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The catecholamine biosynthetic enzyme dopamine β-hydroxylase (DBH): first genome-wide search positions trait-determining variants acting additively in the proximal promoter

Maja Mustapic[1,2,4], Adam X. Maihofer[1], Manjula Mahata[2], Yuqing Chen[2], Dewleen G. Baker[1,3], Daniel T. O'Connor[2] and Caroline M. Nievergelt[1,3,*]

[1]Department of Psychiatry and [2]Department of Medicine, University of California at San Diego, La Jolla, CA 92093, USA, [3]VA San Diego Healthcare System, VA Center of Excellence for Stress and Mental Health (CESAMH), La Jolla, CA 92161, USA and [4]Ruđer Bošković Institute, Zagreb HR-10000, Croatia

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Dopamine beta-hydroxylase (DBH) is the biosynthetic enzyme catalyzing formation of norepinephrine. Changes in DBH expression or activity have been implicated in the pathogenesis of cardiovascular and neuropsychiatric disorders. Genetic determination of DBH enzymatic activity and its secretion are only incompletely understood. We began with a genome-wide association search for loci contributing to DBH activity in human plasma. Initially, in a population sample of European ancestry, we identified the proximal *DBH* promoter as a region harboring three common trait-determining variants (top hit rs1611115, $P = 7.2 \times 10^{-51}$). We confirmed their effects on transcription and showed that the three variants each acted additively on gene expression. Results were replicated in a population sample of Native American descent (top hit rs1611115, $P = 4.1 \times 10^{-15}$). Jointly, *DBH* variants accounted for 57% of DBH trait variation. We further identified a genome-wide significant SNP at the *LOC338797* locus on chromosome 12 as *trans*-quantitative trait locus (QTL) (rs4255618, $P = 4.62 \times 10^{-8}$). Conditional analyses on *DBH* identified a third genomic region contributing to DBH variation: a likely *cis*-QTL adjacent to *DBH* in *SARDH* (rs7040170, $P = 1.31 \times 10^{-14}$) on chromosome 9q. We conclude that three common SNPs in the *DBH* promoter act additively to control phenotypic variation in DBH levels, and that two additional novel loci (*SARDH* and *LOC338797*) may also contribute to the expression of this catecholamine biosynthetic trait. Identification of *DBH* variants with strong effects makes it possible to take advantage of Mendelian randomization approaches to test causal effects of this intermediate trait on disease.

INTRODUCTION

Dopamine β-hydroxylase (DBH) is the final enzyme in norepinephrine biosynthesis, catalyzing the oxidative hydroxylation of dopamine to norepinephrine in the noradrenergic nerve endings of the central and peripheral nervous systems (1). In the bloodstream, DBH enzymatic activity is abundant, emerging from both the sympathetic terminals and the adrenal medullary chromaffin cells (1). As a result of exocytosis, DBH is co-released with norepinephrine from synaptic vesicles into extracellular space and thus can be found in plasma and cerebrospinal fluid (CSF) (2,3). The enzymatic activity of plasma or CSF DBH corresponds to the level of DBH protein, with plasma and CSF DBH correlating highly in humans (4,5). As such, DBH is of high interest to both the neuropsychiatric and cardiovascular field. Changes in DBH activity and/or genetic variants in the *DBH* gene have been implicated in the pathophysiology of major depression (6), ADHD (7,8), Parkinson (9) and Alzheimer's disease (10,11) and PTSD (12,13), potentially through changes in central catecholamine levels, whereas altered sympathoadrenal activity is thought to be implicated in the pathogenesis of hypertension and cardiovascular disease (14,15).

*To whom correspondence should be addressed at: Department of Psychiatry, University of California, San Diego, 9500 Gilman Drive, La Jolla, CA 92093-0737. Tel: +1 8582461895; Email: cnievergelt@ucsd.edu

In family and twin studies plasma DBH (pDBH) activity is highly heritable, relatively stable over time in the same person, and only minimally susceptible to environmental factors such as physical stress or drugs (16). Furthermore DBH activity shows highly variable inter-individual differences which are likely the result of genetic factors (5,17), with heritability estimates accounting for ~80–90% of the variation.

Linkage analysis with non-DNA markers has identified a single quantitative trait locus (QTL) for DBH activity in a region on chromosome 9 (9q34) (18,19) and the *DBH* gene was later mapped to that region (20,21). Sequencing analyses by Zabetian *et al.* (22,23) further characterized the molecular structure of *DBH* and identified a SNP in the promoter region (rs1611115/C-970T/formerly C-1021T), which explained a large ~35–52% inter-individual variation in pDBH activity, while functional polymorphisms (A197T in exon 3, A304S in exon 5 and R535C in exon 11) in the gene did only show a modest putative effect for R535C in these studies (see review in 16). Extended sequencing in the promoter region identified six common SNPs in the proximal promoter and showed functional properties in *in vitro* and *in vivo* experiments for rs1611115 and rs1989787 (C-2073T). A newer linkage study in families confirmed *DBH* as a major contributor of pDBH activity, but also suggested two additional loci, one in close proximity to *DBH* and the second on chromosome 20p12 (24).

Analysis of DBH levels in clinical populations reported racial differences in pDBH activities, with Blacks having lower levels than Whites (25). Genetic studies on the *DBH* locus, initially performed in populations of European ancestry, have then been extended to include subjects of African and Asian descent and confirmed rs1611115 as the polymorphism with the strongest effect (22,26).

Here, we performed the first genome-wide association study (GWAS), with goals to: (1) replicate and extend previous findings on *DBH* locus variation and its effect on pDBH activity, (2) extend the search to identify additional, *trans*-QTLs for pDBH activity levels and (3) expand ancestry studies to include subjects of Native American descent and Hispanic ethnicity. In addition, we further examined functional properties of genetic markers in the *DBH* promoter region displaying peak-association with plasma DBH activity, in transfected chromaffin cells as well as *in vivo*. We show that *DBH* variants with strong effects may be used in a Mendelian randomization (MR) approach to test causal effects of this intermediate trait on disease, such as cardiovascular and neuropsychiatric symptoms and disorders.

