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The C886T Mutation in the *Th* Gene Reduces the Activity of Tyrosine Hydroxylase in the Mouse Brain

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Abstract—Tyrosine hydroxylase (TH) catalyzes hydroxylation of L-tyrosine to L-3,4-dihydroxyphenylalanine, the initial and rate-limiting step in the synthesis of dopamine, noradrenaline, and adrenaline. Mutations in the human *TH* gene are associated with hereditary motor disorders. The common C886T mutation identified in the mouse *Th* gene results in the R278H substitution in the enzyme molecule. We investigated the impact of this mutation on the TH activity in the mouse midbrain. The TH activity in the midbrain of *Mus musculus castaneus* (CAST) mice homozygous for the 886C allele was higher compared to C57BL/6 and DBA/2 mice homozygous for the 886T allele. Notably, this difference in the enzyme activity was not associated with changes in the *Th* gene mRNA levels and TH protein content. Analysis of the TH activity in the midbrain in mice from the F2 population obtained by crossbreeding of C57BL/6 and CAST mice revealed that the 886C allele is associated with a high TH activity. Moreover, this allele showed complete dominance over the 886T allele. However, the C886T mutation did not affect the levels of TH protein in the midbrain. These findings demonstrate that the C886T mutation is a major genetic factor determining the activity of TH in the midbrain of common laboratory mouse strains. Moreover, it represents the first common spontaneous mutation in the mouse *Th* gene whose influence on the enzyme activity has been demonstrated. These results will help to understand the role of TH in the development of adaptive and pathological behavior, elucidate molecular mechanisms regulating the activity of TH, and explore pharmacological agents for modulating its function.

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Keywords: tyrosine hydroxylase, C886T mutation, activity, expression, brain, mice

INTRODUCTION

The dopaminergic system of the brain plays a pivotal role in the regulation of the nervous system, endocrine glands, and adaptive and pathological behavior [1-4]. The nigrostriatal dopaminergic system regulates motor function; its overactivation has been linked to hyperactivity [5-7], while a deficit of its activity is associated with dystonia [8-10] and catalepsy/catatonia [11, 12]. On the other hand, the mesolimbic dopaminergic system is involved in the assessment of signal significance, learning [13-15], and substance addiction [16-19].

In the brain, dopamine (DA) is synthesized from the amino acid L-tyrosine in two steps. First, tyrosine hydroxylase (TH) converts L-tyrosine to L-3,4-dihydroxyphenylalanine (L-DOPA), and then, aromatic amino acid decarboxylase converts L-DOPA to DA. The hydroxylation of L-tyrosine is a key and rate-limiting step determining the DA levels in the brain. Indeed, the *Th* gene knockout reduced DA content in the mouse brain [20, 21]. Some mutations in the human *TH* gene have been associated with childhood parkinsonism [22], increased risk of Parkinson's disease [22, 23], dystonia [24-27], and bipolar disorders [28]. However,

Abbreviations: DA, dopamine; CAST, *Mus musculus castaneus*; L-DOPA, L-3,4-dihydroxyphenylalanine; TH, tyrosine hydroxylase.

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investigating molecular events linking *TH* gene mutations to the nervous system disorders, motor function impairments, and human mental activity is restricted due to social and ethical constraints. Therefore, modeling disturbances caused by mutations in the *Th* gene in laboratory rodents is an important task in modern neuroscience.

The Ensembl genome database (https://www.ensembl.org/index.html) contains information on 21 single-nucleotide polymorphisms (SNPs) in the mouse *Th* gene that lead to the amino acid substitutions in the enzyme molecule. Among those, only one SNP, the C886T mutation resulting in the R278H substitution, has been identified in the *Th* gene of commonly used laboratory mouse strains. The 886T allele is prevalent in most of these strains (C57BL/6, C3H, DBA/2, CBA), whereas the 886C allele was found in the *Mus musculus castaneus* (CAST) subspecies. Previously, it was demonstrated that the G1449A mutation in the mouse *Tph2* gene resulting in the R441H substitution in tryptophan hydroxylase 2 (TPH2), significantly reduces enzyme activity in the mouse brain [29, 30]. Taking into account that TH and TPH2 are related enzymes from the aromatic amino acid hydroxylase family [31], it was reasonable to expect that the C886T mutation decreases the TH activity.

