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Human Bacterial Artificial Chromosome (BAC) Transgenesis Fully Rescues Noradrenergic Function in Dopamine β-Hydroxylase Knockout Mice

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Abstract

Dopamine β-hydroxylase (DBH) converts dopamine (DA) to norepinephrine (NE) in noradrenergic/adrenergic cells. DBH deficiency prevents NE production and causes sympathetic failure, hypotension and ptosis in humans and mice; DBH knockout (Dbh -/-) mice reveal other NE deficiency phenotypes including embryonic lethality, delayed growth, and behavioral defects. Furthermore, a single nucleotide polymorphism (SNP) in the human DBH gene promoter (-970C>T; rs1611115) is associated with variation in serum DBH activity and with several neurological- and neuropsychiatric-related disorders, although its impact on DBH expression is controversial. Phenotypes associated with DBH deficiency are typically treated with L-3,4-dihydroxyphenylserine (DOPS), which can be converted to NE by aromatic acid decarboxylase (AADC) in the absence of DBH. In this study, we generated transgenic mice carrying a human bacterial artificial chromosome (BAC) encompassing the DBH coding locus as well as ~45 kb of upstream and ~107 kb of downstream sequence to address two issues. First, we characterized the neuroanatomical, neurochemical, physiological, and behavioral transgenic rescue of DBH deficiency by crossing the BAC onto a Dbh -/- background. Second, we compared human DBH mRNA abundance between transgenic lines carrying either a “C” or a “T” at position -970. The BAC transgene drove human DBH mRNA expression in a pattern indistinguishable from the endogenous gene, restored normal catecholamine levels to the peripheral organs and brain of Dbh -/- mice, and fully rescued embryonic lethality, delayed growth, ptosis, reduced exploratory activity, and seizure susceptibility. In some cases, transgenic rescue was superior to DOPS. However,
allelic variation at the rs1611115 SNP had no impact on mRNA levels in any tissue. These results indicate that the human BAC contains all of the genetic information required for tissue-specific, functional expression of DBH and can rescue all measured Dbh deficiency phenotypes, but did not reveal an impact of the rs11115 variant on DBH expression in mice.

Introduction

Successful gene therapy, in which introduction of an external DNA construct replaces an absent or malfunctioning gene, will depend in large part on ensuring specific targeting of gene expression to appropriate cell types. The rare human syndrome of dopamine β-hydroxylase (DBH) deficiency results in severe orthostatic hypotension, ptosis, and high levels of circulating dopamine (DA), which reflect the inability of noradrenergic cells to synthesize norepinephrine (NE), resulting in absence of sympathetic noradrenergic tone [1, 2]. Human DBH deficiency results from rare deleterious mutations in the DBH gene, which lead to absent or inadequate expression of DBH protein [3].

Targeted disruption of Dbh in mice produces a precise model of DBH deficiency [4]. The observations that Dbh -/- mice are born in substantially smaller proportions than predicted by Mendelian expectations, and that surviving pups exhibit almost 100% mortality within the first week of life [4], highlight the essential roles of DBH and NE in development and survival. Prenatal and perinatal administration of L-3,4-dihydroxyphenylserine (DOPS), a hydroxylated precursor that is converted to NE by the enzyme aromatic acid decarboxylase (AADC), restores NE synthesis and rescues survival of Dbh -/- animals. The pre-natal mortality associated with the Dbh-/- phenotype arises from cardiovascular instability, which for unclear reasons stabilizes shortly after birth, thus allowing withdrawal of DOPS support. Once DOPS-treated Dbh -/- mice are born, they survive without pharmacological intervention, thereby allowing study of this interesting mutant in adulthood in the absence of NE. Dbh -/- mice have been a useful tool in a variety of investigations of the role of NE in behavior, including neurologically and psychiatrically relevant phenotypes such as arousal [5–7], seizure susceptibility [8], anxiety- and depression-like behaviors [9, 10], learning and memory [11, 12] and a variety of responses to drugs of abuse [13–18].

DBH activity can be measured in human serum, where the wide variation in enzyme activity observed in the population reflects variations in levels of DBH protein derived from sympathetic noradrenergic neurons and neurosecretory cells of the adrenal medulla [19]. Serum DBH level is a genetic trait largely refractory to environmental influences [19–21]. Genotype at -970C>T (rs1611115), a single nucleotide polymorphism (SNP) residing 970 bp upstream of the transcriptional start site of the DBH gene, accounts for 30–50% of the variance in serum DBH levels [22]. The C allele associates with substantially higher serum DBH activity than the T allele, an observation that has been repeatedly replicated in human samples of diverse ancestry [22–25]. However, because this SNP lies in the vast presumptive promoter region that contains many other variants, demonstrating a cause-and-effect relationship has been difficult. A genome-wide association study (GWAS) of serum DBH levels recently demonstrated that -970 C>T associates with variation in serum DBH more strongly than any other marker tested across the genome [26]. The foregoing observations prompted the hypothesis that -970 C>T associates with variation in serum DBH activity because it alters expression of the DBH gene, which should be detectable at the mRNA level. Chen and colleagues [27] tested the function of -970C>T in transient transfection assays of reporter plasmids containing each allele in the context of approximately 3 kb of DBH upstream sequence. Their results supported the hypothesis that -970C>T alters gene expression, but interestingly, yielded data suggesting the T allele
associates with higher reporter expression than the C allele, a result that is opposite to that expected from association studies of human serum DBH.

Barrie and colleagues [28] examined DBH mRNA expression in human tissues, providing evidence that -970C>T associates with variation in DBH mRNA expression in liver (where DBH is presumed to be present by virtue of hepatic sympathetic innervation), with the T allele associating with lower DBH mRNA expression. However, there was no evidence for an association of -970C>T with variation in DBH mRNA expression in the brain or adrenals. Thus, available evidence suggests that the influence of -970C>T on expression of DBH may be specific to sympathetic neurons. Interestingly, the association of variation at -970C>T with DBH expression at the mRNA level in transient transfection assays [25] is in the opposite direction expected from its association with serum DBH activity and in human liver [28]. The reason for this set of observations remains unknown, although it is likely that the transient transfection constructs lacked all of the pertinent regulatory sequences present in vivo.