RESULTS

Genome-wide association study in subjects of European ancestry

An initial GWAS for plasma DBH activity was performed with genotypes of 341 subjects of European ancestry (European Americans, EAs). The mean pDBH level in the 341 EAs was 11.44 IU/L [standard deviation (SD) = 6.95] (Supplementary Material, Fig. S1). Genotypes underwent rigorous quality control and included a final set of 7 871 575 markers obtained by genotyping and imputation. Linear regression under an additive genetic model, incorporating appropriate covariates, resulted in a low genomic control inflation factor of $\lambda_{GC} = 1.002$. A quantile–quantile (QQ) plot is shown in Supplementary Material, Figure S2A. A table with all GWAS results is available in the Supplementary Material, Table S1.

Our analyses identified the *DBH* locus as genome-wide significant with the top hit for a directly genotyped SNP rs1611115 at $P = 7.2 \times 10^{-51}$ (Fig. 1A and Table 1). A regional association plot of the *DBH* locus showed 34 genome-wide significant *DBH* SNPs within the same linkage disequilibrium (LD) block (Fig. 1B). Of these, one SNP was found in an exon (synonymous SNP exm793933, $P = 1.023 \times 10^{-27}$), 22 were intronic and 11 were located upstream of *DBH*, including 3 common SNPs within a 3 kb region of the promoter (rs1076150, rs1989787 and rs1611115, shown in detail in Table 1, top part). Two of these promoter SNPs (rs1989787 and rs1611115) were known to be functional (see 14 and 15) and the functionality of rs1076150 was investigated below. The proportion of variability explained (R^2)

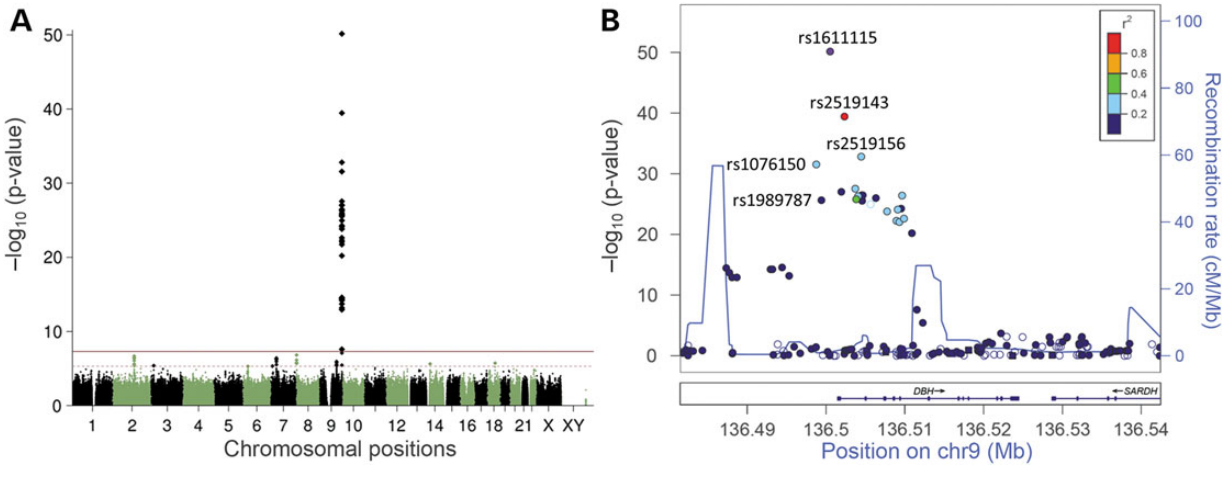

Figure 1. Results of the GWAS of plasma DBH activity in 341 subjects of European origin. (**A**) Manhattan plot showing the $-\log_{10}$(*P*-values) for SNP associations with plasma DBH activity across the genome. The red horizontal line represents the genome-wide significance threshold at $P < 5 \times 10^{-8}$ and the dashed line represents suggestive evidence for association at $P < 5 \times 10^{-6}$. (**B**) Regional association plot, showing significant regions in *DBH* on chromosome 9. Directly genotyped SNPs are indicated by an asterisk (*). The SNPs are color coded based on the linkage disequilibrium with the most significant SNP rs1611115.