We investigated the impact of the C886T mutation in the *Th* gene on the TH activity in the mouse brain and compared the activity of TH in the midbrain (area containing the bodies of DA neurons) in C57BL/6 (886T), DBA/2 (886T), and CAST (886C) mouse strains, as well as the studied the differences in the levels of *Th* gene mRNA and TH protein between the studied mouse strains. We also assessed the contribution of the C886T mutation to the TH activity in the context of other genes and investigated the linkage of the 886T and 886C alleles with the TH activity and protein level in the midbrain of F2 segregating intercross animals obtained by crossbreeding of C57BL/6 and CAST mice.

MATERIALS AND METHODS

Animals. Adult male mice of C57BL/6 (n = 6), DBA/2 (n = 6), and CAST (n = 5) strains, and F2 intercrosses between C57BL/6 and CAST mice (42 males and females) were used in the experiments. The intercrosses were obtained by crossing first-generation hybrids F1(C57BL/6 × CAST) with each other. At the start of the experiment, all mice were 12 weeks old and had the specific pathogen-free (SPF) status that was maintained during the study. The animals were housed under standard SPF conditions at a constant temperature of 23°C and at a 14 h light/10 h dark cycle (lights switched on at 01:00 and switched off at 15:00). The mice had an *ad libitum* access to sterile dry food and water. At the age of 3 weeks, young animals were separated from their mothers and grouped into cages with 4-5 animals of the same sex (Optimice, Animal Care Systems, Inc.). The animals were marked with ear notches, and ear samples obtained during marking were used for DNA extraction and genotyping. Two days before the start of the experiment, the animals were placed into individual cages to minimize the potential influence of group effects on the TH activity. The animals were euthanized using CO_2 asphyxia and decapitated; the midbrains containing the bodies of TH-expressing DA neurons were dissected, frozen in liquid nitrogen, and stored at –80°C until assay.

Genotyping of 886T and 886C alleles. Genomic DNA was isolated from ear punches obtained during animal marking using precipitation with a saturated NaCl solution and then dissolved in sterile water. The concentration of isolated genomic DNA was measured with a NanoDrop 2000 (Thermo Fisher Scientific, USA); genomic DNA was diluted to a concentration of 50 ng/μl. The 886T and 886C alleles were identified by quantitative PCR (qPCR) using an R-402 reagent kit (Syntol, Russia), a forward primer (5′-GTAAGGGACCTCGCATCAGA-3′), and two allele-specific primers: T-allele (5′-CAGCTGGAGGATGTGTCAC<u>A</u>-3′) and C-allele (5′-CAGCTGGAGGATGTGTCAC<u>G</u>-3′). To increase the specificity of detection, we placed A instead of T at position 18 of the allele-specific primers. For allele identification, DNA samples (50 ng) was amplified with the forward primer and one of the allele-specific primers using a CFX96 Touch Real-Time PCR Detection System (Bio-Rad, USA) according to the protocol recommended by the manufacturer (Syntol, Russia): 94°C for 5 min; 40 cycles of 94°C for 15 s, 60°C for 60 s, and 80°C for 2 s (fluorescence measurement). The amount of formed PCR product was determined from the Sybr Green fluorescence. If the allele presents in the DNA sample matched the allele-specific primer, the number of threshold cycle was 24-25; otherwise, it was greater than 28. Each DNA sample was tested 3 times.

Preparation of samples for RT-qPCR and HPLC. To determine the TH activity, TH content, and *Th* gene mRNA level, the midbrain was homogenized in 400 μl of cold 50 mM Tris-HCl (pH 6.0) containing 1 mM dithiothreitol, using a motor driven grinder (Z359971, Sigma-Aldrich, Germany). An aliquot of 100 μl of the homogenate was immediately mixed with 1 ml of ExtractRNA reagent (Eurogene, Russia) for total RNA extraction. The RNA pellet was dissolved in 25 μl of sterile water, treated with RNase-free DNase EM-100 (Biolabmix, Russia) and the optical density was measured using a NanoDrop 2000 spectrophotometer (Thermo Scientific, USA). RNA samples were diluted with sterile water to a concentration of 125 ng/μl and stored at –80°C. The quality of isolated total RNA was assessed by electrophoresis in 1% agarose gel; only samples with clearly visible two ribosomal RNA bands were included in further analysis.