The present study examined the in vivo function of -970C>T within the context of extensive human genomic sequence naturally surrounding the variant. We used bacterial artificial chromosome (BAC) constructs differing in sequence only at -970C>T to restore expression of DBH in Dbh -/- mice, to test three hypotheses. First, that introduction of BAC constructs containing the full human DBH gene and extensive surrounding sequence would rescue DBH expression in Dbh -/- mice, resulting in correction of abnormal phenotypes associated with absence of DBH and NE. Second, that the constructs would drive anatomically appropriate expression of DBH. Third, that BAC constructs containing the C allele at DBH position -970 would drive greater expression of DBH within noradrenergic neurons than those containing the T allele. Our results confirm the first two hypotheses, but not the third.

Materials and Methods

Animals

Dbh -/- mice on a mixed C57BL6/J and 129SvEv background were descendants of those produced by Thomas et al. [4] and maintained on a standard 12 hour light/dark cycle with food and water available ad libitum except during behavioral testing. All procedures were conducted in strict accordance with the NIH Guide for the Care and Use of Laboratory Animals and approved by the Emory University Institutional Animal Care and Use Committee.

Bacterial artificial chromosome cloning

The human DBH gene, on chromosome 9q34.2, has 12 exons and is ~23 kb in length from transcriptional start to transcriptional end. We chose the commercially available RP11-746P3 BAC (~ 175 kb) because it is likely to contain sufficient sequence both upstream (~ 45 kb) and downstream (~ 107 kb) from DBH to include all necessary elements required for correct expression. We obtained the BAC clone from BACPAC Resources, Children’s Hospital Oakland (Oakland, CA), isolated and purified the BAC using the Qiagen Large-Construct kit, and sequenced all exons and intron-exon boundaries for DBH to ensure it retained the full-length gene. We also sequenced the upstream sequence containing position -970 and determined that the original BAC contained a “T” at position -970. We successfully inserted a unique Asil restriction enzyme site into the BAC vector backbone for later linearization. We then created a second, “C” form of the BAC, by converting the “T” to a “C” at position -970 of the DBH gene, using the protocol described by Yang and Shara [29]. Briefly, the BAC was stably transfected into a strain of bacteria carrying recombinatory genes under the control of a temperature-sensitive promoter. We then transformed the cells with our custom-designed oligonucleotide carrying the “C” allele and adjusted the temperature to allow recombination to occur. PCR served
to screen for positive clones. Sequencing confirmed that the “C” and “T” BAC transgene constructs were complete and ready for pronuclear injection.

**Preparation of BAC transgenic mice**

BACs were purified with the Qiagen Large-Construct kit, linearized with AsiI, and isolated on a pulse-field gel. The BAC DNA was then used to generate BAC transgenic mice on a FVB background in the Emory University Mouse Transgenic and Gene Targeting Core Facility (http://www.cores.emory.edu/tmc/index.html) using standard transgenic methods. PCR on tail-snip DNA using human-specific primers corresponding to multiple sites on the transgene confirmed that the human insert within the BAC had fully integrated, and that all exons of human DBH were intact. We used 4 transgenic founders (two with the “C” allele and two with the “T” allele) to establish 4 independent transgenic lines for further characterization. We then crossed each of these lines onto a Dbh -/- background, which will be referred to as Dbh -/- BT lines. We chose one of the “T” lines for extensive behavioral, physiological, and neurochemical analysis. The other “T” line and the 2 other “C” lines were used exclusively to determine the consequences of the -970C>T (rs1611115) polymorphism on mRNA expression.

**Survival of Dbh -/- BT mice**

Dbh -/- BT males were crossed to Dbh +/- females. Offspring were genotyped at weaning (21 days), and the fraction of mice of each genotype was compared to the expected Mendelian ratio. For comparison between the efficacy of genetic and pharmacological rescue of the Dbh -/- lethal phenotype, we also crossed Dbh +/- without the BAC transgene with Dbh +/- females, as described [30]. Briefly, pregnant Dbh +/- females were given the adrenergic receptor agonists isoproterenol and phenylephrine (20 μg/ml each) + vitamin C (2 mg/ml) from E9.5-E14.5, and DOPS (2 mg/ml + vitamin C 2 mg/ml) from E14.5-birth in their drinking water.

**Ptosis**

Ptosis was determined in adult (3–6 months) mice at a fixed distance and measuring the maximum separation between the eyelids, as described [30].

**Novelty-induced locomotor activity**

Mice were placed in locomotion recording chambers (transparent Plexiglas cages placed into a rack with 7 infrared photobeams spaced 5 cm apart; San Diego Instruments Inc., La Jolla, CA), and ambulations (consecutive beam breaks) were recorded for 30 min.

**Flurothyl-induced seizures**

Mice were placed in an air-tight Plexiglas chamber, and the volatile convulsant Bis(2,2,2-trifluoroethyl) ether (flurothyl; Sigma Aldrich, St. Louis, MO) was dripped (20 μl/min) onto filter paper from which it vaporized. Latency to generalized (tonic-clonic) seizure was measured, as described [8].

**Catecholamine measurement by HPLC**

Levels of NE and DA were quantified using high-performance liquid chromatography coupled with electrochemical detection using procedures similar to those described previously [31]. Briefly, mice were euthanized by CO2 asphyxiation, and brain, adrenal, and heart were isolated and frozen on dry ice. Frozen tissue samples were initially prepared by adding 200 μl of ice-cold 0.1 N perchloric acid containing 0.04% sodium metabisulfite and then centrifuged at 13.2
x 1000 r.p.m. for 10 min at 4°C. A 50 μl aliquot of each sample was placed into a microcentrifuge tube and loaded into a refrigerated autosampler (G1329A, Agilent Technologies, Santa Clara, CA), which injected 15 μl of each sample onto an Ultrasound ODS 250 x 4.6 mm column, 5 μm (Beckman Coulter, Fullerton, CA) at a constant flow rate of 1.0 ml/min using a mobile phase consisting of 0.1 mM ethylenediaminetetraacetic acid, 0.8 mM sodium octyl sulfate, 0.7% phosphoric acid, and 5% acetonitrile (pH 2.6). Separated analytes were detected and quantified using a Coulouchem III detector (ESA Inc., Chelmsford, MA), a high sensitivity analytical channel (channel 1, -150 mV; channel 2, +300 mV; model 5011A, ESA Inc.), and a guard cell (+400 mV; model 5020, ESA Inc.). A set of standards containing experimenter-prepared concentrations of NE and DA (50–1000 nM) were analyzed in duplicate along with experimental samples. ChemStation chromatography software (Agilent Technologies) generated chromatograms for each sample analyzed and calculated area under the curve for each peak. Standards were used to generate a standard plot (area under the curve X analyte concentration) from which the estimated concentration in experimental samples was extrapolated.