Table 1. Most significant hits in the genome-wide association study

Allele SNP	CHR	BP	Gene	Location	1/2[b]	EA GWAS Allele 1 freq.	Effect size	SE	*P*	NA descent GWAS Allele 1 freq.	Effect size	SE	*P*	Meta-analysis[a] *Q*	Effect size	*P*
rs1076150[c]	9	136498761	DBH	Upstream	T/C	0.512	−0.947	0.072	**2.74E−32**	0.710	−0.779	0.163	7.67E−06	0.35	−0.920	**1.38E−44**
rs1989787	9	136499412	DBH	Upstream	T/C	0.312	0.924	0.079	**2.13E−26**	0.196	0.747	0.191	1.92E−04	0.39	0.898	**1.50E−34**
rs1611115[c]	9	136500515	DBH	Upstream	T/C	0.248	−1.265	0.070	**7.20E−51**	0.317	−1.195	0.125	**4.10E−15**	0.63	**−1.248**	**4.60E−92**
rs7540659	1	100196119	FRRS1	Intron	T/A	0.341	−0.024	0.095	0.801	0.462	−0.793	0.160	**3.64E−06**	**0.00**	**−0.398**	0.301
rs60674788	2	35027196	CR617033	Downstream	C/G	0.257	−0.403	0.101	8.11E−05	0.354	−0.536	0.179	0.004	0.52	**−0.435**	**7.64E−07**
rs4459781	2	134204665	NCKAP5	Intron	C/T	0.281	−0.499	0.094	**2.15E−07**	0.215	−0.098	0.209	0.641	0.08	**−0.431**	**5.12E−07**
rs77518496	2	143629286	KYNU	Upstream	G/A	0.032	−0.012	0.232	0.959	0.115	1.252	0.253	**3.88E−06**	**0.00**	0.616	0.330
rs2351772	2	204079313	NBEAL1	Intron	C/T	0.418	0.346	0.091	1.71E−04	0.527	0.441	0.149	0.004	0.58	0.372	**1.61E−06**
rs112239800	2	232517876	BC069004	Downstream	G/A	0.102	0.509	0.149	7.38E−04	0.115	1.079	0.268	1.33E−04	0.06	0.645	**7.67E−07**
rs13095328	3	15226050	DIVA	Intron	C/T	0.100	−0.669	0.143	**3.97E−06**	0.059	0.634	0.354	0.077	**0.00**	**−0.058**	0.929
rs3774729[c]	3	63982082	ATXN7	Exon	A/G	0.323	0.000	0.086	0.996	0.290	0.869	0.164	**9.60E−07**	**0.00**	0.424	0.329
rs56030924	3	63995563	AK023371	Intron	A/G	0.286	0.017	0.090	0.848	0.269	0.885	0.164	**6.39E−07**	**0.00**	0.440	0.310
rs831692	3	64003983	PSMD6	Intron	A/G	0.310	0.031	0.089	0.731	0.288	0.893	0.166	**6.65E−07**	**0.00**	0.451	0.296
rs56237630	3	64049375	PRICKLE2	Downstream	A/C	0.145	−0.002	0.119	0.988	0.214	0.941	0.182	**1.52E−06**	**0.00**	0.460	0.329
rs12639432[c]	3	134770520	EPHB1	Intron	T/C	0.302	0.339	0.091	2.44E−04	0.462	0.546	0.150	4.71E−04	0.24	0.395	**4.16E−07**
rs7779937	7	10971712	NDUFA4	Downstream	A/G	0.048	−0.922	0.198	**4.74E−06**	0.016	0.643	0.660	0.333	**0.02**	**−0.266**	0.731
rs13242648	7	35777951	CR595224	Downstream	T/A	0.196	0.554	0.107	**3.92E−07**	0.136	−0.456	0.231	0.051	**0.00**	0.070	0.890
rs12701456	7	35827802	SEPT7	Upstream	C/T	0.196	0.547	0.107	**5.03E−07**	0.132	−0.389	0.231	0.096	**0.00**	0.101	0.828
rs13255006	8	1989315	MYOM2	Upstream	C/G	0.319	0.500	0.093	**1.52E−07**	0.172	−0.034	0.216	0.876	**0.02**	0.268	0.310
rs1338730	9	103520981	MURC	Downstream	C/T	0.402	−0.419	0.085	**1.15E−06**	0.253	−0.404	0.193	0.039	0.94	**−0.417**	**7.40E−08**
rs823919	9	104662606	GRIN3A	Upstream	A/G	0.124	0.582	0.127	6.56E−06	0.172	0.283	0.215	0.191	0.23	0.505	**3.98E−06**
rs7857468	9	136585380	SARDH	Intron	A/C	0.195	0.544	0.099	**8.09E−08**	0.170	0.142	0.236	0.549	0.12	0.484	**1.19E−07**
rs10795764	10	10238394	BC032914	Downstream	C/T	0.434	0.093	0.083	0.265	0.559	0.750	0.133	**2.33E−07**	**0.00**	0.413	0.208
rs870553	10	133970542	JAKMIP3	Intron	G/A	0.010	−1.210	0.417	0.004	0.059	−1.262	0.332	2.75E−04	0.92	**−1.242**	**1.77E−06**
rs112825992	10	134008571	DPYSL4	Intron	T/C	0.009	−1.252	0.450	0.006	0.055	−1.209	0.320	2.92E−04	0.94	**−1.223**	**2.65E−06**
rs4255618	12	131837477	LOC338797	Intron	C/A	0.353	0.388	0.088	1.26E−05	0.322	0.502	0.154	0.002	0.52	0.416	**4.62E−08**
rs8013529[c]	14	23649792	SLC7A8	Intron	G/A	0.139	−0.566	0.118	**2.37E−06**	0.059	0.320	0.328	0.332	**0.01**	**−0.176**	0.689
rs12595689	15	86009293	AKAP13	Intron	C/G	0.085	0.010	0.158	0.952	0.102	−1.198	0.222	**5.88E−07**	**0.00**	**−0.584**	0.333
rs117711052	17	74305308	QRICH2	Upstream	C/G	0.024	1.155	0.278	4.12E−05	0.016	1.567	0.627	0.014	0.55	1.223	**1.48E−06**
rs115172145	17	74310984	PRPSAP1	Intron	C/T	0.024	1.144	0.278	4.77E−05	0.016	1.567	0.627	0.014	0.54	1.213	**1.74E−06**
rs7228140	18	45907244	ZBTB7C	Intron	C/T	0.046	−0.941	0.194	**1.88E−06**	0.071	0.439	0.313	0.165	**0.00**	**−0.273**	0.692
Conditional analysis[d]																
rs7857468	9	136585380	SARDH	Intron	A/C	0.195	0.500	0.065	**2.38E−13**	0.170	0.489	0.155	0.002	0.946	0.498	**1.15E−16**
rs7040170[c]	9	136586367	SARDH	Intron	G/A	0.221	0.439	0.062	**7.82E−12**	0.177	0.456	0.153	0.004	0.918	0.442	**1.31E−14**

[a]Random-effects models were used for SNPs with significant heterogeneity *Q* values (bold), otherwise fixed-effects models.
[b]Allele 1 is the coding allele.
[c]Directly genotyped SNP.
[d]Regression analyses including *DBH* SNPs rs1076150, rs1989787 and rs1611115 as covariates.
P-values in bold meet suggestive ($P < 5.0E-06$) or genome-wide significance ($P < 5.0E-08$).

by the *DBH* gene, based on five highly significant *DBH* SNPs in low LD with each other plus the three (putative) functional promoter SNPs, was 0.569.

No other chromosomal region reached genome-wide significance. However, there were 10 regions which showed suggestive evidence ($P < 5 \times 10^{-6}$) in EAs. For each of these, the SNP with the lowest *P*-value is presented in Table 1 (middle part) and Supplementary Material, Figure S3A.