The remaining 300 µl of the homogenate was centrifuged for 15 min at 12,700 rpm (4°C). The supernatant was transferred to clean tubes, and protein concentration was determined by the Bradford method using the Bio-Rad Protein Assay kit (Bio-Rad). The supernatant was stored at –80°C and used for the TH activity and protein assays.

TH activity was assessed using the HPLC method developed by us for determining the activity of TH in brain tissues based on the rate of L-DOPA synthesis*. A 15-µl aliquot of the TH-containing supernatant was incubated for 15 min at 37°C with 0.3 mM L-tyrosine (Sigma-Aldrich), 0.3 mM artificial cofactor 6,7-dimethyl-5,6,7,8-tetrahydropterin (DMPH4, Sigma-Aldrich)**, 0.3 mM *m*-hydroxybenzylhydrazine decarboxylase inhibitor (Sigma-Aldrich), and 5 units of catalase (Sigma-Aldrich) in a final volume of 25 µl. The incubation was stopped by adding 75 µl of 0.6 M $HClO_4$. Protein was precipitated by centrifugation for 15 min at 14,000 rpm. The clear supernatant was diluted two-fold with ultrapure water to reduce the acid concentration to a detector-safe level of 0.3 M. Synthesized L-DOPA was determined by HPLC on a Luna C18(2) column (length, 100 mm; diameter, 4.6 mm; 5 µm particle size, Phenomenex, USA) equipped with a Zorbax SB-C8 guard column (length, 12.5 mm; diameter, 4.6 mm; 5 µm particle size, Agilent, USA) using an LC-20AD chromatograph (Shimadzu Corporation, Japan). The mobile phase (pH 3.2) contained 13.06 g of KH_2PO_4, 200 µl of 0.5 M Na_2EDTA, 300 mg of sodium 1-octanesulfonate, 940 µl of concentrated H_3PO_4, and 130 ml of methanol (13%) in 1 liter. The mobile phase flow rate was 0.6 ml/min. The concentration of synthesized L-DOPA was determined using a DECADE II™ electrochemical detector and a VT-03 glassy carbon electrode (3 mm; Antec, Netherlands). The temperature of the column and the detector was 40°C. Under these conditions, the retention time of L-DOPA on the column was 4 min. The area of the L-DOPA peak was determined with the LabSolution LG/GC v. 5.54 software (Shimadzu Corporation, Japan) using L-DOPA standards (25, 50, and 100 pmol). The activity of TH was expressed in pmol of L-DOPA synthesized per minute per mg protein (measured by the Bradford method).

Assessment of *Th* gene mRNA level was carried out by RT-qPCR. cDNA was synthesized on the isolated RNA using random hexanucleotide primers and R01 kit (Biolabmix, Russia). The content of *Th* gene cDNA was determined by qPCR using R-402 kit (Syntol, Russia) and forward (5′-CCGTACACCCTGGCCATTGATG-3′) and reverse (5′-ATGAAGGCCAGGAGGAATGCAGG-3′) primers specific to the nucleotide sequence of the mouse *Th* gene exon in the following regime: 94°C for 5 min; 40 cycles of 94°C for 15 s, 64°C for 60 s, 80°C for 2 s (fluorescence measurement) [31]. The *Polr2a* housekeeping gene was used for normalization after amplification with the forward (5′-GTTGTCGGGCAGCAGAATGTAG-3′) and reverse (5′-TCAATGAGACCTTCTCGTCCTCC-3′) primers specific to the nucleotide sequence of the mouse *Polr2a* gene exon in the following regime: 94°C for 5 min, 40 cycles of 94°C for 15 s, 63°C for 60 s, 80°C for 2 s (fluorescence measurement) [32]. Mouse genomic DNA standards (25, 50, 100, 200, 400, 800, 1600, and 3200 copies of mouse genomic DNA per 1 µl) were used for the threshold cycle calibration. The level of the *Th* gene expression was expressed as the number of copies of cDNA of this gene per 100 copies of *Polr2a* gene cDNA [33].