**In situ hybridization**

To assess anatomic specificity of DBH expression, we employed in situ hybridization. C57Bl6/J wild-type (WT) (Jackson Laboratories, Bar Harbor, ME) and Dbh-/- BT mice were euthanized by CO2 asphyxiation, and brains were removed and frozen on dry ice. Sixteen-micrometer coronal sections containing the substantia nigra/ventral tegmental area (SN/VTA) and locus coeruleus (LC) were cut on a cryostat and mounted onto Fisher Superfrost slides (Fisher Scientific, Houston, TX). Slides were stored at -80°C until assayed. Tissue preparation and labeling of the mouse TH and human DBH oligonucleotides was performed as described previously [32]. The mouse TH oligonucleotide probe was a 48 base probe complementary to nucleotides 1351–1398 of the TH mRNA [33]. The human DBH oligonucleotide is composed of two different 51-base oligonucleotides (regions 478–529 and 1339–1390) of the human DBH sequence [34]. Each oligonucleotide was 3’-end-labeled with [33P]dATP (New England Nuclear, Boston, MA) using terminal deoxyribonucleotidyl transferase (Invitrogen, Piscataway, NJ) and then purified with Illustra MicroSpin G-25 Columns (GE Healthcare, Piscataway, NJ). The mouse TH hybridization buffer contained 0.35 X 10^6 cpm/50 μl, and the human DBH hybridization buffer contained 1.32 X 10^6 cpm/50 μl. Slides were washed as described in detail in previously published work for the oligonucleotide probes [32, 35] and apposed to film (Eastman Kodak, Rochester, NY) at room temperature for 18 hours for mouse TH and 4 days for human DBH.

**Reverse-transcription PCR**

Mice were euthanized by CO2 asphyxiation, and brain (LC microdissected), adrenal, heart, liver, and lung were isolated and frozen on dry ice. Tissue was homogenized in TRIzol, and RNA was extracted as previously described including column purification and DNase treatment to remove residual gDNA [36]. Due to the small amount of tissue available for the adrenal samples, 1 μg of glycogen was added to aid in RNA recovery. We measured the RNA integrity via Bioanalyzer (Agilent Technologies) for a subset of samples for each tissue type and genotype group. Concentrations were measured using the RNA or DNA quantitation reagent on the Qubit Fluorometer (Life Technologies) and diluted to equal concentrations. cDNA was synthesized via reverse transcription with SuperScript III (Invitrogen), oligo-dT, and gene-specific primers for human DBH and SARDH.

**Quantitative real-time PCR (qRT-PCR)**

mRNA expression was measured by qRT-PCR with a 7500 Fast Real-Time PCR System (Life Technologies) with the following primers: DBH_F: 5’GACGCCTGGAGTGACCAGAA,
DBH_R_RNA: 5’CAGTGACCAGGAAACGGCAGTTC. Reactions were prepared in duplicate in 10 ul volumes with Fast SYBR Green Master Mix (Applied Biosystems). After PCR, amplification plots were inspected, threshold values were set to 0.2 using the 7500 Software v2.0.5 (Applied Biosystems) and threshold cycle numbers (Ct) were obtained. We also designed multiple 150–190 bp product primer sets along the BAC to test for the presence of copy number variation. The mean Ct for the four sets was used to normalize the RNA expression values for each mouse. The primers were BAC2F: 5’GCCTGCCCTCTGCAAAC, BAC2R: 5’CCTGGGTGGG ACTTGGAAC, BAC9F: 5’TGTCCACTTGGACAGCACGC, BAC9R: 5’AGGAGCTTGAGAAAA CGGGA, BAC31F: 5’TGCACCACTGTGGCAACC, BAC31R: 5’CGACTTTGCTTTGCT GC, BAC37F: 5’GCTGTATTACCCACGCA and BAC37R: 5’GAAATGATGCTGGTGG TG. For these experiments, DNA template was extracted from brain via overnight digestion, ethanol precipitation, and phenol chloroform extraction [37].

Results

The human RP11-746P3 BAC contains regulatory elements sufficient to drive anatomically accurate human DBH mRNA expression in the mouse

DBH mRNA is normally expressed exclusively in noradrenergic/adrenergic cells, while tyrosine hydroxylase (TH) mRNA is expressed in all catecholaminergic neurons. Fig 1 shows in situ hybridization micrographs from wild-type C57Bl6/j and Dbh -/- BT mice. As expected, mouse TH is expressed in both midbrain DA neurons and LC neurons of Dbh -/- BT mice. By contrast, human DBH mRNA is restricted to LC neurons in the brain and the adrenal medulla of Dbh -/- BT mice, and completely absent from wild-type animals.

BAC transgenesis restores normal catecholamine levels to Dbh -/- mice

DBH is required for the conversion of DA to NE in noradrenergic and adrenergic cells. To determine whether BAC-driven expression of human DBH mRNA resulted in DBH function, NE and DA levels were measured in brain and peripheral organs of DBH-competent control (Dbh +/- - mice), DBH-deficient (Dbh -/-), and transgenic (Dbh -/- BT) mice. As described before by us and others [17, 36, 39], Dbh -/- mice lack NE and have elevated DA levels in all tissues examined, while the presence of the BAC transgene increased NE and decreased DA close to control levels (Fig 2, Fig 3). One-way ANOVA showed a significant effect of mouse genotype (referring here to Dbh genotype with or without BAC transgenesis) for brain (NE: F2,21 = 13.20, p<0.001; DA: F2,19 = 9.95, p<0.001), adrenal (NE: F2,21 = 22.38, p<0.0001; DA: F2,19 = 7.57, p<0.01), and heart (NE: F2,19 = 13.20, p<0.001; DA: F2,13 = 11.04, p<0.01). Tukey’s post-hoc tests revealed that NE was significantly reduced and DA was significantly elevated in Dbh -/- mice compared to Dbh +/- controls, while Dbh -/- BT mice had catecholamine content that was significantly different than Dbh -/- mice and comparable to controls.