Replication of the GWAS in subjects of Native American ancestry

To replicate our findings we performed a second GWAS on subjects of Native American descent (NAs), including subjects with varying degrees of NA admixtures as typically seen in Hispanic subjects ($n = 91$). The mean pDBH level in 93 NAs was 10.2 IU/l (SD = 6.94) and was not significantly different from pDBH levels in EAs ($P > 0.29$). The genomic control inflation λ_{GC} was 1.009 (a QQ-plot is shown in Supplementary Material, Fig. S2B). A table with all GWAS results is available in the Supplementary Material, Table S2. Replicating our results in EAs, we confirmed the *DBH* locus to be highly significant, with the same top hit rs1611115 at $P = 4.10 \times 10^{-15}$ (Table 1 and Supplementary Material, Fig. S4A). A regional association plot of the *DBH* locus showed an additional five intronic genome-wide significant SNPs within the same LD-block (Supplementary Material, Fig. S4B). The proportion of variability explained (R^2) by the *DBH* locus, based on four independent (LD < 0.5), highly significant *DBH* SNPs (including the three promoter SNPs), was 0.57.

We did not identify other genome-wide significant regions in this small NA population. Eight other loci showed suggestive evidence for association with pDBH activity ($P < 5 \times 10^{-6}$). For each of these regions the SNP with the lowest *P*-value is presented in Table 1 (middle part) and Supplementary Material, Figure S3B.

Meta-analysis of EA and NA GWAS

An inverse variance weighted meta-analysis of the EA and NA GWAS results indicated no significant heterogeneity (*Q*) at the *DBH* locus and resulted in highly significant associations for the promoter region of this locus with the top hit rs1611115 at $P = 4.60 \times 10^{-92}$, as well as rs1076150 (T-2734C) and rs1989787, at $P = 1.38 \times 10^{-44}$ and $P = 1.50 \times 10^{-34}$, respectively (Table 1, right side and Supplementary Material, Fig. S5A). A complementary pooled analysis (mega-analysis) of the EA and NA subjects for the three promoter SNPs showed comparable results (Supplementary Material, Fig. S5B). A C to T transition progressively diminished pDBH activity for rs1076150 and rs1611115, while increasing pDBH activity for rs1989787. In each case, SNP allele effects on trait seemed to be additive, with intermediate effects for SNP heterozygotes, confirmed by the fact that recessive and dominant genetic models were less significant than the additive model for these three SNPs (data not shown).

In addition to the *DBH* locus, the meta-analysis showed a genome-wide significant association for intronic SNP rs4255618 in *LOC338797* on chromosome 12 ($P = 4.62 \times 10^{-8}$). A BLAST search (on BLASTN_2.2.28+ at NCBI) of the RNA-coding region (~20 kb) of this uncharacterized locus showed no homology to *DBH*. In addition, seven new loci reached suggestive evidence for association in the meta-analysis (top hits for these loci are shown in Table 1). The proportion of variability explained (R^2) by the *DBH* locus, based on seven highly significant *DBH* SNPs in low LD with each other (including the three promoter SNPs), was 0.57. Adding rs4255618 in *LOC338797* to the *DBH* model significantly increased R^2 to 0.59 (LR test $P = 6.09 \times 10^{-5}$) in a joint analysis of EA and NA subjects.

Conditional analysis on the *DBH* locus

Because of the strong effect of the *DBH* locus on pDBH activity, we repeated the GWAS conditioned on the three *DBH* peak functional promoter SNPs rs1076150, rs1989787, and rs1611115 in EAs, NAs and the meta-analysis to test for additional, *DBH*-independent loci (Supplementary Material, Fig. S6). The *SARDH* locus, adjacent to *DBH* and previously showing suggestive evidence for association, became genome-wide significant in EAs with an imputed top hit for rs7857468 ($P = 2.38 \times 10^{-13}$). Rs7857468 replicated in NAs with a nominally significant $P = 0.002$, resulting in a meta-analysis *P*-value $= 1.15 \times 10^{-16}$ (Table 1, bottom part). Results for the most significant directly genotyped SNP in *SARDH* (rs7040170, $P = 1.31 \times 10^{-14}$) are also shown. Regional association plots of the conditioned GWAS results in EAs and NAs for the *DBH* and neighboring *SARDH* loci are shown in Supplementary Material, Figure S6B and D. Adding the *SARDH* SNP to the *LOC338797* and *DBH* model significantly increased R^2 to 0.648 (LR test $P = 8.13 \times 10^{-16}$) in a joint analysis of EA and NA subjects. The conditional analysis did not result in stronger results for the loci showing suggestive evidence in the primary analyses (Supplementary Material, Fig. S6A and C).

Functional analysis of variant C-2734T and four naturally occurring haplotypes in the *DBH* promoter

Functional analyses of the promoter variants rs1611115 and rs1989787 have previously been published by our group (14,15). Here we extend these analyses to the third promoter variant rs1076150, identified in the GWAS with a highly significant effect. Using the same six common promoter SNPs (minor allele frequency MAF > 0.05) as in previous work, we constructed luciferase promoter plasmids for four common, naturally occurring six-SNP haplotypes from the BAC promoter insert. The promoter activity of these four natural haplotypes (HAPs 1–4), measured as a function of luciferase expression in chromaffin cells is shown in Supplementary Material, Figure S7. We found that genotypic variations showed a significant overall effect ($F = 33.8$, $P < 0.001$), with haplotypes showing different *DBH* promoter/luciferase reporter activities (expressed as Firefly/Renilla ratio). To evaluate the individual effect of the rs1076150 SNP we constructed mutant variants on balanced backgrounds for two of the four haplotypes (HAP2 and HAP4), differing only at the desired −2734 position. When compared with the T allele, the C allele displayed higher expression on two different backgrounds (HAP2: $P = 0.0047$ and HAP4: $P = 0.0098$) (Fig. 2).

Bioinformatics of variant promoter motifs

In order to further investigate the functional properties of the *DBH* promoter variant rs1076150, we used bioinformatics tools (CONSITE and MotifLab) for the analysis of regulatory sequences. Both tools predicted that at position −2734 (upstream from the translation start site), SNP rs1076150 disrupted a binding motif for the transcription factor Snai1. As indicated in Supplementary Material, Figure S8, the match and binding score for the C-allele were predicted to be higher than for the T allele, possibly resulting in different expression levels of the DBH protein. For a complete characterization of the DBH promoter region, the computational molecular predictions and proposed mechanistic consequences of disrupted transcription factor binding motifs for the other two functional promoter variants rs1611115 and rs1989787 were added in Supplementary Material, Figure S8.

