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Report

Loss-of-Function Mutation in Tryptophan Hydroxylase-2 Identified in Unipolar Major Depression

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Summary

Dysregulation of central serotonin neurotransmission has been widely suspected as an important contributor to major depression. Here, we identify a (G1463A) single nucleotide polymorphism (SNP) in the rate-limiting enzyme of neuronal serotonin synthesis, human tryptophan hydroxylase-2 (*hTPH2*). The functional SNP in *hTPH2* replaces the highly conserved Arg441 with His, which results in ~80% loss of function in serotonin production when hTPH2 is expressed in PC12 cells. Strikingly, SNP analysis in a cohort of 87 patients with unipolar major depression revealed that nine patients carried the mutant (1463A) allele, while among 219 controls, three subjects carried this mutation. In addition, this functional SNP was not found in a cohort of 60 bipolar disorder patients. Identification of a loss-of-function mutation in hTPH2 suggests that defect in brain serotonin synthesis may represent an important risk factor for unipolar major depression.

Introduction

The neurotransmitter serotonin (5-hydroxytryptamine [5-HT]) has been implicated in a variety of physiological functions in both peripheral and central nervous systems (CNS) (Lucki, 1998; Veenstra-VanderWeele et al., 2000; Malhotra et al., 2004). Many neuropsychiatric disorders, such as depression (Lesch, 2004; Malhotra et al., 2004), schizophrenia (Veenstra-VanderWeele et al., 2000), autism (Veenstra-VanderWeele and Cook, 2004), aggression and suicidal behavior (Arango et al., 2003), and attention-deficit/hyperactivity disorder (ADHD) (Gainetdinov et al., 1999; Quist and Kennedy, 2001), are considered to be related to dysfunction in serotonergic neurotransmission in the CNS. Therefore, the brain 5-HT system is a major target for tricyclic antidepressants, selective serotonin reuptake inhibitors (SSRIs), monoamine oxidase inhibitors (MAOIs), and psychostimulants (Lucki, 1998; Gainetdinov and Caron, 2003; Blier and Abbott, 2001; Gordon and Hen, 2004; Malhotra et al., 2004). Numerous studies have suggested associations between various neuropsychiatric disorders and genes that modulate central serotonergic neurotransmission, such as the 5-HT transporter (SERT, 5-HTT) (Lesch et al., 1996; Caspi et al., 2003; Murphy et al., 2003), 5-HT receptors (Lucki, 1998; Arango et al., 2003; Bonasera and Tecott, 2000; Gordon and Hen, 2004; Malhotra et al., 2004), and monoamine oxidases (Shih et al., 1999), as well as the rate-limiting enzymes in 5-HT synthesis, tryptophan hydroxylases (TPH1 and TPH2) (Arango et al., 2003; Harvey et al., 2004).

TPH1 and TPH2 belong to the superfamily of aromatic amino acid hydroxylases, which also includes tyrosine hydroxylase (TH) and phenylalanine hydroxylase (PAH). These four enzymes share considerable structural similarity and require the same cofactors for function (Fitzpatrick, 1999; Jiang et al., 2000). In contrast to TPH1, which controls most of peripheral 5-HT synthesis (Cote et al., 2003; Walther et al., 2003), TPH2 was recently discovered and found to be neuronal specific and predominantly expressed in brain serotonergic neurons originating from raphe nuclei (Walther et al., 2003; Patel et al., 2004). We have previously identified a functional (C1473G) single nucleotide polymorphism (SNP) which results in the replacement of proline with arginine at position 447 in mouse Tph2 (Zhang et al., 2004). Expression of mutant Tph2 (P447R) in PC12 cells revealed an ~55% decrease in 5-HT levels as compared to wild-type Tph2. Moreover BALB/cJ and DBA/2 inbred mice carrying the homozygous mutant (1473G) alleles showed 50%–70% reduction in the rate of synthesis of cortical and striatal 5-HT accompanied by ~40% reduction in 5-HT tissue content when compared to C57Bl/6 and 129X1/SvJ inbred mice carrying homozygous wild-type (1473C) alleles. Interestingly, these strains of mice display significantly different behaviors and responses to antidepressants (Lucki et al., 2001). These observations provided direct evidence for the fundamental role of Tph2 in brain 5-HT synthesis (Zhang et al., 2004) and raised the interesting possibility that similar mutations in human *TPH2* (*hTPH2*) may affect brain 5-HT homeostasis in certain neuropsychiatric conditions.