BAC transgenesis rescues embryonic lethality, growth delay, ptosis, novelty-induced locomotor activity, and seizure susceptibility associated with DBH deficiency

We next determined whether the restoration of normal catecholamine levels by BAC transgenic expression of human DBH rescues physiological and behavioral phenotypes associated with DBH deficiency. Approximately 95% of Dbh -/- mice die during embryogenesis or during the first few days of life unless adrenergic receptor agonists and DOPS are added to the drinking water of the pregnant dam [4]. Table 1 shows the numbers of viable offspring born to Dbh -/- BT
males crossed with $Dbh^{+/−}$ dams and untreated with DOPS. The proportion of $Dbh^{-/-}$ offspring lacking the transgene was far below Mendelian expectations ($\chi^2$, 3 d.f. = 36.7, $p < 0.0001$), whereas the BAC construct fully rescued viability in $Dbh^{-/-}$ BT offspring, with the proportions of the three viable genotypes not differing from one-third for each genotype ($\chi^2$, 2 d.f. = 2.21, $p > 0.05$).

Fig 1. A human BAC transgene drives specific $Dbh$ expression to the locus coeruleus and adrenal gland. Shown are representative examples of human dopamine β-hydroxylase (DBH) and mouse tyrosine hydroxylase (TH) mRNA expression in adrenal gland and brain sections from C57Bl6/J wild-type and BAC transgenic mice containing the noradrenergic locus coeruleus (LC, corresponding to Figure 75 in the Mouse Brain Atlas) and the dopaminergic substantia nigra pars compacta/ventral tegmental area (SN/VTA, corresponding to Figure 55 in the Mouse Brain Atlas[38]).

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p = 0.331). By contrast, crosses between Dbh -/- male and Dbh +/- females that received adrenergic agonists + DOPS during pregnancy resulted in 71 Dbh +/- and 43 Dbh -/- viable progeny (38%), as compared to 32 Dbh +/- BT and 38 Dbh -/- BT viable offspring (54%; expected

Fig 2. A human BAC transgene restores normal central and peripheral NE levels to Dbh -/- mice. Dbh +/-, Dbh -/-, and Dbh -/- BT littermates were assessed for tissue NE levels in the (A) brain, (B) heart, and (C) adrenal by HPLC. Shown is mean ± SEM ng of NE per mg tissue. N = 7–9 per genotype.

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proportions 50% for each set of genotypes; $p = 0.03$, Fisher’s Exact Test) in the crosses of $Dbh^{-/-}$ male and untreated $Dbh^{+/+}$ females.

Fig 3. A human BAC transgene restores normal central and peripheral DA levels to $Dbh^{-/-}$ mice. $Dbh^{+/+}$, $Dbh^{-/-}$, and $Dbh^{-/-}$ BT littersmates were assessed for tissue DA levels in the (A) brain, (B) heart, and (C) adrenal by HPLC. Shown is mean ± SEM ng of DA per mg tissue. N = 4–9 per genotype.

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Fig 4 shows comparisons of weaning weight (4A), the degree of ptosis (4B), novelty-induced locomotion (4C) and latency to seizure after exposure to flurothyl (4D) in Dbh -/- BT mice, Dbh -/- mice born to DOPS-treated dams from which DOPS was withheld after birth, or NE-competent Dbh +/- littermates (which prior studies have shown do not differ from wild-type, Dbh +/- mice; [4, 8, 40]). One-way ANOVAs showed significant effects of genotype for

Table 1. Viable offspring born to a Dbh +/- female by DBH -/- BT male cross*.

| Offspring genotype | Observed Count | Observed proportion | Expected Proportion+ |
|--------------------|----------------|---------------------|---------------------|
| DBH +/-            | 45             | 0.385               | 0.25                |
| DBH +/- BT         | 32             | 0.274               | 0.25                |
| DBH -/-            | 2              | 0.017               | 0.25                |
| DBH -/- BT         | 38             | 0.325               | 0.25                |

*χ², 3 d.f. = 36.7, p < 0.0001.

+Mendelian expectation assuming no impact of genotype on survival.

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Fig 4. A human BAC transgene rescues Dbh +/- developmental, physiological, and behavioral phenotypes. Dbh +/-, Dbh -/-, and Dbh -/- BT littermates were assessed for (A) weaning weight, (B) ptosis, (C) novelty-induced locomotor activity, and (D) seizure susceptibility. Shown is mean ± SEM (A) weight in grams, (B) mm eye opening, (C) ambulations in 30 min, and (D) latency to flurothyl-induced generalized seizure. N = 6–8 per genotype. *p<0.05, **p<0.01, ****p<0.0001.

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weaning weight (F2,25 = 14.68, p<0.0001), ptosis (F2,21 = 11.07, p<0.001), novelty-induced locomotion (F2,17 = 11.13, p<0.001), and seizure susceptibility (F2,19 = 6.80, p<0.01). Tukey’s posthoc tests revealed that Dbh -/- mice weighed less and had reduced eye opening, exploratory activity, and latency to generalized seizure, and BAC transgenesis fully rescued each of the Dbh -/- phenotypes.

The -970C>T (rs1611115) human polymorphism does not affect BAC transgenic human DBH expression in the mouse

To determine the specific impact of the -970C>T (rs1611115) human polymorphism on human DBH gene expression, we compared mRNA expression in 4 independent Dbh -/- BT mouse lines differing only at that single base (2 “C” lines, 2 “T” lines). After controlling for transgene copy number, no significant genotype differences in human DBH mRNA abundance were detected for the LC, adrenal, heart, liver, or lung (Fig 5).

