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RHESUS MONKEY TRYPTOPHAN HYDROXYLASE-2 CODING REGION HAPLOTYPES AFFECT mRNA STABILITY

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Abstract—Tryptophan hydroxylase-2 (TPH2) synthesizes neuronal 5-HT and its genetic variance is associated with numerous behavioral traits and psychiatric disorders. This study characterized the functional significance of two nonsynonymous single nucleotide polymorphisms (SNPs) (C74A and G223A) in rhesus monkey *TPH2* (m*TPH2*). Four haplotypes of m*TPH2* were cloned into pcDNA3.1 and stably transfected into PC12 cells. The levels of mTPH2 mRNA and protein were assessed by quantitative real-time PCR and Western blot, respectively, while the intracellular 5-HT was measured by enzyme-linked immunosorbent assay (ELISA). The variant A-A haplotype showed significantly higher levels of mTPH2 mRNA and protein, as well as significantly higher 5-HT production than the wild-type C-G haplotype, while the other two variant haplotypes (C-A and A-G) also tended to produce more 5-HT than C-G haplotype when stably expressed in PC12 cells. Both C74A and G223A were predicted to change mRNA secondary structure, and analysis of the mRNA stability showed that the wild-type C-G haplotype mRNA degrades more quickly than mRNAs of the mutant m*TPH2* haplotypes in both stable PC12 and transient HEK-293 cells. This study demonstrates that nonsynonymous SNPs in m*TPH2* can affect mRNA stability. Our findings provide an additional mechanism by which nonsynonymous SNPs affect TPH2 function, and further our understanding of TPH2 gene expression regulation.

Key words: TPH2, 5-HT, nonsynonymous polymorphisms, gene expression, nonhuman primate.

5-HT is a major central neurotransmitter involved in many brain functions, and dysregulation of the central 5-HT system contributes to a wide spectrum of psychiatric disorders, including depression, autism, schizophrenia, drug abuse and addiction, suicide, and attention-deficit/hyperactivity disorder (ADHD). Accordingly, a number of pharmaceuticals that target the 5-HT system are widely used for the treatment of various psychiatric disorders. The synthesis of 5-HT is initiated by the hydroxylation of the amino acid (AA) tryptophan, which is the rate-limiting step catalyzed by tryptophan hydroxylase (TPH). Two isoforms of TPH (TPH1 and TPH2) have been identified to date, among which TPH2 is exclusively expressed in the brain in a circadian rhythm while TPH1 is primarily expressed in peripheral tissues (Walther and Bader, 2003; Walther et al., 2003; Côté et al., 2003; Zhang et al., 2004). Thus, genetic variations affecting the gene expression or enzymatic activity of TPH2 may alter 5-HT neurotransmission and thereby influence behavioral traits, drug response, and vulnerability to psychiatric disorders.

Previous studies of our laboratory and other groups have demonstrated that specific polymorphisms in the 5′-regulatory and coding regions of human *TPH2* (h*TPH2*) can affect gene expression or protein enzymatic activity (Chen et al., 2008; Scheuch et al., 2007; Lin et al., 2007; Cichon et al., 2008). In particular, a single nucleotide polymorphism (SNP) (G-703T) has been reported to predict amygdala responsiveness (Brown et al., 2005; Canli et al., 2005), emotional processing (Herrmann et al., 2007), personality traits and disorders related to emotional dysregulation (Gutknecht et al., 2007; Reuter et al., 2007b). Moreover, there have been numerous studies linking h*TPH2* variants to various psychiatric diseases, such as major depression (Zhou et al., 2005), suicidality (Lopez et al., 2007), autism (Coon et al., 2005), ADHD (Sheehan et al., 2005), and drug addiction (Reuter et al., 2007a; Nielsen et al., 2008). In addition, the antidepressant response to the selective 5-HT reuptake inhibitors (SSRIs) in patients is associated with h*TPH2* genetic variance (Tzvetkov et al., 2008). Interestingly, a functional nonsynonymous SNP (C1473G or P447R) that causes striking loss of enzymatic activity was identified in mouse *TPH2* (Zhang et al., 2004), making this rodent a useful experimental model for TPH2 research. This SNP in mouse *TPH2* differentiates not only the aggressive behavior (Kulikov et al., 2005), but also the antidepressant effect of the SSRI citalopram (Crowley et al., 2005; Cervo et al., 2005).

