

Report

A Quantitative-Trait Analysis of Human Plasma-Dopamine β -Hydroxylase Activity: Evidence for a Major Functional Polymorphism at the *DBH* Locus

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Dopamine- β -hydroxylase (D β H) catalyzes the conversion of dopamine to norepinephrine and is released from sympathetic neurons into the circulation. Plasma-D β H activity varies widely between individuals, and a subgroup of the population has very low activity levels. Mounting evidence suggests that the *DBH* structural gene is itself the major quantitative-trait locus (QTL) for plasma-D β H activity, and a single unidentified polymorphism may account for a majority of the variation in activity levels. Through use of both sequencing-based mutational analysis of extreme phenotypes and genotype/phenotype correlations in samples from African American, European American (EA), and Japanese populations, we have identified a novel polymorphism (–1021C→T), in the 5' flanking region of the *DBH* gene, that accounts for 35%–52% of the variation in plasma-D β H activity in these populations. In EAs, homozygosity at the T allele predicted the very low D β H-activity trait, and activity values in heterozygotes formed an intermediate distribution, indicating codominant inheritance. Our findings demonstrate that –1021C→T is a major genetic marker for plasma-D β H activity and provide new tools for investigation of the role of both D β H and the *DBH* gene in human disease.

Dopamine- β -hydroxylase (D β H [MIM 223360]) catalyzes the conversion of dopamine to norepinephrine. It is localized in catecholamine-containing vesicles of adrenergic and noradrenergic cells (Oka et al. 1967; Kemper et al. 1987). D β H protein is released in response to stimulation (Viveros et al. 1968), and D β H activity, derived largely from sympathetic nerves, can be measured in human plasma (Weinshilboum and Axelrod 1971a; Weinshilboum 1978). Plasma (or serum) activity levels vary widely among individuals (Weinshilboum et al. 1973) but are stable within individuals over time (Fahn-

drich et al. 1982). Family and twin studies have indicated that the majority of interindividual variability in plasma-D β H activity is genetically determined (Ross et al. 1973; Weinshilboum et al. 1973; Oxenstierna et al. 1986).

In a large study of European Americans (EAs), Weinshilboum et al. (1975) identified a subgroup, consisting of 3%–4% of the population, who have very low levels of plasma-D β H activity. Parents of these individuals have an average enzyme activity that is intermediate between the very low D β H-activity subgroup and a population selected at random. Pedigree segregation analyses of these families suggested that the very low D β H-activity trait is monogenically inherited by an autosomal codominant mechanism (Weinshilboum 1983). Weinshilboum (1978) postulated the existence, at an unspecified locus, of a functional low-activity allele, DBH^L, with a frequency of ~20% in the EA population. A single locus was estimated to account for 46%–75% of the

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plasma D β H–activity variance in the population (Elston et al. 1979; Goldin et al. 1982; Wilson et al. 1990). Studies using protein-phenotype markers showed strong evidence for linkage between a major locus controlling plasma-D β H activity and the ABO blood-group locus (Goldin et al. 1982; Wilson et al. 1988). The *DBH* gene was subsequently cloned, mapped to chromosome 9q34, and shown, by molecular studies, to be linked to ABO (Lamouroux et al. 1987; Craig et al. 1988; Gelernter et al. 1991; Perry et al. 1991). Molecular markers at the *DBH* locus were then shown to associate with variation in both plasma-D β H activity (Wei et al. 1997; Cubells et al. 1998, 2000) and cerebrospinal-fluid (CSF) levels of immunoreactive D β H protein (Cubells et al. 1998). Taken together, these findings strongly suggest that *DBH* itself is the major quantitative-trait locus (QTL) for plasma-D β H activity—and that it contains the DBH^L allele. The molecular identity of the DBH^L allele remains to be determined.