Discussion

DBH catalyzes conversion of DA to NE within noradrenergic and adrenergic vesicles in the central and peripheral nervous systems and in the adrenal medulla. Absence of DBH leads to a syndrome characterized by inadequate sympathetic tone, leading to severe orthostatic hypotension, ptosis and other manifestations of sympathetic failure. Central nervous system manifestations are more subtle in humans, as there are no dramatic psychiatric or neurological manifestations reported in patients with DBH deficiency. However, these patients are chronically medicated with NE-promoting drugs such as DOPS to control the cardiovascular phenotypes, which may also prevent the manifestation of behavioral changes. Although small studies have suggested a variety of links between differences in DBH genotype and/or plasma levels and other human disorders, (reviewed in [41]) more recent genome-wide association studies have not provided evidence for replicable associations of DBH genotype to human neurological or psychiatric disorders (e.g., [42], [43], [44]).

Dbh knockout mice have been used to assess brain disorders potentially associated with DBH deficiency. Mice in which targeted disruption of the Dbh gene results in absence of DBH and NE display a cardiovascular syndrome highly similar to the human DBH deficiency syndrome [9, 30, 45]. Moreover, several phenotypes with relevance to neurological and neuropsychiatric disorders, including increased seizure susceptibility [8], age-related motor impairment [46], learning and memory deficits [11, 12], decreased arousal/exploration [5, 11], altered antidepressant drug responses [10], and changes in cocaine-induced behaviors [18] occur in Dbh -/- mice. The current study demonstrates that introduction of the human DBH gene by BAC transgenesis restores the deficits in noradrenergic function resulting from absence of Dbh expression, as manifested in organismic, behavioral and neurochemical phenotypes.

The BAC construct drove anatomically specific expression within noradrenergic neurons and the adrenal medulla, demonstrating that the human sequence context surrounding DBH was capable of directing the murine transcriptional machinery in a cell-specific manner. That observation was not guaranteed for two reasons. First, while conventional transgenes using small fragments of the human DBH promoter are able to drive gene expression in noradrenergic and adrenergic cells, these constructs also result in widespread ectopic expression in non-catecholaminergic neurons and organs [47–49]. Second, the degree of sequence identity between non-coding regions of DBH and Dbh are relatively modest. For example, comparison of the proximal 2000 bp immediately 5’ to the translational start site (ATG) of human DBH (GRC 38/hg 38) and mouse Dbh (GRC38/mm10) using ALIGN [50] (http://atlas.igh.cnrs.fr/bin/align-guess.org) reveals only 52.8% sequence identity.
Fig 5. The -970 C-T polymorphism does not affect Dbh mRNA abundance. 
Dbh -/- BT mice carrying either the C allele (2 independent lines, pooled) or the T allele (2 independent lines, pooled) at position -970 were assessed for mRNA abundance in the (A) brain, (B) adrenal, (C) heart, (D) liver, and (E) lung by qRT-PCR. Shown are individual and mean ± SEM threshold cycle numbers (ΔCt) normalized to copy number. N = 6–11 per allele.

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In contrast to conventional Dbh -/- mice, which lack NE completely and have elevated DA levels due to DA production in noradrenergic/adrenergic cells, Dbh -/- BT mice had NE and DA levels comparable to controls. In general, transgenic rescue of catecholamine abundance was superior to that achieved via DOPS administration. A thorough characterization of DOPS-induced neurochemical changes in Dbh -/- mice was reported by Thomas and colleagues [30]. They showed that a single injection of DOPS (1 mg/g, s.c.) is sufficient to transiently restore NE to wild-type levels in most peripheral organs. However, NE rescue was only partial in most areas of brain and completely deficient in adrenal. Repeated DOPS treatment (1 mg/g, s.c. every 12 hours, 7 injections total) provides extra benefit in some brain regions (e.g. frontal cortex) but not others (e.g. midbrain, cerebellum). Moreover, because DOPS bypasses the requirement for DBH by relying on a different enzyme for NE synthesis (aromatic amino acid decarboxylase; AADC) rather than correcting its deficiency, pharmacological rescue does not attenuate the excessive production of DA in noradrenergic/adrenergic cells. By contrast, the BAC transgene almost completely reversed both the NE deficiency and DA surplus in all tissues examined, including the adrenal gland. Another important difference is that because AADC is expressed in dopaminergic and serotonergic neurons as well as noradrenergic/adrenergic cells, NE production and release following DOPS administration lacks anatomical specificity. The BAC transgene drives DBH expression, and thus NE production and release, in a pattern indistinguishable from the endogenous condition.

Most aspects of physiology and behavior in Dbh -/- mice were rescued comparably by either DOPS or transgenesis; for example, both approaches normalized ptosis and seizure susceptibility [8, 30], while the impact of DOPS on novelty-induced locomotor activity and postnatal growth in Dbh -/- mice has not been examined. These results suggest that partial DBH or NE deficiency does not have a demonstrative impact on these phenotypes, an idea supported by the lack of any deficit observed in Dbh +/- mice that have half the normal copies of Dbh but close to normal catecholamine levels. The one exception was pup survival, where the transgene was far superior. Notably, in that case DOPS was administered at a low dose via drinking water to pregnant dams, a paradigm that restores NE levels to only ~10% of normal [4].

Contrary to our original hypothesis, DBH expression in mice carrying the “T” allele at position -970 did not differ appreciably from that in “C” mice. This observation is in line with the report of Barrie and colleagues [28] that expression of mRNA encoding DBH differed only in the liver of humans, but not in the brain, strongly suggesting that -970C>T only impacts human expression of the DBH gene within sympathetic noradrenergic neurons [28], and is consistent with the finding that the majority of the serum enzyme arises from the sympathetic nervous system [19]. However, even liver DBH expression did not significantly differ between carriers of the “T” and “C” alleles in Dbh -/- BT mice. These results suggest that the transcriptional machinery sensitive to the -970C>T region of the human promoter is not present in mouse noradrenergic sympathetic neurons, or if it is present, the transcriptional machinery in mouse does not respond to the sequence surrounding rs1611115 in the same way the human machinery does. Consistent with that idea is that the sequence immediately surrounding rs1611115 (AGTCTACTTG[CT]GAGACAGACA, where underlined bases are identical to human sequence and bold shows the base corresponding to rs1611115). Alternatively, despite all of the human association evidence to the contrary [22, 25, 26, 28], it is possible that -970C>T is not the functional polymorphism accounting for the substantial difference in serum DBH levels associated with genotype at this SNP, or that it requires cooperation with distal enhancer sites not present in the transgene. Despite our failure to detect a gene expression difference, this is, to our knowledge, the first use of BAC transgenesis in mice.
to elucidate the potential function of a neuronal non-coding human SNP, an approach that should prove useful for other variants identified in association studies.