Rhesus monkeys share genetic, physiological, and behavioral similarities with humans, and therefore have an advantage over rodents to model the pathophysiology of human diseases, especially in the field of neuroscience and neuropsychiatry. A working hypothesis in our laboratory is that rhesus monkeys harbor functionally parallel, though often non-identical, polymorphisms that mimic in effect human genetic variations, making it feasible to utilize rhesus monkey as a model to clarify genetic factors influencing disorder-related phenotypes and to serve as a preclinical platform for the development of individualized medication. For example, a nonsynonymous SNP C77G (P26R) in rhesus monkey mu opioid receptor (OPRM1) functionally parallels a non-identical

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Abbreviations: AA, amino acid; ADHD, attention deficit hyperactivity disorder; ANOVA, analysis of variance; CDSN, corneodesmosin; CSTA, cystatin A; DRB, 5,6-dichlororibofuranosyl benzimidazole; DRD2, dopamine receptor D2; ELISA, enzyme-linked immunosorbent assay; FBS, fetal bovine serum; h*TPH2*, human *TPH2*; MDR1, multidrug resistance polypeptide 1; m*TPH2*, rhesus monkey *TPH2*; OPRM1, mu opioid receptor; SNP, single nucleotide polymorphism; SSRI, selective 5-HT reuptake inhibitor; TPH, tryptophan hydroxylase.

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SNP A118G (N40D) in human OPRM1 and in both cases, the SNP associates with common phenotypes (Kroslak et al., 2007; Bond et al., 1998; Miller et al., 2004; Barr et al., 2007). As the first step toward building a nonhuman primate model for TPH2 pharmacogenetics, we have recently identified a constellation of polymorphisms across the rhesus monkey *TPH2* (m*TPH2*) locus, including two coding SNPs (C74A and G223A) causing AA substitutions (P25H and G75S, respectively), with the minor allele frequencies being 0.019 and 0.054, respectively (Chen et al., 2006). Both AA substitutions locate in the N-terminal regulatory region of TPH2, and both sites are highly conservative across diverse species, especially for the 75G which is 100% conservative for both TPH1 and TPH2 across 11 species (shown in Fig. 1), suggesting that both SNPs might be functionally significant. In the present study, we set out to investigate the functional effect of the two nonsynonymous SNPs of m*TPH2*.

EXPERIMENTAL PROCEDURES

Plasmid construction

We have previously cloned the full-length coding region of m*TPH2* (GenBank accession no. NM_001039946) from raphe nucleus (Chen et al., 2006). All four theoretic haplotypes (C-G, C-A, A-G, and A-A) for the two nonsynonymous SNPs (C74A and G223A) of m*TPH2* were constructed. The C-G and C-A haplotypes were directly obtained during the cloning of m*TPH2* cDNA, because the animal from which the cDNA was derived happened to be heterozygous for the G223A polymorphism. These two haplotypes were cloned into the pcDNA3.1/V5-His-TOPO® vector (Invitrogen, Carlsbad, CA, USA), and the resultant constructs were used as the template to generate the other two haplotypes (A-G and A-A) carrying the 74A allele. Two mutagenic primers, mTPH2-m1f (5′-ctggattcagcagtgcAcgaagagcatcagctac-3′) and mTPH2-m1r (5′-gtagctgatgctcttcgTgcactgctgaatccag-3′), were employed to generate the 74A substitution by using the QuickChange® Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA) according to the manufacturer's instruction. All constructs were sequence-verified and correct orientation was confirmed.