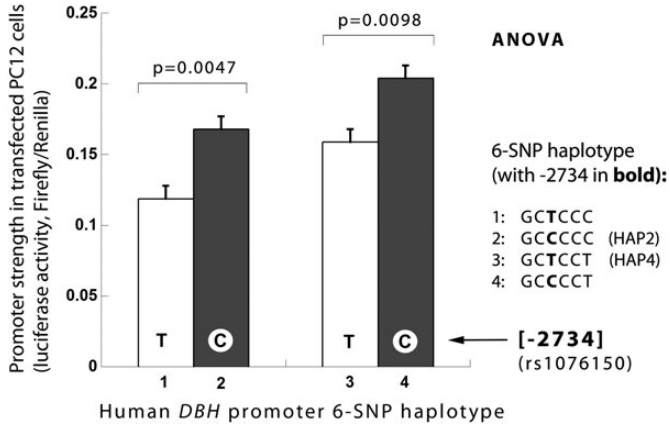

Figure 2. *In vitro* effects of human *DBH* promoter variant C-2734T (rs1076150): Balanced mutants on two haplotype backgrounds (HAP2, HAP4) yield consistent (C > T) effects on transcription in chromaffin cells. Strength of the promoter variants is shown as luciferase activity in PC12 cell type (mean ± SEM). *P*-values are result of C versus T variant comparison for each haplotype background by ANOVA.

In vivo effects of functional *DBH* promoter haplotypes on human pDBH activity

We further evaluated the directional effects of the three functional SNPs (rs1076150 → rs1989787 → rs1611115) in the *DBH* promoter region (which showed the highest associations with pDBH activity in the GWAS) in a haplotype analysis in the combined 434 EA and NA subjects. First, we considered haplotype homozygotes for the four naturally occurring diploid haplotypes (Fig. 3A), and noted significant differences in pDBH activity with a plasma activity rank order of: CTC>CCC>TCC>TCT ($P = 1.84 \times 10^{-29}$). Finally we analyzed the effects of haplotype copy number on pDBH activity for the four haplotypes (Fig. 3B). The results were internally consistent with those for haplotype homozygotes, showing that increasing CTC copy number progressively elevated pDBH activity ($P = 7.49 \times 10^{-32}$), with reciprocal effects for haplotype TCT copy number ($P = 2.96 \times 10^{-66}$). Corresponding individual SNP effects are also shown in Supplementary Material, Figure S5A.

Application of the MR test using genetic variants in DBH

PTSD re-experiencing symptoms were assessed post-deployment in 402 subjects with available pDBH levels and ranged from 0 to 29 (mean = 5.87). Re-experiencing symptoms were significantly associated with pDBH (beta = 0.13, $P = 0.012$), making a MR analysis applicable. The MR estimate of the association of pDBH and re-experiencing symptoms was significant (beta = 0.21, $P = 0.002$), indicating that pDBH is a causal component in the development of re-experiencing symptoms.

DISCUSSION

Dopamine β-hydroxylase as an essential part of the catecholamine biosynthetic pathway, converts dopamine to norepinephrine. DBH is encoded by a single gene located on chromosome 9q34 and its enzymatic activity is expressed both in plasma

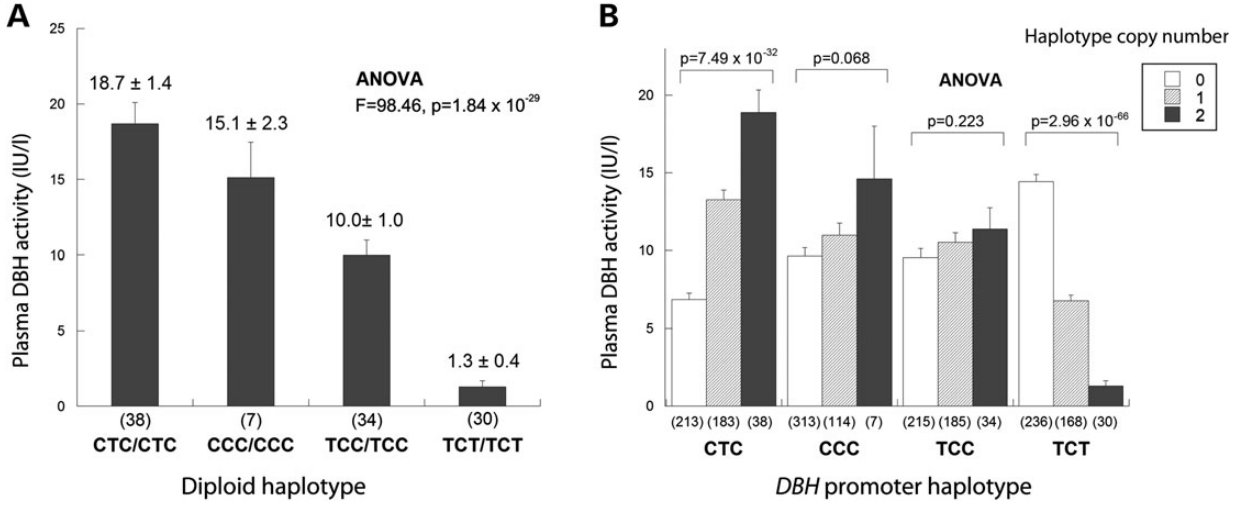

Figure 3. *In vivo* effects of *DBH* promoter functional variants T-2734C (rs1076150), C-2073T (rs1989787) and C-970T (rs1611115) on plasma DBH activity (IU/l). (**A**) *DBH* promoter diploid haplotype (rs1076150 → rs1989787 → rs1611115) effect on pDBH activity (IU/l). Only subjects homozygous for a given haplotype (rs1076150 → rs1989787 → rs1611115) are shown. (**B**) Effect of *DBH* promoter haplotype (rs1076150 → rs1989787 → rs1611115) copy number (0, 1, or 2 copies per genome) on pDBH activity (IU/l, adjusted mean ± SEM).

and CSF. The effects of this *cis*-QTL on plasma, serum and/or CSF DBH activity have been previously investigated in isolation (14,22,24), but to date no genome-wide association studies have been reported on DBH activity. Here, we present the first GWAS of plasma DBH levels and further characterize transcriptional control of the *DBH* gene.