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**Author Contributions**

Conceived and designed the experiments: JFC DW. Performed the experiments: JPS ESB DFM WS TB KM TAS LCL KES AM PC DLP PS. Analyzed the data: JFC JPS ESB DFM WS TAS LCL KES KM PC DLP PS DW. Contributed reagents/materials/analysis tools: ESB WS TB KM PS DW. Wrote the paper: JFC ESB WS DFM DLP PS JPS DW.

**References**

1. Man in 't Veld AJ, Boomsma F, Moleman P, Schalekamp MA. Congenital dopamine-beta-hydroxylase deficiency. A novel orthostatic syndrome. Lancet. 1987; 1(8526):183–8. Epub 1987/01/24. PMID: 2880016.

2. Robertson D, Haile V, Perry SE, Robertson RM, Phillips JA, 3rd, Biaggioni I. Dopamine beta-hydroxylase deficiency. A genetic disorder of cardiovascular regulation. Hypertension. 1991; 18(1):1–8. Epub 1991/07/01. PMID: 1677640.

3. Kim CH, Zabetian CP, Cubells JF, Cho S, Biaggioni I, Cohen BM, et al. Mutations in the dopamine beta-hydroxylase gene are associated with human norepinephrine deficiency. Am J Med Genet. 2002; 108(2):140–7. Epub 2002/02/22. doi: 10.1002/ajmg.10196 [pii]. PMID: 11857564.

4. Thomas SA, Matsumoto AM, Palmiter RD. Noradrenaline is essential for mouse fetal development. Nature. 1995; 374(6523):643–6. Epub 1995/04/13. doi: 10.1038/374643a0 PMID: 7715704.

5. Hunsley MS, Palmiter RD. Altered sleep latency and arousal regulation in mice lacking norepinephrine. Pharmacol Biochem Behav. 2004; 78(4):765–73. Epub 2004/08/11. doi: 10.1016/j.pbb.2004.05.008 S0091-3057(04)00167-4 [pii]. PMID: 15301933.

6. Mitchell HA, Bogenpohl JW, Liles LC, Epstein MP, Bozyczko-Coyne D, Williams M, et al. Behavioral responses of dopamine beta-hydroxylase knockout mice to modafinil suggest a dual noradrenergic-dopaminergic mechanism of action. Pharmacol Biochem Behav. 2008; 91(2):217–22. Epub 2008/08/16. S0091-3057(08)00269-4 [pii] doi: 10.1016/j.pbb.2008.07.014 PMID: 18703079.

7. Swoap SJ, Gutilla MJ, Liles LC, Smith RO. Weisnhenker D. The full expression of fasting-induced torpor requires beta 3-adrenergic receptor signaling. J Neurosci. 2006; 26(1):241–5. Epub 2006/01/10. 26(1)/241 [pii] doi: 10.1523/JNEUROSCI.3721-05.2006 PMID: 16396993.

8. Szot P, Weisnhenker D, White SS, Robbins CA, Rust NC, Schwartzkroin PA, et al. Norepinephrine-deficient mice have increased susceptibility to seizure-inducing stimuli. J Neurosci. 1999; 19(24):10985–92. Epub 1999/12/14. PMID: 10594079.

9. Swoap SJ, Weisnhenker D, Palmiter RD, Garber G. Dbh(-/-) mice are hypotensive, have altered circadian rhythms, and have abnormal responses to dieting and stress. Am J Physiol Regul Integr Comp Physiol. 2004; 286(1):R108–13. Epub 2003/09/13. doi: 10.1152/ajpregu.00405.2003 00405.2003 [pii] PMID: 12969876.

10. Cryan JF, O’Leary OF, Jin SH, Friedland JC, Ouyang M, Hirsch BR, et al. Norepinephrine-deficient mice lack responses to antidepressant drugs, including selective serotonin reuptake inhibitors. Proc Natl Acad Sci U S A. 2004; 101(21):8186–91. Epub 2004/05/19. doi: 10.1073/pnas.0401080101 0401080101 [pii]. PMID: 15148402.

11. Hammerschmidt T, Kummer MP, Terwel D, Martinez A, Gorji A, Pape HC, et al. Selective loss of noradrenaline exacerbates early cognitive dysfunction and synaptic deficits in APP/PS1 mice. Biol Psychiatry. 2013; 73(5):454–63. Epub 2012/08/14. S0006-3223(12)00546-X [pii] doi: 10.1016/j.biopsych.2012.06.015 PMID: 22883216.

12. Murchison CF, Zhang XY, Zhang WP, Ouyang M, Lee A, Thomas SA. A distinct role for norepinephrine in memory retrieval. Cell. 2004; 117(1):131–43. Epub 2004/04/07. S0006-3223(04)002594 [pii] PMID: 15066288.
13. Weinshenker D, Ferrucci M, Busceti CL, Biagioni F, Lazzari G, Liles LC, et al. Genetic or pharmacological blockade of noradrenaline synthesis enhances the neurochemical, behavioral, and neurotoxic effects of methamphetamine. J Neurochem. 2008; 105(2):471–83. Epub 2007/11/29. JNC5145 [pii] doi: 10.1111/j.1471-4159.2007.05145.x PMID: 18042179.

14. Weinshenker D, Miller NS, Blizinsky K, Laughlin ML, Palmiter RD. Mice with chronic norepinephrine deficiency resemble amphetamine-sensitized animals. Proc Natl Acad Sci U S A. 2002; 99(21):13873–7. Epub 2002/10/09. doi: 10.1073/pnas.212519999 212519999 [pii] PMID: 12370425.