Cell culture and transfection

The neuroendocrine PC12 cell line was employed for a series of experiments in this study. This cell line can endogenously synthesize dopamine and norepinephrine, but not 5-HT (Greene and Tischler, 1976), suggesting that it possesses essential elements for monoamine synthesis and could be capable of synthesizing 5-HT if TPH2 were exogenously expressed. Cells were maintained in Ham's F12 medium supplemented with 15% fetal bovine serum (FBS) and 2.5% horse serum at 37 °C in an atmosphere of 5% CO_2. The day before transfection, cells were cultured in tissue culture flasks ($\sim 2\times10^6$ cells/flask) containing 10 ml of growth medium. Transfection of the pcDNA3.1/mTPH2 constructs (16 μg for each) was performed with Lipofectamine™ 2000 (Invitrogen) according to the manufacturer's instruction. PC12 cells transfected with the empty pcDNA3.1 vector were used as the negative control. Stable transfectants were selected in the presence of 500 μg/ml of G418 (GIBCO) for 6 weeks with medium change on every 4th or 5th day. Stable PC12 cell lines thus obtained were maintained in growth medium containing 250 μg/ml of G418.

HEK-293 cells transiently transfected with the m*TPH2* haplotypes were additionally used for the analysis of mRNA stability. HEK-293 cells were cultured in Dulbecco's modified Eagle's medium containing 10% FBS, 100 U/ml penicillin, 100 μg streptomycin, and 0.1 mM non-essential AAs in an atmosphere of 5% CO_2 at 37 °C. Cells were seeded in six-well plates at approximately 5×10^5 cells/well and transfections were performed the next day using 2.4 μg of construct.

RNA isolation and quantitative real-time PCR for mRNA analysis

Total RNA was extracted from the stable PC12 or transient HEK-293 cells using Tri-zol reagent (Invitrogen). For the analysis of mRNA stability, 5,6-dichlororibofuranosyl benzimidazole (DRB, 100 μM) (Sigma-Aldrich, St. Louis, MO, USA) was added (24 h after transfection for HEK-293 cells) and cells were lysed at 0, 1, 2, 4, and 6 h after DRB treatment, followed by isolation of RNA. Total RNA was then reverse transcribed into cDNA using Superscript™ III reverse transcriptase and oligo-dTs (Invitrogen), and synthesized cDNA was diluted to 50 ng/μl for use. To avoid DNA contamination, RNA samples were treated with RQ1 RNase-free DNase I (Promega) for 1 h at 37 °C. Assays were performed in triplicate. Real-time PCR was performed on a Roche LightCycler 2.0 system (Roche Diagnostics, Indianapolis, IN, USA) using 50

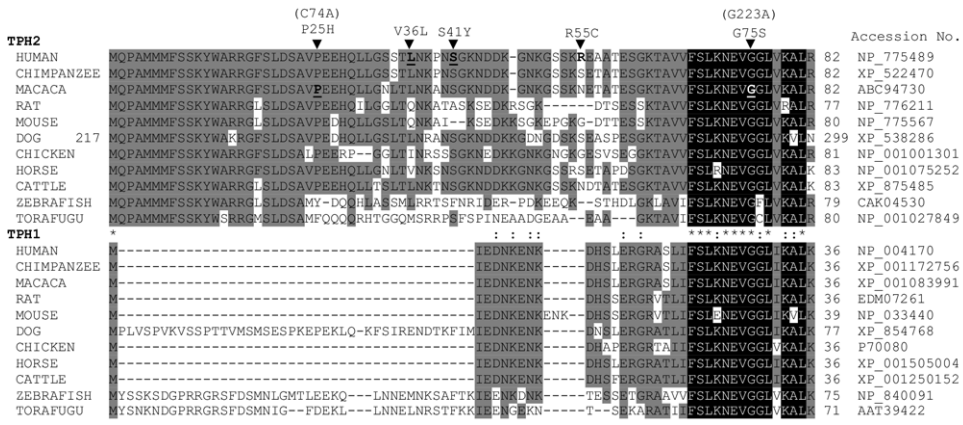

Fig. 1. Alignment of the proximal *N*-terminal of the two TPH isoforms (TPH2 and TPH1) of 11 species. AA substitutions caused by non-synonymous SNPs in rhesus monkey (P25H and G75S) and human (V36L, S41Y, and R55C) TPH2 are underlined and indicated by arrows. * One hundred percent conservative between TPH2 and TPH1; # the dominant AA is the same between TPH2 and TPH1. The 75G is 100% conservative between the 11 species for both TPH2 and TPH1, while the 25P shows 82% conservative between the 11 species for TPH2. The human S41Y was recently reported to be functionally significant (Lin et al., 2007).