Altered plasma-D β H activity has been reported in a variety of psychiatric and neurological disorders (Weinshilboum and Axelrod 1971b; Lieberman et al. 1972; Fujita et al. 1978; Matuzas et al. 1982; Nagatsu et al. 1982). Given the evidence summarized above, *DBH* may therefore be an important disease-causing or disease-modifying candidate gene. Disease-related variation in D β H activity could be interpreted more readily if the molecular basis of heritable variation in plasma D β H were known. Comprehensive studies examining the relationship between polymorphic variation at *DBH* and plasma-D β H activity have not been published. Here we report a sequencing-based mutational analysis of the *DBH* gene from subjects with extreme plasma-D β H phenotypes. We then examine genotype/phenotype correlations in samples from African American (AA), Japanese (Jp), and EA populations.

Plasma and DNA samples from 58 unrelated AA and 174 unrelated EA subjects (self-identified population groups; those of mixed or other heritage were excluded) were collected during the course of several ongoing genetic studies. The groups included both healthy individuals and those with psychiatric and substance-abuse disorders. As discussed elsewhere (Cubells et al. 1998), sampling from a variety of diagnostic groups is unlikely to obscure fundamental genetic influences on plasma-D β H activity. Plasma and DNA samples were obtained from 53 healthy, unrelated ethnic-Japanese subjects recruited at the Institute for Comprehensive Medical Sciences, Fujita Health University, Toyoake, Japan. All subjects gave informed consent for participation in molecular-genetic studies.

D β H activity was assayed in plasma samples from all subjects, as described elsewhere (Cubells et al. 1998). The *DBH* gene is ~23 kb in length and contains 12 exons (Kobayashi et al. 1989). The genomic sequence is publicly available (Genbank accession numbers AC000404

and AC001227). We searched for potential functional polymorphisms by sequencing a total of 6,443 bp of *DBH*, including the proximal 1,468 bp of the 5' upstream area, all exons (2,744 bp), and 2,182 bp of intronic sequence, spanning ≥ 49 bp flanking each exon. A minimum of eight individuals were selected to represent extreme values of D β H activity. This group included at least four individuals (two EA and two Jp) with very low activity (defined here as <2.5 nmol octopamine/min/ml plasma [hereafter expressed as “nmol/min/ml”]), two EAs with near-average activity, and two EAs with activity >2 SD above the mean. Twelve subjects, eight (six EA and two Jp) with very low activity, were included for the sequencing of the proximal 1.0 kb of the promoter region and of exon 1. Sequencing was performed, with minor modifications, as described elsewhere (Zabetian et al. 2000). PCR primer sequences and reaction conditions are available on request.

All newly detected polymorphisms that appeared to aggregate according to D β H activity in the sequenced subset of samples, as well as all previously described non-synonymous polymorphisms, were selected as functional candidates and were genotyped in the entire AA, EA, and Jp samples. The average rate of PCR nonamplification for each population-specific genotype group was 1.6%, and the maximum was 4.0%. Genotypes were determined by RFLPs, by digestion of PCR products with the appropriate restriction enzymes, as shown in table 1. RFLP genotyping was confirmed by the sequencing of representative samples of each polymorphism.

An initial plot of genotypes at –1021C→T and of population-specific variance against mean D β H activity produced three approximately straight lines passing through the origin (data not shown), suggesting that a square-root transformation would be appropriate for stabilization of the variance. Maximum likelihood was therefore used to fit a Box-Cox power transformation (Box and Cox 1964) and to test equality of the power parameter to 0.5, on the assumption of normality and a common variance. On the basis of the result of this test, an analysis of variance of square-root D β H activity was used to test for genotype effects, population effects, and genotype \times population interaction. In addition, deviation from additive gene action was tested and was found to be nonsignificant. Because heritability can be estimated more accurately if Hardy-Weinberg equilibrium (HWE) can be assumed, deviations from HWE were assessed using the HWSIM program (Cubells et al. 1997; also see the HWSIM Web site). Since some of the analyses contained small numbers of observations in some cells, *P* values for all analyses were estimated empirically, with Monte Carlo simulations (10,000 iterations in each case) based on observed allele frequencies. Significance levels were estimated as the proportion of times that the simulated distribution reached or exceeded the observed deviation from HWE. Herita-