15. Gaval-Cruz M, Schroeder JP, Liles LC, Javors MA, Weinshenker D. Effects of disulfiram and dopamine beta-hydroxylase knockout on cocaine-induced seizures. Pharmacol Biochem Behav. 2008; 89(4):556–62. Epub 2008/03/11. S0091-3057(08)00057-9 [pii] doi: 10.1016/j.pbb.2008.02.009 PMID: 18329701.

16. Weinshenker D, Rust NC, Miller NS, Palmiter RD. Ethanol-associated behaviors of mice lacking norepinephrine. J Neurosci. 2000; 20(9):3157–64. Epub 2000/04/25. PMID: 10777779.

17. Olson VG, Heusner CL, Bland RJ, During MJ, Weinshenker D, Palmiter RD. Role of noradrenergic signaling by the nucleus tractus solitarius in mediating opiate reward. Science. 2006; 311(5763):1017–20. Epub 2006/02/18. 311/5763/1017 [pii] doi: 10.1126/science.1119311 PMID: 16484499.

18. Schank JR, Ventura R, Puglisi-Allegra S, Alcaro A, Cole CD, Liles LC, et al. Dopamine beta-hydroxylase knockout mice have alterations in dopamine signaling and are hypersensitive to cocaine. Neuropsychopharmacology. 2006; 31(10):2221–30. Epub 2006/01/06. 1301000 [pii] doi: 10.1038/sj.npp.1301000 PMID: 16395294.

19. Weinshilboum R, Axelrod J. Serum dopamine-beta-hydroxylase activity. Circ Res. 1971; 28(3):307–15. Epub 1971/03/01. PMID: 4925832.

20. Weinshilboum RM, Raymond FA, Elvenack LR, Weidman WH. Serum dopamine-ß-hydroxylase: Sibling-sibling correlation. Science. 1973; 181:943–5. PMID: 4730445.

21. Weinshilboum RM, Schorott HG, Raymond FA, Weidman WH, Elveback LR. Inheritance of very low serum dopamine-beta-hydroxylase activity. Am J Hum Genet. 1975; 27(5):573–85. PMID: 1163533.

22. Zabetian CP, Anderson GM, Buxbaum SG, Ichinose H, Nagatsu T, et al. A quantitative-trait analysis of human plasma-dopamine beta-hydroxylase activity: evidence for a major functional polymorphism at the DBH locus. Am J Hum Genet. 2001; 68(2):515–22. PMID: 11170900.

23. Kohnke MD, Zabetian CP, Anderson GM, Kolb W, Gaertner I, Buchkremer G, et al. A genotype-controlled analysis of plasma dopamine beta-hydroxylase activity and -1021C/T polymorphism of DBH gene in healthy and alcoholic subjects: evidence for alcohol-related differences in noradrenergic function. Biol Psychiatry. 2002; 52(12):1151–8. Epub 2002/12/19. S0006322302014270 [pii] PMID: 12488060.

24. Mustapic M, Pivac N, Kozaric-Kovacic D, Dezeljin M, Cubells JF, Muck-Seler D. Dopamine beta-hydroxylase (DBH) activity and -1021C/T polymorphism of DBH gene in combat-related post-traumatic stress disorder. Am J Med Genet B Neuropsychiatr Genet. 2007; 144B(8):1087–9. Epub 2007/09/14. doi: 10.1002/ajmg.b.30526 PMID: 17853400.

25. Chen Y, Wen G, Rao F, Zhang K, Wang L, Rodriguez-Flores JL, et al. Human dopamine beta-hydroxylase (DBH) regulatory polymorphism that influences enzymatic activity, autonomic function, and blood pressure. J Hypertens. 2010; 28(1):76–86. Epub 2009/12/17. doi: 10.1097/HJH.0b013e3283323cb87 PMID: 20009769.

26. Mustapic M, Malhofer AX, Mahtata M, Chen Y, Baker DG, O’Connor DT, et al. The catecholamine biosynthetic enzyme dopamine beta-hydroxylase (DBH): first genome-wide search positions trait-determining variants acting additively in the proximal promoter. Hum Mol Genet. 2014; 23(23):6375–84. Epub 2014/07/06. ddu332 [pii] doi: 10.1093/hmg/ddu332 PMID: 24986918.

27. Chen Y, Zhang K, Wen G, Rao F, Sanchez AP, Wang L, et al. Human dopamine beta-hydroxylase promoter variant alters transcription in chromaffin cells, enzyme secretion, and blood pressure. Am J Hypertens. 2011; 24(1):24–32. Epub 2010/09/04. ajh2010186 [pii] doi: 10.1093/ajh/hpj090 PMID: 20814407.

28. Barrie ES, Weinshenker D, Verma A, Pendergrass SA, Lange LA, Ritchie MD, et al. Regulatory polymorphisms in human DBH affect peripheral gene expression and sympathetic activity. Circ Res. 2014; 115(12):1017–25. Epub 2014/10/19. CIRCRESAHA.116.304398 [pii] doi: 10.1161/CIRCRESAHA.116.304398 PMID: 25326128.

29. Yang Y, Sharan SK. A simple two-step, ‘hit and fix’ method to generate subtle mutations in BACs using short denatured PCR fragments. Nucleic Acids Res. 2003; 31(15):e80. Epub 2003/07/31. PMID: 12886532.

30. Thomas SA, Marck BT, Palmiter RD, Matsumoto AM. Restoration of norepinephrine and reversal of phenotypes in mice lacking dopamine beta-hydroxylase. J Neurochem. 1998; 70(6):2468–76. Epub 1998/05/29. PMID: 9603211.
31. Schroeder JP, Cooper DA, Schank JR, Lyle MA, Gaval-Cruz M, Ogbonmwan YE, et al. Disulfiram attenuates drug-primed reinstatement of cocaine seeking via inhibition of dopamine beta-hydroxylase. Neuropsychopharmacology. 2010; 35(12):2440–9. Epub 2010/08/26. npp.2010.127 PMID: 20736996.