The determination of protein quantity via by Western blotting was conducted as described in [34]. Proteins were separated by SDS-PAGE in 10% PAG (10 µg of total protein per lane) and transferred onto a membrane. The membrane was stained with rabbit polyclonal anti-TH antibodies (1 : 500; ab112, Abcam, UK). Rabbit polyclonal antibodies to GAPDH (1 : 2000, ab9485, Abcam) and mouse monoclonal antibodies to GAPDH (1 : 10,000, HC301, TransGen, China) served as internal controls. The content of TH protein (60 kDa) was normalized to the content of GAPDH (37 kDa) and expressed in relative units.

Statistical analysis of the results was performed using Statistica 9.0 (StatSoft, Inc.). The levels of the *Th* gene mRNA, TH protein content, and TH activity in the midbrain were expressed as mean ± SEM and analyzed using one-way (interstrain differences) and two-way (F2) ANOVA followed by the intergroup comparison using the Fisher's least significant difference (LSD) method. The segregation ratio of the TT, TC, and CC genotypes among the F2 intercrosses was tested against the 1 : 2 : 1 ratio using the Pearson χ^2-test. The significance level was set at 0.05.

RESULTS

Comparison of the *Th* gene expression levels, TH protein content, and TH activity in the midbrain of C57BL/6 (886T), DBA/2 (886T), and CAST (886C) male mice. Mice from the three strains exhibited differences in the TH activity in the midbrain [$F(2,14) = 21.68$, $p < 0.001$]. Thus, the TH activity in the midbrain of CAST mice was significantly higher than in C57BL/6 ($p < 0.001$) and DBA/2 mice ($p < 0.001$) (Fig. 1). However, no differences between the strains were found in the levels of *Th* gene transcripts [$F(2,13) = 2.67$, $p = 0.11$]

* Since no traces of endogenous L-DOPA were detected in the supernatant, no tissue control samples were used.

** Because the retention time of L-DOPA (4 min) coincided with the retention times of the 5,6,7,8-tetrahydrobiopterin and 6-methyl-5,6,7,8-tetrahydropterin cofactors, only DMPH4 was used as a cofactor.

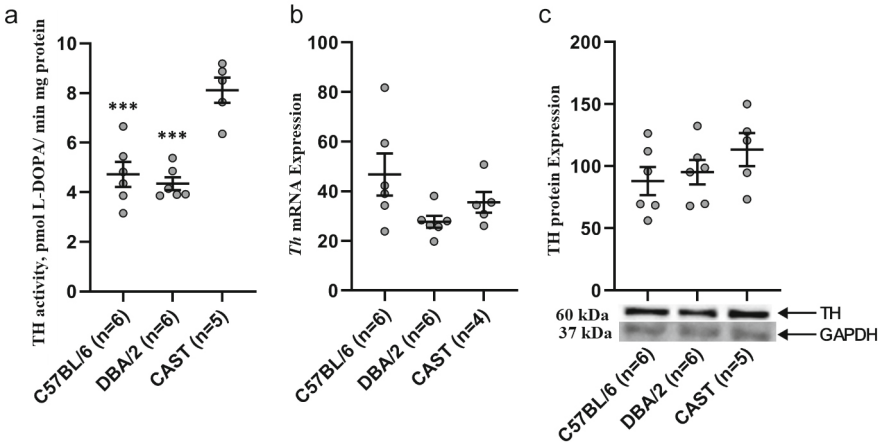

Fig. 1. TH activity (a), *Th* gene mRNA level (b), and TH protein content (c) in the midbrain of C57BL/6 (TT), DBA/2 (TT), and (CAST) (CC) mouse strains. Individual values are presented along with means ± SEM. Expression of the *Th* gene was normalized to the *Polr2a* gene expression; TH protein levels were normalized to the GAPDH levels; *** $p < 0.001$ vs. CAST mice.