Table 1
Allele Frequencies of *DBH* Polymorphisms in Three Population Samples

LOCATION	POLYMORPHISM ^a	AMINO ACID CHANGE	RESTRICTION SITE FOR GENOTYPING	MEAN ± BINOMIAL SE, FOR ALLELE FREQUENCY IN		
				AA (2n=116)	EA (2n=348)	Jp (2n=106)
5' Flanking	−1021C→T	...	<i>MwoI</i> ^b	.198 ± .037	.224 ± .022	.160 ± .036
Exon 3	499G→C ^c	E167Q	<i>BsrI</i>	ND ^d	ND	ND
Exon 3	589G→A ^c	A197T	<i>BstUI</i>	.129 ± .031	.083 ± .015	.144 ± .035
Exon 3	675G→C ^c	K225N	<i>Apal</i> ^b	ND	ND	ND
Exon 4	706G→C ^c	E236Q	<i>BanII</i>	ND	ND	ND
Exon 4	826G→A ^c	D276N	<i>BsiEI</i>	.052 ± .021	.003 ± .003	ND
Intron 4	IVS4+601T→C	...	<i>BsmBI</i> ^b	.348 ± .045	.524 ± .034	.837 ± .036
Exon 5	908T→C ^c	L303P	<i>BsII</i>	ND	ND	ND
Exon 5	910G→T ^c	A304S	<i>MwoI</i>	.147 ± .033	.062 ± .013	.010 ± .010
Exon 11	1603C→T ^c	R535C	<i>BstUI</i>	.035 ± .017	.043 ± .011	ND

^a Nucleotide positions are numbered according to either cDNA sequence, for exons, or genomic sequence, for the 5' flanking region, beginning at the A of the ATG initiator Met codon; positions for introns are numbered according to the genomic sequence, starting from the G of the donor-site invariant GT.

^b Artificial restriction site.

^c From Halushka et al. (1999).

^d ND=not detected in sample.

^e From Kobayashi et al. (1989).

bility was estimated, under the assumption of HWE, as follows: $b^2 = [2p(1-p)\delta^2]/[2p(1-p)\delta^2 + s^2]$, where the additive gene effect is δ , the gene frequency is p , s^2 is the estimate of the common variance about the genotype mean, and its standard error is obtained by application of the large-sample “delta” method (DeGroot 1989), by double differentiation of the log likelihood to obtain the variance-covariance matrix of δ and s^2 . A pooled estimate of heritability was also calculated under the assumption of HWE, as a weighted average of the individual population heritabilities, with weights inversely proportional to their variances and summing to 1.

The plasma D β H-activity data are summarized in figure 1. There was a small but statistically significant difference, in mean activity values, between EAs and Jps ($P = .003$), but there were no differences between AAs and either Jps or EAs ($P > .1$). In the EA group, a cluster of individuals with activity values <2.5 nmol/min/ml was identified by visual inspection of frequency histograms (fig. 1), and was defined as the “very low D β H-activity” subgroup. This corresponds closely with the threshold of <2.6 nmol/min/ml defined by Weinshilboum et al. (1975) for the very low D β H-activity subgroup (corrected, for differences in methodology, by a factor of 18.9, per Weinshilboum [1978]). This subgroup constituted 7.5% of EAs and 3.8% of Jps in the present study. In the AA group, however, no discrete cluster of very low levels was readily discernible, with 5.2% of the activity values <2.5 nmol/min/ml.

The sequencing of the eight individuals representing phenotypic extremes revealed 18 single-nucleotide polymorphisms (SNPs) not previously reported (ID numbers obtained from the Database of Single Nucleotide Poly-

morphisms). Fourteen of these were located in introns, four in the 5' flanking region, and none in the coding sequence. The most proximal SNP in the 5' flanking region occurred at position −496. Two of these SNPs—one located in the 5' flanking region (−1021C→T), the other in intron 4 (IVS4+601T→C)—associated with the very low D β H-activity subgroup in the sequenced samples and were included in further genotyping studies of the complete sample populations.