Here we report the identification of a functional (G1463A) SNP in *hTPH2*, which replaces a highly conserved Arg441 with His and results in an ~80% reduction in activity when expressed in cell culture systems. Furthermore, we identified nine subjects carrying this functional SNP in a cohort of 87 unipolar major depression patients. These data provide a potential molecular mechanism for aberrant 5-HT function in neuropsychiatric disorders.

Results

In order to explore whether functional SNP(s) could be identified in *hTPH2*, we screened 11 exons of *hTPH2* by sequence analysis in 48 genomic DNA samples randomly selected from a cohort of ~300 individuals from a study of psychosocial and behavioral risk in lower

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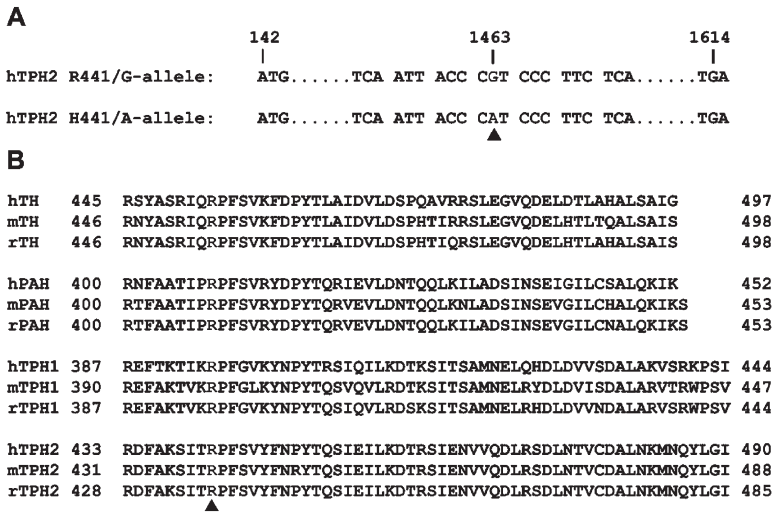

Figure 1. (G1463A) SNP in *hTPH2* and Partial Sequence Alignment of TH, PAH, TPH1, and TPH2

(A) The (G1463A) polymorphism in *hTPH2*. The G/A polymorphism is highlighted with an arrowhead. Nucleotide numbers are shown for the start and stop codons of *hTPH2* as well as the site of polymorphism. (B) Sequence alignment of the C-terminal regions of tyrosine hydroxylase (TH), phenylalanine hydroxylase (PAH), TPH1 and TPH2 in human (h), mouse (m), and rat (r). The highly conserved arginine residues are highlighted with an arrowhead. Numbers indicate positions of amino acids.

social-economical characteristic groups (Williams et al., 2003). Among these samples, one novel (G1463A) SNP in the coding region of *hTPH2* was identified, which replaced Arg441 (CGT) with His (CAT) (Figure 1A). Sequence alignment among TH, PAH, TPH1, and TPH2 in human, mouse, and rat revealed that Arg441 is highly conserved among members of this family (Figure 1B). Interestingly, an R408W mutation at the corresponding position in PAH (Figure 1B) abolishes PAH enzyme activity and leads to phenylketonuria (PKU), the most severe form of hyperphenylalaninemia. Furthermore, this R408W mutation has been identified in ~10% of the PKU patients, representing the most prevalent pathogenic mutation in PAH (Scriver et al., 2003).