32. Szot P, White SS, Veith RC. Effect of pentylenetetrazol on the expression of tyrosine hydroxylase mRNA and norepinephrine and dopamine transporter mRNA. Brain Res Mol Brain Res. 1997; 44(1):46–54. Epub 1997/02/01. S0169328X96002173 [pii]. PMID: 9030697.

33. Grima B, Lamouroux A, Blanot F, Biguet NF, Mallet J. Complete coding sequence of rat tyrosine hydroxylase mRNA. Proc Natl Acad Sci U S A. 1985; 82(2):617–21. Epub 1985/01/01. PMID: 2857492.

34. Kobayashi K, Kurosawa Y, Fujita K, Nagatsu T. Human dopamine beta-hydroxylase gene: two mRNA types having different 3’-terminal regions are produced through alternative polyadenylation. Nucleic Acids Res. 1989; 17(3):1089–102. Epub 1989/02/11. PMID: 2922261.

35. McMillan PJ, White SS, Franklin A, Greenup JL, Leverenz JB, Raskind MA, et al. Differential response of the central noradrenergic nervous system to the loss of locus coeruleus neurons in Parkinson’s disease and Alzheimer’s disease. Brain Res. 2011; 1373:240–52. Epub 2010/12/15. S0006-3223(10)02654-5 [pii] doi:10.1016/j.brainres.2010.12.015 PMID: 21147074.

36. Anderle P, Nielsen CU, Pinsonneault J, Krog PL, Brodin B, Sadee W. Genetic variants of the human dipeptide transporter PEPT1. J Pharmacol Exp Ther. 2006; 316(2):636–46. Epub 2005/11/01. jpet.105.094615 [pii] doi:10.1124/jpet.105.094615 PMID: 16258023.

37. Miller SA, Dykes DD, Polesky HF. A simple salting out procedure for extracting DNA from human nucleated cells. Nucleic Acids Res. 1988; 16(6):1215. Epub 1988/02/11. PMID:334216.

38. Paxinos G, and Franklin KBJ. The mouse brain in stereotaxic coordinates. San Diego, CA: Academic Press; 2001.

39. Bourdelat-Parks BN, Anderson GM, Donaldson ZR, Weiss JM, Bonsall RW, Emery MS, et al. Effects of dopamine beta-hydroxylase genotype and disulfiram inhibition on catecholamine homeostasis in mice. Psychopharmacology (Berl). 2005; 183(1):72–80. Epub 2005/09/16. doi:10.1007/s00213-005-0139-8 PMID:16163519.

40. Mitchell HA, Ahem TH, Liles LC, Javors MA, Weinschenker D. The effects of norepinephrine transporter inactivation on locomotor activity in mice. Biol Psychiatry. 2006; 60(10):1046–52. Epub 2006/08/09. S0006-3223(06)00538-5 [pii] doi:10.1016/j.biopsych.2006.03.057 PMID:16893531.

41. Cubells JF, Zabetian CP. Human genetics of plasma dopamine beta-hydroxylase activity: applications to research in psychiatry and neurology. Psychopharmacology (Berl). 2004. PMID:15088079.

42. Schizophrenia Working Group of the Psychiatric Genomics C. Biological insights from 108 schizophrenia-associated genetic loci. Nature. 2014; 511(7510):421–7. doi:10.1038/nature13595 PMID:25056061; Pubmed Central PMCID: PMCPMC4112379.

43. Major Depressive Disorder Working Group of the Psychiatric GC, Ripke S, Wray NR, Lewis CM, Hamilton SP, Weissman MM, et al. A mega-analysis of genome-wide association studies for major depressive disorder. Mol Psychiatry. 2013; 18(4):497–511. 10.1038/mp.2012.21. 22472876; Pubmed Central PMCID: PMCPMC3837431. doi:10.1038/mp.2012.21 PMID:22472876.

44. Nalls MA, Pankratz N, Lit CM, Do CB, Hernandez DG, Saad M, et al. Large-scale meta-analysis of genome-wide association data identifies six new risk loci for Parkinson’s disease. Nat Genet. 2014; 46(9):989–93. doi:10.1038/ng.3043 PMID:25064009; Pubmed Central PMCID: PMCPMC4146673.

45. Thomas SA, Palmiter RD. Thermoregulatory and metabolic phenotypes of mice lacking noradrenaline and adrenaline. Nature. 1997; 387(6628):94–7. Epub 1997/05/01. doi:10.1038/38709a0 PMID:9139628.

46. Rommelfanger KS, Edwards GL, Freeman KG, Liles LC, Miller GW, Weinschenker D. Norepinephrine loss produces more profound motor deficits than MPTP treatment in mice. Proc Natl Acad Sci U S A. 2007; 104(34):13804–9. 0702753104 [pii] doi:10.1073/pnas.0702753104 PMID: 17702867.

47. Mercer EH, Hoyle GW, Kapur RP, Brinster RL, Palmiter RD. The dopamine beta-hydroxylase gene promoter directs expression of E. coli lacZ to sympathetic and other neurons in adult transgenic mice. Neuron. 1991; 7(5):703–16. Epub 1991/11/01. 0896-6273(91)90274-4 [pii] doi:10.1016/0896-6273(91)90274-4 PMID:1742021.

48. Steiner RA, Hohmann JG, Holmes A, Wrenn CC, Cadd G, Jureus A, et al. Galanin transgenic mice display cognitive and neurochemical deficits characteristic of Alzheimer’s disease. Proc Natl Acad Sci U S A. 2001; 98(7):4144–9. Epub 2000/03/22. doi:10.1073/pnas.061445598 061445598 [pii] PMID:11259657.

49. Hoyle GW, Mercer EH, Palmiter RD, Brinster RL. Cell-specific expression from the human dopamine beta-hydroxylase promoter in transgenic mice is controlled via a combination of positive and negative regulatory elements. J Neurosci. 1994; 14(5 Pt 1):2455–63. Epub 1994/05/01. PMID:8182421.
50. Pearson WR, Wood T, Zhang Z, Miller W. Comparison of DNA sequences with protein sequences. Genomics. 1997; 46(1):24–36. Epub 1997/12/24. S0888-7543(97)94995-8 [pii] doi: 10.1006/geno.1997.4995 PMID: 9403055.