Four of the eight previously described nonsynonymous SNPs were not detected in any of the three population samples, and only two (589G→A and 910G→T) occurred in all three samples (table 1). There were no deviations from HWE for genotypes at any of the polymorphisms in either the AA group or the Jp group. In the EA sample, a small but statistically significant ($P = .034$) deviation from HWE was seen in genotypes at −1021C→T, but at none of the other polymorphisms. However, this observed P value does not reach significance after application of the Bonferroni correction for three tests.

The −1021C→T polymorphism was strongly associated with untransformed D β H-activity values in all three population samples ($P < .001$). Because of significant heterogeneity of variance between genotype groups, an appropriate transformation function was estimated, as described above. The value of the power parameter was .5083, whether separate genotypic means or additive gene effects were fitted, and the nonadditivity (dominance variance) was not significant ($P = .72$). This estimate was not significantly different ($P = .9$) from .5, the square-root function. Table 2 gives the population-/genotype-specific means (and the standard errors of the mean) for square-root D β H activity. An analysis of var-

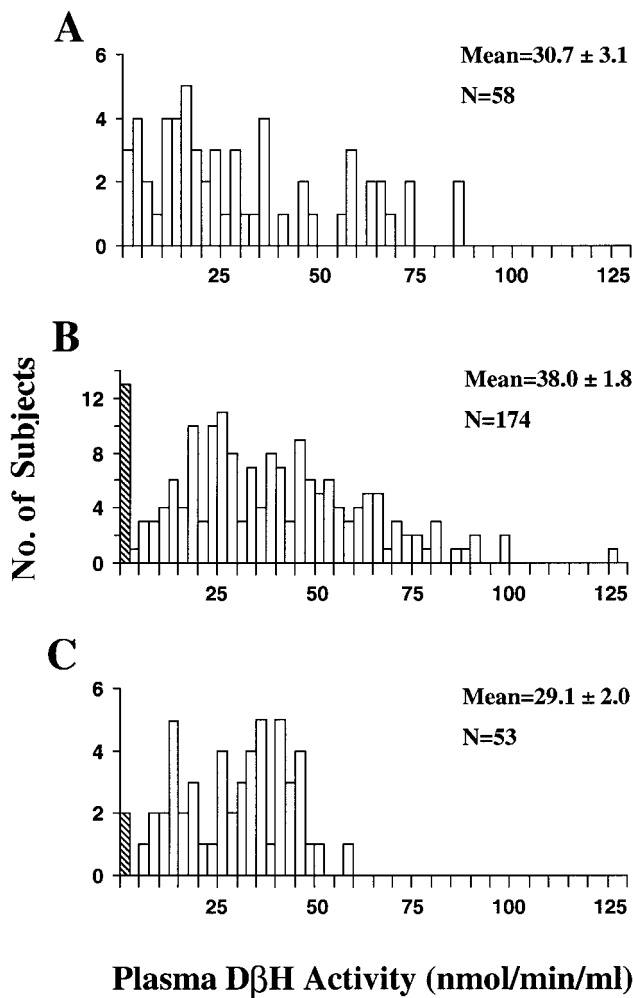


Figure 1 Distribution of plasma-DβH activity in AA (A), EA (B), and Jp (C). Subjects are grouped in successive 2.5-nmol/min/ml increments. The hatched bar indicates the results for the very low DβH-activity group. The EA and Jp mean activity values differed significantly ($P < .01$, one-way ANOVA; followed by Dunnett C post-hoc test).

iance of square-root DβH activity indicated significant effects of genotype at $-1021C \rightarrow T$ ($P < .001$) and of population ($P < .001$), but not of genotype \times population interaction ($P = .51$). The proportion of variability (R^2) due to genotype at $-1021C \rightarrow T$ was .35, .51, and .52 for the AAs, EAs, and Jps, respectively. Addition of 1603C \rightarrow T to the model resulted in a small but significant ($P = .0024$) increase of .02 in R^2 . The added contribution of the remaining four polymorphisms detected in our population samples was not significant ($P > .05$).