To analyze the functional consequence of the R441H mutation in hTPH2 on 5-HT synthesis, we transfected either hemagglutinin (HA)-tagged wild-type (wt) or R441H mutant hTPH2 into pheochromocytoma (PC12) cells and measured 5-HT levels as described (Xu et al., 2000; Zhang et al., 2004). Previously, we established a direct correlation between 5-HT levels measured in PC12 cells transfected with *Tph2* genetic variants and the rate of 5-HT synthesis in the brains of inbred mice carrying these *Tph2* variants (Zhang et al., 2004). While here we did not measure TPH2 enzyme activity directly, we found that 5-HT levels in PC12 cells expressing the R441H mutant were ~80% lower as compared to PC12 cells expressing wt hTPH2 (Figure 2A), indicating a severe loss of function in the ability of the mutant enzyme to synthesize 5-HT. Because humans may carry heterozygous (1463G/1463A) or homozygous (1463A/1463A) alleles and since oligomerization is required for the activity of this family of enzymes (Yang and Kaufman, 1994; Fitzpatrick, 1999), we tested whether the R441H mutant could exert a dominant-negative effect and/or affect oligomerization of hTPH2. We first measured 5-HT levels in PC12 cells transfected with plasmids encoding wt hTPH2, R441H mutant hTPH2, or the combination of both plasmids. These experiments revealed an additive, not dominant-negative, effect of the R441H mutant on 5-HT synthesis (Figure 2B). We then performed immunoprecipitation studies in PC12 cells expressing wt-wt, wt-R441H, or R441H-R441H hTPH2 tagged with either FLAG or HA epitopes. As shown in Figure 2C, the R441H mutant hTPH2 was able to form homo- (R441H-R441H) or hetero- (wt-R441H) oligomers to a similar extent as compared to wt hTPH2, suggesting that the R441H mutation does not affect dimerization of the subunits. In fact, the equivalent R408W mutation in PAH has been found to affect protein folding, causing a complete loss of protein expression and enzymatic activity as compared to wt PAH (Pey et al., 2003). However, the arginine to histidine mutation in hTPH2 did not markedly affect hTPH2 protein expression, at least when expressed in PC12 cells (Figure 2A, lower panel), indicating that it may result in a perturbation that leads to a milder attenuation of function than the mutant PAH.

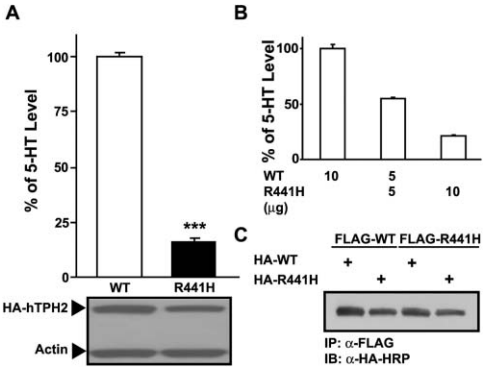

Figure 2. Biochemical Properties of hTPH2 (R441H) Mutant

(A) 5-HT levels in PC12 cells expressing mutant R441H hTPH2 were lower than those in PC12 cells expressing wt hTPH2 ($n = 8$ experiments), whereas no differences in dopamine levels were observed. 5-HT levels were normalized to the respective hTPH2 expression levels (mutant enzyme expression was ~75% of wt levels). (B) Additive, not dominant-negative, effect of R441H hTPH2. 5-HT levels were measured in PC12 cells transfected with plasmids expressing wt (10 μg), wt (5 μg) + R441H (5 μg), or R441H (10 μg) ($n = 4$ experiments). (C) Immunoprecipitation of hTPH2. PC12 cells transfected with wt or mutant R441H hTPH2 were subjected to immunoprecipitation (IP) with anti(α)-FLAG antibody and immunoblotted (IB) with anti(α)-HA antibody conjugated to horse radish peroxidase (HRP). (All data are presented as means ± SEMs. Statistical significance of all data presented is analyzed by Student's t test: ***$p < 0.001$.)

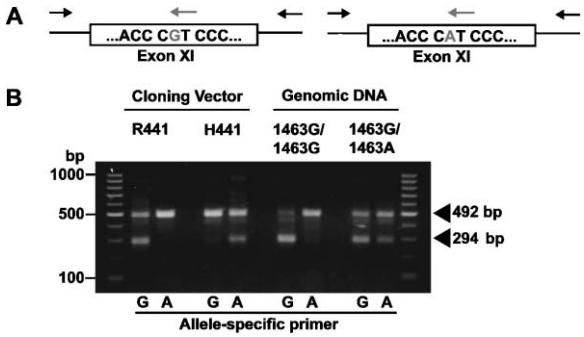

Figure 3. Allele-Specific Genotyping of (G1463A) SNP in *hTPH2*

(A) The (1463G) or (1463A) allele in *hTPH2*. Positive control primers and allele-specific primers for genotyping are shown as black and gray arrows, respectively. (B) Genotyping of (G1463A) polymorphism in human genomic DNA samples and cloning vectors of partial genomic DNA carrying either (1463G) or (1463A) allele. PCR products for positive control (492 bp) and allele-specific products (294 bp) are indicated with arrowheads.