Our GWAS was first performed in subjects of EA ancestry. We replicated the *DBH* locus as major contributor to pDBH activity, explaining ~57% of the variability in EAs. As found by others, rs1611115 was the most significant polymorphism in this gene (22), with a $P < 7.2 \times 10^{-51}$, by far exceeding the genome-wide significance threshold of $P < 5 \times 10^{-8}$, and another 33 SNPs (some of them with independent effects) at this locus met genome-wide significance. No other loci were found to be genome-wide significant in this relatively small sample of 341 EAs, but 10 loci reached suggestive evidence of association with pDBH at $P < 5 \times 10^{-6}$ and await further replication in larger datasets. However, none of these loci were located on 20p12, a *trans*-QTL suggested in a linkage study by (24). The often poor correspondence between the susceptibility loci identified in genetic linkage and genome-wide association studies may be due in part to allelic heterogeneity, which reduces power in GWAS compared to linkage analyses (27).

Genetic association studies on the DBH locus have compared the three main ancestry groups from Europe, Africa and Asia. EAs were reported to have higher mean pDBH levels as compared to Japanese (22) and Africans from Nigeria (14,25). The promoter SNP rs1611115 was consistently reported as the most significant candidate SNP in *DBH* across studies and ancestral groups (14,22,23,28). Here, we extend this work to include subjects of genetically determined Native American descent, typically self-identifying as either Native American or Hispanic in our study. We found no difference in pDBH activity levels between our EA and NA subjects. The GWAS replicated the DBH locus with the same top hit (rs1611115 at $P = 4.1 \times 10^{-15}$) and consistent effect size estimates ($R^2 = 0.59$ and 0.57 in EAs, respectively) in this even smaller sample of 93 subjects.

Increasing our power to detect additional loci by combining the relatively small number of EA and NA subjects in a meta-analysis, we identified *LOC338797* (rs4255618) on chromosome 12q at $P = 4.62 \times 10^{-8}$, meeting the traditional genome-wide significance threshold of 5×10^{-8}. However, genotype imputations based on 1000 Genomes Project reference data are increasing the effective number of independent tests and more stringent thresholds have recently been suggested (e.g. 1×10^{-8} for all common SNPs) (29). Irrespective of the specific threshold selected, the relevance of *LOC338797* and all findings showing suggestive evidence of association have to be confirmed through independent replication of these results. *LOC338797* seems to encode a 4-exon, previously uncharacterized 1794-base lncRNA, but the RNA-coding region bears no homology to *DBH* itself, and its role in DBH remains to be determined. However, adding *LOC338797* to our genetic model of DBH only marginally increased the percent trait variability explained (from 57 to 59% in the combined analysis).

An additional analysis conditioned on the *DBH* locus promoter SNPs, to mask its strong effect on trait, identified sarcosine dehydrogenase *SARDH*, a gene adjacent to *DBH,* as an apparently independent, genome-wide significant hit in EAs. Its top hit rs7857468 was nominally replicated in NAs, leading to an overall *P*-value of 1.15×10^{-16}, and further improving our model to explain 65% of overall variability in pDBH activity. *SARDH* encodes an enzyme localized to the mitochondrial matrix that catalyzes the oxidative demethylation of sarcosine. Even though adjacent to (and within 86.6 kb of) DBH, the conditional peak *SARDH* markers displayed little LD with the DBH promoter, as judged by marker-on-marker LD ($R^2 < 0.2$) as well as a cM/Mb recombination boundary peak (Supplementary Material, Fig. S6B and D). However, analysis of the local chromosomal region by Chromatin conformation capture (or Hi-C, (30)) in human ES cells as well IMR-90 fibroblasts revealed that both *DBH* and *SARDH* inhabit the same topological domain, bounded by insulator/barrier (CTCF motif) elements. Thus, it is conceivable that the *SARDH* region harbors a 3′ transcriptional enhancer for *DBH* expression.

Mechanisms underlying DBH expression and secretion into plasma and CSF have invoked continuing interest among a broad range of investigators. One genetic variant in particular (rs1611115) has been widely investigated and ultimately documented (14) as a functional variant in the *DBH* promoter (14,22). We previously conducted systematic polymorphism discovery across the human *DBH* locus, and probed the functional consequences of two promoter variants (rs1989787 and rs1611115). We showed that rs1611115 disrupted consensus transcriptional motifs for *n-MYC* and *MEF-2* (14) and rs1989787 for *c-FOS* (15), and that trans-activation of these variants by the corresponding transcription factors resulted in changes in *DBH* expression. The effects of variant rs1076150 on transcription reported here are novel, and allowed us to evaluate the effects upon gene expression of all three functional variants simultaneously. Here, we present an overview of properties of all three major functional variants in the proximal *DBH* promoter (Supplementary Material, Fig. S8). We found additive effects of each functional SNP upon DBH secretion into plasma (Supplementary Material, Fig. S5), and noted that the activity of contributory SNP alleles summated to give rise to a spectrum of promoter haplotype activities (Fig. 3A and B).

Genetic variants in *DBH* and/or pDBH activity have been directly implicated in mechanisms leading to increased susceptibility to disease. As the final enzyme in norepinephrine biosynthesis, DBH plays a role in differential availability of dopamine and norepinephrine. Consequently, DBH is involved in mechanisms underlying disorders associated with changes in the noradrenergic system (31–35). For example, our most significant *DBH* variant (rs1611115) is influencing heritable ‘intermediate phenotypes’ (e.g. autonomic and renal traits) as physiological risk traits in later development of hypertension (e.g. the T allele was found to decrease urine epinephrine excretion and basal blood pressure) (14,15) and progressive renal disease (36). In addition, biological and genetic studies suggest associations of low DBH levels with psychotic symptoms, and with mental disorders such as schizophrenia, depression, attention deficit hyperactivity disorder and alcoholism (see review 16). However, large GWAS on cardiovascular and psychiatric disorders (e.g. as reported by Ricopili) did not replicate strong effects for genetic variants in *DBH*.