The heritabilities \pm SE of square-root DβH activity under the assumption of HWE and additive gene action were $h^2 = .31 \pm .12$, for AAs; $.44 \pm .09$, for EAs; and $.52 \pm .28$, for Jps ($h^2 = .50$ for EAs, when there is no assumption of HWE). The overall (mean \pm SEM) her-

itability was $.40 \pm .07$ when data from all three populations were pooled. Histograms of square-root DβH activity versus genotype at $-1021C \rightarrow T$ are presented in figure 2. In EAs, the TT genotype was strongly associated ($P < .0001$) with the very low DβH-activity trait, since 12 of the 13 subjects with the trait were TT homozygotes. The remaining individual was heterozygous at $-1021C \rightarrow T$ but was the only subject in any of the three populations who was homozygous at the nonsynonymous exon 11 polymorphism 1603C \rightarrow T. Four TT homozygotes had transformed activity values that exceeded the very low DβH-activity threshold (<1.58 square-root nmol/min/ml), but all were below the population mean (5.74 square-root nmol/min/ml). Transformed activity values in subjects who were heterozygous at $-1021C \rightarrow T$ formed a distribution intermediate between the two homozygous groups in the EA and Jp samples, in a way consistent with autosomal codominant inheritance of the very low DβH-activity trait (fig. 2).

Several previous population-based studies have found associations between polymorphisms in the *DBH* gene and plasma-DβH activity, in individuals of European heritage (Wei et al. 1997; Cubells et al. 1998, 2000). These polymorphisms have included one in the 5' flanking region ($-4784(-)4803del$) and a synonymous SNP in exon 2 (444G \rightarrow A). However, the allele frequencies and distributions of genotype-specific mean activities for each of these polymorphisms are not compatible with those predicted for the *DBH*^L allele, and neither of these previous allelic associations holds in any sample except the EA sample (J.F.C., C.P.Z., G.M.A., and J.G., unpublished observations). Therefore, these previous as-

Table 2
DβH Activity Values Specific to Genotypes at $-1021C \rightarrow T$ in Three Population Samples

SAMPLE AND GENOTYPE (<i>n</i>)	DβH Activity (nmol/min/ml)	MEAN ± SEM		<i>h</i> ^{2 a}
		Square-Root DβH Activity (square-root nmol/min/ml)		
AA:				
TT (4)	6.0 ± 4.2	2.07 ± .76		
CT (15)	15.8 ± 3.7	3.62 ± .44		
CC (39)	39.0 ± 3.7	5.96 ± .30		
Overall				.31 ± .12
EA:				
TT (16)	4.1 ± 2.1	1.47 ± .36		
CT (46)	25.2 ± 2.0	4.80 ± .22		
CC (112)	48.1 ± 2.1	6.74 ± .15		
Overall				.44 ± .09
Jp:				
TT (1)	.4	.66		
CT (15)	15.0 ± 1.7	3.79 ± .21		
CC (37)	35.6 ± 1.8	5.85 ± .20		
Overall				.52 ± .28

^a Under the assumption of HWE.