Based on the studies in PC12 cells, we then searched for this functional (1463A) allele in human populations with 5-HT-related psychiatric disorders. First, we established a PCR-based genotyping method using a modified amplification refractory mutation system (ARMS)-PCR (Ye et al., 2001) with one allele-specific primer and two positive control primers flanking the site of the (G1463A) SNP (Figure 3A) (Zhang et al., 2004). As shown in Figure 3B, only G-allele-specific primers gave rise to an allele-specific PCR product (294 bp) in a genomic DNA sample carrying homozygous wt 1463G/1463G alleles, while both G- and A-allele-specific PCR products were detected in a genomic DNA sample carrying heterozygous 1463G/1463A alleles. To further validate this allele-specific PCR genotyping approach, we amplified genomic DNA using the two positive control primers mentioned above to clone the PCR fragment (492 bp) into a cloning vector and then confirmed by sequence analysis. Cloning vectors carrying either the (1463G) or (1463A) allele were then used as templates to confirm the specificity of the genotyping method (Figure 3B).

We next used ARMS-PCR to screen a cohort of unipolar major depression patients ($n = 87$), a cohort of bipolar disorder patients ($n = 60$), and control subjects ($n = 219$) for the (G1463A) SNP in *hTPH2*. Using this approach, we identified nine unipolar major depression patients and three control subjects who carried the mutant (1463A) allele. No carriers of the mutant allele were found among bipolar disorder patients (Table 1). The frequency of the mutant (1463A) allele in unipolar major depression patients versus control subjects was statistically significant ($\chi^2 = 13.31$, $p < 0.001$). No statistical difference in the frequency of this mutant allele was present between bipolar disorder patients and control subjects. Genotypes of samples identified carrying the (1463A) allele and additional samples from the control subjects ($n = 24$) were then confirmed by sequence analysis of Exon XI of *hTPH2*. Among the nine unipolar major depression patients identified, six patients carried heterozygous (1463G/1463A) alleles and three patients carried homozygous (1463A/1463A) alleles (Table 1). Furthermore, seven of them had a family history of mental illness or drug and alcohol abuse, six had suicidal ideation or attempt, four had generalized anxiety symptoms, and interestingly, seven patients exhibited lack of responsiveness to SSRI, while the other two patients were responsive only to highest doses of SSRI (Table 1). The three control subjects carrying the 1463A allele were not diagnosed as having unipolar major depression according to the criteria of the Diagnostic and Statistical Manual of Mental Disorders (DSM-IV), but they displayed clinical symptoms of comorbid conditions. One of the three control subjects carrying homozygous mutant (1463A) alleles had generalized anxiety symptoms, while the other two carrying heterozygous (1463G/1463A) alleles had mild depression and a family history of mental illness or drug and alcohol abuse (Table 1), suggesting a potentially higher susceptibility for certain neuropsychiatric disorders in the presence of the mutant (1463A) allele.

Discussion

In this study, we report the identification of a mutation in human TPH2 that results in ~80% loss of function in the activity of the enzyme. Moreover, nine subjects from a cohort of 87 unipolar major depression patients were found to carry the mutant (1463A) allele. These findings suggest that deficiency in brain 5-HT synthesis may be an important risk factor for unipolar major depression.

We previously characterized a functional (C1473G) SNP in mouse Tph2 that exhibited an ~55% reduction in activity when expressed in PC12 cells and an ~50% decrease in the rate of brain 5-HT synthesis and tissue content in mice carrying the mutant allele (Zhang et al., 2004). The highly conserved Pro residue corresponding to this functional (C1473G) SNP in mice is Pro449 in human TPH2, which is in close proximity to the (G1463A) SNP (Arg441) reported in the present study, suggesting that this region of hTPH2 is critical for enzyme function. Importantly, when the R441H mutant hTPH2 was expressed in PC12 cells, 5-HT levels were reduced by ~80% as compared to wt hTPH2, indicating a more severe loss-of-function phenotype as compared to the mouse Tph2 (P447R) mutant. Therefore, this human R441H mutation in hTPH2 may exert an even more profound effect on brain 5-HT homeostasis.