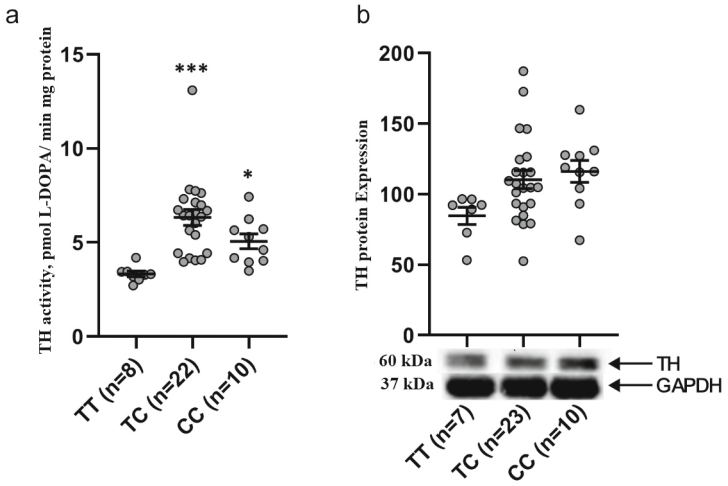

Fig. 2. TH activity (a) and protein levels (b) in the midbrain of F2 intercrosses with the TT, TC, and CC genotypes. The data for males and females of the same genotype are combined. Individual values are presented along with means ± SEM. The TH protein level was normalized to the GAPDH protein level. * $p < 0.05$, *** $p < 0.001$ vs. TT.

and TH protein content [F(2,14) = 1.23, $p = 0.32$] in the midbrain (Fig. 1).

TH protein level and TH activity in the midbrain of F2 intercrosses. The number of males and females with the TT, TC, and CC genotypes among the F2 intercrosses (42 animals) is presented in the table. For statistical analysis, the data for males and females with the same genotype were combined. The obtained values closely correspond to the expected distribution of 1 : 2 : 1 ($\chi^2(1) = 0.805$, $p > 0.05$).

Two-way ANOVA indicated a substantial contribution of the "genotype" factor to the TH activity in the midbrain of F2 animals [F(2,34) = 9.47, $p < 0.001$]. However, no influence of the "gender"› factor [F(1,34) < 1] and interaction between the factors [F(2,34) = 1.74, $p = 0.19$] on the TH activity in the midbrain of F2 mice was detected. This allowed us to combine the values obtained for males and females of the same genotype in order to increase the sample size for each genotype. The activity of TH in the midbrain was lower in individuals with the TT genotype compared to those with the TC ($p < 0.001$) and CC ($p = 0.02$) genotypes (Fig. 2). No differences between the genotypes were observed in the TH protein levels [F(2,38) = 2.22, $p = 0.12$] (Fig. 2).

Distribution of the TT, TC, and CC genotypes among the males and females from the F2 intercross offspring obtained by crossing F1 hybrids (C57BL/6 × CAST)

Genotype	Males	Females	Males + Females
TT	5	3	8
TC	13	10	23
CC	5	6	11

DISCUSSION

This study provides the first experimental evidence of the influence of the prevalent C886T mutation in the *Th* gene on the TH activity in the midbrain of laboratory mice. The midbrain was chosen for examination because TH is expressed in the bodies of dopaminergic and noradrenergic neurons located in the midbrain and then distributed via axonal transport to other brain structures receiving projections from these neurons. First, we compared the activity of TH in the midbrain of mice with the TT (C57BL/6 and DBA/2) and CC (CAST) genotypes. CAST mice demonstrated an increased activity of this enzyme compared to TT mice. Although the C886T mutation is located in the TH catalytic domain and theoretically cannot influence expression of the *Th* gene, we could not rule out that the increased enzyme activity observed in CAST mice may be due to some unknown genetic factors upregulating expression of the *Th* gene and/or TH protein level. To test this assumption, we measured expression of the *Th* gene and TH protein levels in the midbrain of C57BL/6, DBA/2, and CAST mice and found no differences in these parameters. It can be hypothesized that the differences in the enzyme activity among these three strains are not associated with the regulation of the *Th* gene expression and/or TH protein stability.