The large proportion of DBH heritability that can be explained by a small number of genetic markers, in combination with the potentially important role of this intermediate phenotype for both psychiatric and cardiovascular disorders is unique and

may represent a useful methodological tool to develop and test genetic epidemiological methods (37,38). To this end, we have applied genetic markers in *DBH* to the MR approach to investigate a potential causal effect of the pDBH and PTSD association previously reported (12,13). Our preliminary results on the effect of pDBH on PTSD re-experiencing symptoms indeed support this causal relation, but these findings will need to be confirmed in larger studies.

In conclusion, a first GWAS on pDBH activity identified the *DBH* gene as the principal locus determining pDBH levels in both EA and NA populations, explaining 57% of the variability. Two additional novel loci, *SARDH* and *LOC338797*, explaining combined an additional 8% of overall variability, were identified here and will have to be replicated in independent studies. Compared with other GWAS studies, the effects reported here were detected in relatively small datasets. Future studies on larger datasets may discover additional loci of smaller effects. Further, we demonstrated the potential application of strong genetic predictors of intermediate phenotypes such as DBH to the investigation of the disease etiology in the context of PTSD.

In perspective, the characterization of DBH activity and its underlying genetic regulation has positioned us uniquely for future studies of 'intermediate phenotypes', potentially leading to discovery of causal variants in complex genetic traits and disorders such as found in the psychiatric and cardiovascular fields.

MATERIALS AND METHODS

Subjects and biological sample collection

Participants were recruited from the Marine Resiliency Study (MRS), a large, prospective study of post-traumatic stress disorder (PTSD) involving active-duty United States Marines bound for deployment to Iraq or Afghanistan (39). The protocols for these studies were approved by the University of California-San Diego Institutional Review Board (IRB Protocols #070533, #110770X), and all subjects provided written informed consent to participate. Here we evaluated a subgroup of the MRS with available genotype and pDBH activity phenotype data, including 532 healthy, unrelated males from four different battalions (cohorts) assessed at pre-deployment. Following a 7-month deployment to a combat zone, post-traumatic stress symptoms were evaluated using a structured diagnostic interview, the Clinician Administered PTSD Scale (CAPS; (40–43)). Inter-rater reliability in MRS for the CAPS total score was high (Intraclass correlation coefficient = 0.99). Re-experiencing symptoms (CAPS-B symptom cluster) were used here. Initially, ethnicity and race were established by self-report, including information on geographic origin of both parents. The cohort studied here included 86% Caucasian and 22% Hispanic subjects, with a mean ($\pm$SD) age of 22.41 $\pm$ 3.23 years (range 18–41), typical for the overall MRS participants.

Blood was sampled from an antecubital vein for preparation of heparinized plasma (for assay of pDBH activity) and EDTA-anticoagulated blood (for preparation of genomic DNA). Heparinized blood from lithium heparin tubes was kept on ice prior to centrifugation and plasma was stored at -70°C prior to thawing for assays in batch. Genomic DNA was prepared from 1–2 ml blood leukocytes and diluted to a standard concentration of 50 ng/μl for genotyping.

Genotyping, quality control procedures and genotype imputations

Genotyping of 2585 DNA samples (532 with pDBH activity measures) was carried out by Illumina (http://www.illumina.com/) using the HumanOmniExpressExome array (HOEE 8v1_A) with 951 117 loci. Initial allele calling was performed by Illumina in GenomeStudio (V2011.1) and resulted in a sample success rate of 99.65%, a locus success rate of 99.86%, a genotype call rate of 99.88%, with reproducibility including 28 replicate DNA sample pairs of >99.99%. Additional data cleaning was performed in PLINK v1.07 (44) using standard procedures. SNPs were excluded if the call rate was <95%, if they violated Hardy–Weinberg Equilibrium ($P < 1 \times 10^{-6}$), or if they showed plate effects (P-value $< 1 \times 10^{-8}$ for any one plate or $< 1 \times 10^{-4}$ for two or more plates). Sample ID was confirmed by evaluating concordance between 31 overlapping genotypes from the HOEE array and those from an initial 'fingerprinting' panel including 41 ancestry-informative markers (AIMs) (45), resulting in the exclusion of one sample (overall concordance rate >0.99). Unexpected familial relationships were identified using pairwise identical-by-descent estimation and two subjects from sib-pairs were removed. Sample heterozygosity was between 0.211 and 0.302 and no excessive high or low samples were identified. The final dataset included 851 541 markers genotyped in 2548 individuals with a genotyping rate of >0.998.

Imputations were performed with standard protocols using the default parameters in IMPUTE2 v2.2.2, using 1000 Genomes Phase 1 integrated variant set haplotypes for the autosomes and the interim set for the X chromosome. Prior to imputation, genetic markers that had exceedingly rare alternative alleles (minor allele frequency MAF < 0.0002) were excluded. Next, genomes were divided into ~5 Mb segments, and phasing and imputed genotypes were calculated for each. Imputed markers with low imputation quality values (Info value $\leq$0.5) were excluded. GTOOL v0.7.0 was used to convert genotype probabilities into calls for markers with probabilities >90% (genotypes were called missing if the posterior probability of any genotype was $\leq$90%), resulting in a total of 24 068 319 successfully imputed polymorphic markers, and a total of 24 919 860 genotyped and imputed markers for association analyses.

Ancestry assessment and control for genetic background heterogeneity

Ancestry was determined using genetic information as described in (45). In brief, genotypes of 1783 AIMs were used to determine a subject's ancestry at the continental level for the seven geographic regions Africa, Middle East, Europe, Central/South Asia, East Asia, Americas and Oceania. Ancestry estimates were determined using STRUCTURE v2.3.2.1. (46) at $K = 7$, including prior population information of the HGDP reference set (47). Based on these ancestry estimates, MRS subjects included here were placed into two main ancestral groups: subjects with >95% European ancestry were grouped with EAs ($N = 341$); and subjects with >5% Native American ancestry (and <10% African, and <5% each Central Asian, East Asian and Oceanic ancestry) as Native American descendants (NAs) ($N = 93$). A very wide range of Native American ancestry

proportions is typical for subjects of self-reported Hispanic and Native American ethnicity/race (e.g. (48,49). Subjects with other ancestral backgrounds were not analyzed here ($N = 98$).