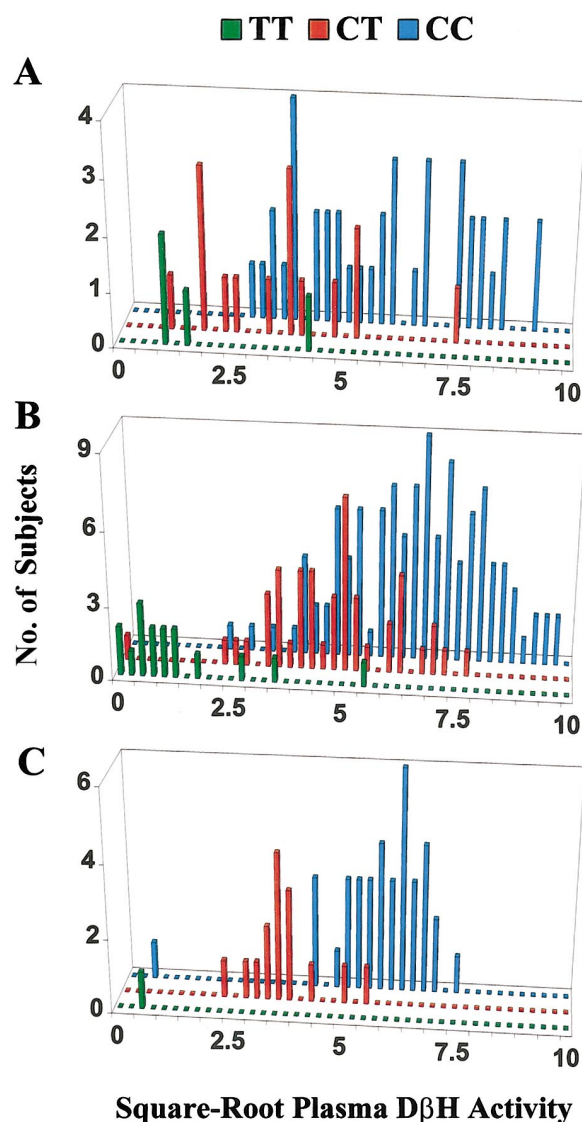


Figure 2 Distribution of square-root plasma-D β H activity, by genotype, at *DBH* polymorphism $-1021C \rightarrow T$ in AA (A), EA (B), and Jp (C). Green bars denote T/T homozygotes, red bars denote C/T heterozygotes, and blue bars denote C/C homozygotes. A single C/C homozygote with an extreme activity value of 11.2 square-root nmol/min/ml was omitted from the EA distribution plot, for the purpose of graphical clarity.

sociations appear to arise from linkage disequilibrium (LD) between the examined alleles and nearby functional variants. In contrast, $-1021C \rightarrow T$ accounts for one third or more of the variance in plasma-D β H activity, in all three of the ethnically diverse populations examined in the present study. Such cross-population consistency in genotype-phenotype relationships supports a causal association between a polymorphism and trait outcome, rather than an association based solely on LD (Risch 2000). Moreover, the frequency ($.224 \pm .022$) of the T

allele in the EA sample examined here is very close to the predicted frequency (.20) for DBH^L (Weinshilboum 1978). The mean \pm SEM and range (38.0 ± 1.8 and 0–125 nmol/min/ml) of activity values for EAs in this study are similar to those obtained in Weinshilboum et al.'s (1975) study of 227 unrelated healthy adult EAs (36.1 ± 1.5 and 0–129.6 nmol/min/ml, corrected as described above). Thus, the present study provides the strongest evidence yet for the molecular identity of the DBH^L allele, predicted some 25 years ago to account for a large proportion of the genetic variation in human plasma-D β H activity.

Several additional considerations suggest that $-1021C \rightarrow T$ may itself be the major functional polymorphism at the *DBH* locus, accounting for variation in plasma-D β H activity:

1. The DBH^L allele appears to lower plasma-D β H activity by diminishing the levels of circulating D β H protein, rather than by decreasing the activity of homo-specific enzymes (Dunnette and Weinshilboum 1976). A 5'-upstream polymorphism such as $-1021C \rightarrow T$, differentially regulating *DBH* transcription, would readily account for this type of biochemical phenotypic variation.
2. Reporter-gene experiments in transgenic mice suggest that the region between base pair -600 and base pair -1100 contains elements critical for human *DBH*-gene expression (Hoyle et al. 1994). To date, specific *cis*-acting motifs, including CREB- and Phox2a/2b-binding sites, have only been identified in more-proximal 5' flanking regions (Kim et al. 1998).
3. $-1021C \rightarrow T$ is located in a noradrenergic cell-type-specific DNase I hypersensitive site of the *DBH* gene (Ishiguro et al. 1993).

These observations do not directly demonstrate a functional role for $-1021C \rightarrow T$, nor do they rule out the possibility that LD with an as-yet-unidentified functional variant accounts for the strong association with D β H activity. However, these converging lines of evidence suggesting the testable hypothesis that $-1021C \rightarrow T$ alters transcriptional regulation of *DBH* expression warrant further study.

