelic variation in DBH will affect the protein structure (and, possibly, function) of D β H within central noradrenergic neurons, as well as in CSF and serum. Understanding the details of such effects could provide clues to the role of DBH in heritable aspects of noradrenergic transmission, and as a causative or modifying gene in psychiatric and medical illness.

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