To study a linkage between the 886C and 886T alleles with a high and low TH activity, we produced F2 intercrosses with different genotypes (TT, TC, and CC) and investigated the relationship between the TH activity and genotype of these mice. The distribution of the TT, TC, and CC genotypes among F2 corresponded well to the expected Mendelian segregation of 1 : 2 : 1, which, in turn, may serve as an evidence of the absence of the effect of the C886T mutation on mouse survival. The TH activity in the brains of F2 mice with the TC and CC genotypes was higher than in the mice with the TT genotype. This result not only demonstrated the linkage of high enzyme activity with the 886C allele but also indicated that the C886T polymorphism in the *Th* gene is a major genetic factor determining the TH activity in mouse brain. Indeed, the influence of this factor on the TH activity is so significant that segregation by a large number of mutations, which distinguish C57BL/6 and CAST mice but theoretically may affect the TH activity, could not mask the effect of the C886T mutation. At the same time, no linkage of this mutation with the TH protein level was found. This result can be considered as an additional experimental evidence that the C886T polymorphism does not affect expression of the TH protein and/or its stability.

A question arises regarding possible molecular mechanisms underlying reduction in the TH activity caused by the R278H substitution. The most obvious mechanism involves a decrease in the level of active enzyme due to the reduced stability and lifetime of the mutant protein. In this case, the TH activity in heterozygotes should be equal to the arithmetic mean of TH activities in the two homozygotes. Recently, it has been demonstrated that the C1473G mutation in the mouse *Tph2* gene, resulting in the P447R substitution in TPH2, reduces the stability and the lifetime of the enzyme and, therefore, decreases the number of active enzyme molecules [35]. It has been shown that the TPH2 activity in the brain of 1473CG heterozygotes is equal to the arithmetic mean of the enzyme activities in homozygous 1473CC and 1473GG mice [36]. We found no differences in the TH activity in the mice with the 886TC and 886CC genotypes, indicating complete dominance of the 886C allele. This inheritance pattern contradicts the hypothesis of the influence of the C886T mutation on the level of TH protein. Since TH, like all aromatic amino acid hydroxylases, is a tetramer consisting of four subunits, it can be assumed that the presence of a "normal" subunit (allele 886C) in heterozygous animals somehow corrects the negative effect of the "defective" subunit (allele 886T) on the activity of the entire tetramer. However, elucidating the exact mechanism underlying the effect of the R278H substitution on the TH activity requires special investigation using recombinant TH molecules.

Since TH is a key enzyme in the synthesis of DA, a neurotransmitter that regulates motor activity, it can be assumed that the 886C allele is associated with the increased levels/metabolism of DA and motor activity. However, at present, it is impossible to verify these assumptions due to a significant uncontrolled genetic variability of these traits. To study the association of the C886T mutation with DA levels and motor activity, it is necessary to significantly reduce the proportion of uncontrolled variations in these traits by conducting at least 10 consecutive backcrosses of heterozygous individuals (886CT) onto C57BL/6 (886TT) mice.

In this pilot study, we have undoubtedly demonstrated that the C886T polymorphism is a key factor determining the activity of TH in the mouse brain. The C886T mutation is the first naturally occurring common mutation in the mouse *Th* gene for which the effect on the enzyme activity has been shown. This opens up new possibilities for experimental modeling of the influence of functional mutations in the *Th* gene on the physiological functions under normal and pathological conditions.

Contributions. K.A.V. developed the concept and supervised the study; A.I., R.I.A., K.A.V., and M.V.S. conducted the experiments; B.D.V. and K.A.V. discussed the results; K.A.V. wrote the manuscript; B.D.V. and K.A.V. edited the manuscript.

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Ethics declarations. All procedures were conducted following international guidelines for the care and use of laboratory animals (National Institute of Health Guide for the Care and Use of Laboratory Animals, NIH Publications No. 80023, 1996) and the decree of the Ministry of Health of the Russian Federation dated 01.04.2016 No. 119n "On the Approval of the Rules of Good Laboratory Practice" (registered on 15.08.2016 No. 43232). Animal housing conditions and experimental procedures were approved by the Ethics Committee of the Institute of Cytology and Genetics, Siberian Branch of the Russian Academy of Sciences. The authors of this work declare that they have no conflicts of interest.

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