In the EA sample, genotypes at $-1021C \rightarrow T$ deviated slightly from HWE, with a greater number of TT homozygotes being observed than were expected, possibly because of a sampling bias due to the inclusion of subjects with psychiatric illnesses. Regardless of the source of the deviation, if the TT genotype is associated with the very low D β H-activity trait, then one would expect to see that trait in a larger proportion of individuals within the sample; and this is precisely what was observed: the very low D β H-activity subgroup constituted a larger proportion of our EA sample (7.5%) than it did in Weinshilboum et al.'s (1975) study of adult EAs (3.1%).

The clinical significance of plasma-D β H activity levels has been the subject of controversy for some time. Initially,

some 3 decades ago, it was hoped that plasma-D β H activity might both provide a direct measure of noradrenergic and adrenergic synaptic activity and be a sensitive indicator of sympathetic function. This idea proved overly simplistic, since it later became apparent that the wide individual variation in activity levels is primarily genetically determined, with estimates of heritability being .61–.98 (Elston et al. 1979; Oxenstierna et al. 1986; Wilson et al. 1990). Also, even extreme manipulations of sympathetic function, such as cold-pressor stimulation and strenuous exercise, had minimal effects on plasma-D β H levels, despite producing large increases in circulating catecholamines (Winer and Carter 1977; Peronnet et al. 1985). Yet, these findings do not exclude the role of plasma D β H as an indicator of long-term trends in—or of functional reserve of—the sympathetic nervous system. Our findings in the present study suggest that low plasma-D β H levels result from diminished expression of the *DBH* gene associated with the –1021T allele. Homozygosity at this allele, resulting in very low plasma-D β H activity, might likewise limit the maximum potential production of D β H enzyme within noradrenergic neurons. Normally, this may have no significant physiological consequences. However, in disease states in which degeneration of the sympathetic nervous system occurs, such as in idiopathic Parkinson Disease (IPD) (Wakabayashi and Takahashi 1997), this may be an important issue; for example, a growing body of evidence suggests that mild autonomic dysfunction in IPD is quite common but that severe symptoms are rare (Turkka et al. 1986; Magalhaes et al. 1995; Druschky et al. 2000). Individuals with IPD who are homozygous for the DBH^T allele may have a decreased sympathetic functional reserve and a diminished capacity to compensate for the loss of adrenergic neurons. These patients might thus have an earlier onset of—and more severe symptoms of—autonomic dysfunction. Our results make this hypothesis directly testable.

Within in the field of psychiatry, there has been a great deal of interest in *DBH* as a disease-modifying gene and in plasma-D β H activity as an intermediate trait. Many conflicting reports on the relationship between plasma/CSF D β H levels and psychiatric disorders have been published (Fujita et al. 1978; Meltzer et al. 1980; Mathew et al. 1981; Matuzas et al. 1982; Arató et al. 1983). Since much of the individual variation in plasma-D β H levels is under genetic control, and since researchers previously had been unable to account for the genetic component of the variance in plasma D β H, such conflicting results are not surprising. Identification of the putative functional polymorphism –1021C→T will allow future clinical studies to evaluate whether D β H-activity differences in disease groups represent differences in genotype frequencies at the *DBH* locus. In addition, controlling for the large proportion of plasma-D β H variance that is accounted for by the *DBH* genotype may unmask

changes in D β H activity that result from illness, medical treatment, or the influences of other genetic loci. Our demonstration of the –1021C→T polymorphism as a major genetic marker for plasma-D β H activity will allow the role of the *DBH* gene in human disease to be evaluated in a new light.

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Electronic-Database Information

Accession numbers and URLs for data in this article are as follows:

Database of Single Nucleotide Polymorphisms (dbSNP), <http://www.ncbi.nlm.nih.gov/SNP/> (for NCBI-assay ID numbers for 18 new SNPs detected in this study [accession numbers 2418871–2418888])

GenBank, <http://www.ncbi.nlm.nih.gov/Genbank/index.html> (for *DBH* genomic sequences [accession numbers AC000404 and AC001227])

HSWIM, <http://info.med.yale.edu/genetics/kkidd/programs.html>

Online Mendelian Inheritance in Man (OMIM), <http://www.ncbi.nlm.nih.gov/Omim/> (for D β H [MIM 223360])

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