Unipolar major depression is a brain disorder with 2%–19% population prevalence and 40%–70% heritability with complex, polygenic, and epistatic genetic factors (Lesch, 2004). Previous linkage studies have provided evidence that there is a sex-specific disposition locus for major depression on chromosome 12q22–12q23.2, confirming chromosome 12q as one of the candidate regions for neuropsychiatric disorder susceptibility (Abkevich et al., 2003). Human *TPH2* is located on chromosome 12q21.1 and, as we report here, a severe loss-of-function R441H mutation in hTPH2 has been identified in 9 out of 87 unipolar major depression patients tested. Our results that 78 unipolar major depression patients in this study carry wt (1463G) alleles are not surprising, because depression is heterogeneous in origin and genes other than *hTPH2* or other functional SNP(s) in *hTPH2* may also contribute to vulnerability to

Table 1. Summary of Subjects Carrying Functional (G1463A) SNP in *hTPH2*

Unipolar Major Depression Patients	Patient I.D.	Sex	Age	Allele	Family History	Suicidality	Anxiety	SSRI Response	Note
n = 87	1202	F	72	A/A	−	+	−	+	Sertraline 200 mg
	1294	M	80	G/A	+	+	−	−	
	1496	M	74	A/A	−	−	+	+	Sertraline 100 mg
	1745	M	71	G/A	+	−	+	−	
	1747	M	82	G/A	+	+	−	−	
	1839	F	69	G/A	+	−	−	−	
	1851	F	65	G/A	+	+	−	−	
	1902	F	77	A/A	+	+	+	−	
	1975	M	64	G/A	+	+	+	−	
Control Subjects									
n = 219	1174	F	76	A/A	−		+		
	1541	F	80	G/A	+		−		mild depression
	1996	F	75	G/A	+		−		mild depression

Family history: family history for mental illness, drug, and alcohol abuse. Suicidality: suicidal ideation or attempt. Anxiety: three or more anxiety symptoms. All patients listed above were Caucasians, except subject 1541, who was African American. The mutant (1463A) allele was not found in a cohort of 60 bipolar disorder patients.

unipolar major depression. In fact, emerging evidence indicates that genetic variance plays a critical role in different clinical responses to psychotropic drugs (Malhotra et al., 2004). For example, a growing number of studies have suggested a relationship, although with conflicting results, between a polymorphism in the promoter region of SERT (Lesch et al., 1996) and efficacy of SSRI to treat depression (Malhotra et al., 2004). Intriguingly, in our study, reduced responsiveness to SSRI in unipolar major depression patients was found in subjects carrying the functional (G1463A) SNP in *hTPH2*, suggesting a role of brain 5-HT synthesis in the efficacy of SSRI treatment. It will be also important in future studies to examine whether loss-of-function mutations in hTPH2 may play a role in the paradoxical adverse effects in response to SSRI treatment, such as suicidality (March et al., 2004) and SSRI-exacerbated mania and psychosis (Malhotra et al., 2004).

The three control subjects carrying the (1463A) allele in *hTPH2* were not included in the cohort of unipolar major depression patients based on criteria of DSM-IV diagnosis. However, the fact that these subjects had generalized anxiety symptoms or mild depression and a family history of mental illness or drug and alcohol abuse further underscores the complexity and polygenetic nature of neuropsychiatric disorders. It is possible that functional SNPs in *hTPH2*, including this (G1463A) SNP, may be present as an important predisposing factor in the pathophysiology of several neuropsychiatric disorders. It is also noteworthy that the functional (G1463A) SNP was not found in a small cohort of bipolar disorder patients in this study. A recent genetic association study using a bipolar disorder French-Canadian pedigree has identified SNPs in noncoding regions of *hTPH2*, suggesting a potential role of *hTPH2* in bipolar disorder (Harvey et al., 2004). Therefore, further studies will be needed to elucidate the relationship between hTPH2 function and bipolar disorder.

Although (G1463A) is the first functional SNP identified in human *TPH2*, it is likely that additional functional SNP(s) could be found in *hTPH2*. In support of this idea, numerous studies have shown that there are more than 400 mutations in the closely related enzyme PAH that cause various degrees of hyperphenylalaninemia, and over 300 mutations identified are missense mutations in the coding regions (Scriver et al., 2003). In fact, we recently found two additional SNPs in the coding regions of *hTPH2* that are currently under investigation (data not shown).