GWAS was performed separately in 341 EAs and 93 NAs. To control for additional genetic background heterogeneity within the two ancestral groups, and varying degrees of EA admixture within the NAs, principal component analyses (PCA) implemented in the EIGENSTRAT software (50) based on 10 000 random, autosomal SNPs were performed. The first 3 Eigenstrat-derived PCAs were included each as covariates in the association analyses.

Functional effects of trait-associated *DBH* promoter variants (rs1076150, rs1989787, rs1611115): promoter/luciferase reporter activity assays

Human *DBH* promoter/reporter plasmids were constructed from BAC genomic clone (RP11-317B10) obtained from CHORI (http://bacpac.chori.org) as described before. The *DBH* promoter region (extending distally from −3000 to +51 bp) containing six common polymorphic sites was excised from the BAC clone and inserted into the upstream/polylinker region of firefly luciferase reporter plasmid pGL3-Basic (Promega; Madison, WI, USA). Common naturally occurring haplotypes and additional variants were made by site-directed mutagenesis (QuikChange, Stratagene (Agilent), Santa Clara, CA, USA), verified by dideoxy sequencing, and co-transfected with Renilla luciferase expression plasmid pRL-TK (Herpes simplex virus thymidine kinase promoter driving Renilla luciferase, Promega) as a transfection efficiency control, into PC12 pheochromocytoma cells (at ∼50–60% confluence, 1 day after 1:4 splitting) as previously described (14). Firefly and Renilla luciferase activities in cell lysates were measured 16 h post-transfection, and results were presented as Firefly/Renilla luciferase activity ratio ('Stop & Glo'; Promega, Madison, WI, USA).

Biochemical properties of plasma DBH

Plasma DBH activity was measured in 25 μl of heparinized plasma by a modified Nagatsu/Udenfriend spectrophotometric method (51), and reported as IU/l (IU/l = μmol/min/l plasma at 37°C, protocol available online at http://hypertension.ucsd.edu/). This method is based on a conversion of the synthetic DBH substrate tyramine by DBH (in the presence of Cu^{2+}, N-ethylmaleimide and fumarate) to octopamine, which is then oxidized to parahydroxybenzaldehyde by sodium periodate. The oxidation is terminated by sodium metabisulfite, and the end product parahydroxybenzaldehyde is quantified by its absorbance at 330 nm in the ultraviolet spectrum. The mean plasma DBH activity inter-assay coefficient of variation was 12.8%. The mean plasma DBH level in 532 subjects was 10.86 IU/l (SD = 6.77) and ranged from 0.01 to 37.41 IU/l (Supplementary Material, Fig. S1).

Bioinformatic analyses

Computational prediction and motif discovery for transcription factors in the promoter region of *DBH* where candidate SNPs were positioned was made using web interface tools CONSITE (52) and graphical interface MotifLab (53), available at (http://asp.ii.uib.no:8090/cgi-bin/CONSITE/consite/) and (http://tare.medisin.ntnu.no/motiflab/), respectively. For both tools, predictions were based on position weight matrices for binding sites annotated in JASPAR and TRANSFAC databases. Motifs from consensus sequences, whose score was higher than 80% for binding to a motif containing a target SNP, were considered candidates.

Statistical analyses

Plasma DBH levels were square-root transformed to conform to normality ($P > 0.74$, Kolmogorov–Smirnov test). GWAS of transformed plasma DBH levels was performed in EA ($N = 341$) and NAs ($N = 93$) separately using linear regression under an additive genetic model with covariates age, cohort (three dummy coded variables), and three PCAs as implemented in PLINK. SNPs were pruned to a minor allele frequency (MAF) ≥ 0.01 in the combined dataset, which resulted in the inclusion of 7 871 575 SNPs. Genome-wide significance was set to $P < 5 \times 10^{-8}$ and suggestive evidence for association was considered at $P < 5 \times 10^{-6}$. Meta-analyses on the EA and NA results were performed in PLINK, using a fixed-effects model for SNPs with no significant heterogeneity (I) and a random-effects model when heterogeneity was significant (Cochrane's Q statistic). Conditional analyses on the *DBH* locus were performed to identify additional genetic associations by including the three *DBH* peak promoter SNPs rs1076150, rs1989787 and rs1611115 as additive covariates. Percent variability explained (R^2) by a SNP or multiple SNPs in a gene were calculated using a linear regression in R 3.0.0, using the—clump function in PLINK to generate a list of highly significant SNPs in low LD for each gene with genome-wide significant SNPs. QQ plots and Manhattan plots were made using R 3.0.0. LocusZoom 1.2 (54) was used to construct regional association plots, including recombination information from HapMap phase II CEU. SG-ADVISER (http://genomics.scripps.edu/ADVISER/) was used for SNP annotations.

Analysis of variance (ANOVA) was used to compare luciferase reporter activity between different *DBH* haplotypes *in vitro*, and linear regression models and ANOVA based on an additive genetic model, with age, cohort and three PCAs as covariates were used for *in vivo* experiments to test for associations of haplotypes with DBH enzymatic activity in plasma using IBM SPSS Statistics, v.20.

Associations between pDBH levels, CAPS total score and symptom cluster B were tested in the combined EA ($N = 341$) and NA ($N = 93$) sample. To account for the non-normal distribution of CAPS scores, a zero-inflated negative binomial distribution (ZINB) regression was used (55), with additional covariates age, cohort (three dummy coded variables), and three PCAs based on continental ancestry. Associations between *DBH* SNPs and CAPS scores were tested under an additive genetic model.

Instrumental variable analysis. To demonstrate the utility of strong genetic effects on intermediate phenotypes for application to a MR approach, an association of pDBH with post-deployment PTSD re-experiencing symptoms was tested, using a ZINB regression (55), with additional covariates age, cohort and PCs. Following the determination of a significant

association, the DBH SNP with the strongest effect (rs1611115) on pDBH was used as an instrument to test if pDBH is in the causal pathway to disease development (i.e. PTSD). MR estimates for the effect of pDBH on CAPS were then derived using a control function approach (56) an ordinary least squares regression of pDBH levels on rs1611115 was performed, including covariates age, cohort and PCs, followed by a ZINB regression of the CAPS score on pDBH, including the residuals from the first regression and age, cohort and PCs as covariates.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at *HMG* online.

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