In conclusion, our present and previous biochemical studies in human and mice (Zhang et al., 2004) provide a potential molecular mechanism underlying dysfunction in 5-HT neurotransmission in the brain. Clearly, further large-scale genetic studies are needed to confirm these observations and to investigate in detail the inheritance and penetrance of this functional (G1463A) SNP in *hTPH2* in unipolar major depression. Furthermore, detailed kinetic and biochemical characterization of the mutant enzyme as well as direct measurements of brain 5-HT status in affected subjects will ultimately be needed. It will also be of interest to test how this mutation may interact with other genetic alterations previously associated with depression. Finally, it will be worth exploring the presence of this and other functional mutations of *hTPH2* in other neuropsychiatric conditions, such as generalized anxiety disorder, schizophrenia, suicidality, autism, ADHD, and drug abuse.

Experimental Procedures

Research Samples

Subjects in the original cohort of ~300 healthy adults were evaluated to rule out neuropsychiatric disorders. They were recruited from the community for participating in a study of psychosocial and behavioral risk in lower social-economical characteristic groups (Williams et al., 2003). Subjects investigated in detail in this study were participants in the NIMH sponsored Conte Center for the Neuroscience of Depression at Duke University. Subjects gave informed consent prior to entry into the study. The study was approved by the Institutional Review Board. The interview procedures used a structured interview Duke Depression Evaluation Schedule (DDESE). This interview uses the Diagnostic Interview Schedule (DIS) (Bosworth et al., 2002). At baseline enrollment, 87 unrelated subjects had a DSM-IV diagnosis of unipolar major depression and were 60 years of age or older; they did not have to be currently depressed to participate in this study. These subjects were not diagnosed with any other

psychiatric disorders. Sixty additional subjects had a DSM-IV diagnosis of bipolar disorder. The control group consisted of 219 subjects without any reported personal history of mental illness, including unipolar major depression, bipolar disorder, or any other major neuropsychiatric disorders or substance abuse. Among the total 366 subjects tested in this study, 331 were Caucasians, 28 were African Americans, 6 were Asians, 1 was Hispanic, and 41% were male subjects. Blood was collected from these subjects, and DNA was extracted.

Sequencing Analysis

Primers for sequencing the 11 exons of the human *TPH2* gene were generated using ExonPrimer (http://ihg.gsf.de/ihg/ExonPrimer.html), a perl script for the design of intronic primers for PCR amplification of exons. RepeatMasker and BLAT (Kent, 2002) algorithms were run on the input sequences to ensure primer specificity. Primers were obtained from IDT (Coralville, IA). PCR and cycle sequencing (cycle sequence kit version v1.1, Applied Biosystems) were performed on the MJ Tetrad 225 (Waltham, MA). Sequence was generated on an ABI 3730 (Applied Biosystems). Sequence data files were uploaded into the PolyPhred program (Nickerson et al., 1997) for quality analysis and SNP identification.

Genotyping and PCR Conditions

ARMS-PCR genotyping was performed on human genomic DNA samples and cloning vectors carrying either the (1463G) or (1463A) allele. The PCR reactions were carried out with primers for positive control (hOuter/Forward [5′-ATGTGTGAAAGCCTTTGACCCAAAGACA] and hOuter/Reverse [5′-TGCGTTATATGACATTGACTGAACTGCT]) plus either G-allele- (5′-TAGGGATTGAAGTATACTGAGAAGGCAC) or A-allele- (5′-TAGGGATTGAAGTATACTGAGAAGGCAT) specific primers. The PCR conditions were as follows: 1 cycle (5 min at 94°C) and 40 cycles (30 s at 94°C, 30 s at 63°C, 30 s at 72°C) using *Taq* DNA polymerase (Fisher). Genotyping and sequencing experiments were carried out with investigators blinded to the clinical history of patients.

Neurochemical Assessments

To determine dopamine and 5-HT levels in PC12 cells, five million cells were homogenized in 200 μl 0.1 M $HClO_4$, centrifuged, and filtered. Supernatants were then analyzed by HPLC using electrical chemical detection (Xu et al., 2000; Zhang et al., 2004).

GenBank Accession Numbers

TPH1 (human, NM_004179; mouse, NM_009414; rat, P09810), *TPH2* (human, NM_173353; mouse, NM_173391; rat, NM_173839), *TH* (human, NM_000360; mouse, NM_009377; rat, NM_012740), *PAH* (human, NM_000277; mouse, NM_008777; rat, NM_012619).

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