Published in final edited form as:

Neuroscience. 2008 August 13; 155(2): 485–491. doi:10.1016/j.neuroscience.2008.05.050.

Rhesus Monkey Tryptophan Hydroxylase-2 Coding Region Haplotypes Affect mRNA Stability

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Abstract

Tryptophan hydroxylase-2 (TPH2) synthesizes neuronal serotonin and its genetic variance is associated with numerous behavioral traits and psychiatric disorders. This study characterized the functional significance of two nonsynonymous SNPs (C74A and G223A) in rhesus monkey *TPH2* (*mTPH2*). Four haplotypes of *mTPH2* 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 serotonin (5-HT) was measured by 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 *mTPH2* haplotypes in both stable PC12 and transient HEK-293 cells. This study demonstrates that nonsynonymous SNPs in *mTPH2* 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.

Keywords

TPH2; serotonin; nonsynonymous polymorphisms; gene expression; nonhuman primate

Serotonin (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 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

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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 lab and other groups have demonstrated that specific polymorphisms in the 5'-regulatory and coding regions of human *TPH2* (*hTPH2*) 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 promoter 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 *hTPH2* 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 serotonin reuptake inhibitors (SSRIs) in patients is associated with *hTPH2* 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 lab 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 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 towards building a nonhuman primate model for TPH2 pharmacogenetics, we have recently identified a constellation of polymorphisms across the rhesus monkey *TPH2* (*mTPH2*) locus, including two coding SNPs (C74A and G223A) causing amino acid (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 *mTPH2*.

Experimental procedures

Plasmid construction

We have previously cloned the full-length coding region of *mTPH2* (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 *mTPH2* were constructed. The C-G and C-A haplotypes were directly obtained during the cloning of *mTPH2* 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), 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'-ctggattcagcagtcAcgaagagcatcagctac-3') and mTPH2-m1r (5'-gtagctgatgctcttcgTgcactgtgaatccag-3'), were employed to generate the 74A substitution by using the QuickChange[®] Site-Directed Mutagenesis Kit (Stratagene, LA Jolla, CA) 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 et al., 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% FBS and 2.5% horse serum at 37°C in an atmosphere of 5% CO₂. The day before transfection, cells were cultured in tissue culture flasks (~2×10⁶ 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 mTPH2 haplotypes were additionally used for the analysis of mRNA stability. HEK-293 cells were cultured in Dulbecco's Modified Eagle's Medium containing 10% fetal bovine serum (FBS), 100 U/ml penicillin, 100 µg streptomycin, and 0.1 mM non-essential amino acids in an atmosphere of 5% CO₂ at 37°C. Cells were seeded in 6-well plates at approximately 5×10⁵ 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-dichlororibofuransyl benzimidazole (DRB, 100µM) (Sigma-Aldrich, St Louis, MO) was added (24 h after transfection for HEK-293 cells) and cells were lysed at 0, 1, 2, 4, and 6 hr 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 hr at 37°C. Assays were performed in triplicate. Real-time PCR was performed on a Roche LightCycler 2.0 system (Roche Diagnostics, Indianapolis, IN) using 50 ng of cDNA for each reaction. The Taqman[®] Master kit in combination with the Universal Probe Library (generously supplied by Roche Diagnostics, USA and Penzberg, Germany) was used to assess mTPH2 gene expression. Primers mTPH2-#15F (5'-caagcaagaagggaactg-3') and mTPH2-#15R (5'-ttgtcagaaaggcatgctt-3'), as well as the universal probe #15 for the qRT-PCR was designed using the Probe Library Assay Design Center (<http://www.roche-applied-science.com/sis/rtpcr/upl/adc.jsp>). Quantification of the mTPH2 mRNA was based on an external standard curve using serial 10-fold dilutions (10⁷~10⁰ copies) of the pcDNA3.1/mTPH2 construct. For comparison of gene expression between haplotypes, calculated mRNA levels were used to perform the statistics and then expressed relative to the C-G wild-type haplotype, while for mRNA stability analysis the mTPH2 mRNA levels were

expressed relative to the value at 0 hr (non-treatment) of each haplotype. The PCR reactions were run in duplicate and the entire experiment was performed on 2 or 3 independent occasions.

Western Blotting

Protein was extracted from stable PC12 cells simultaneously with RNA isolation and dissolved in 1% SDS. The protein concentration was determined by Bradford assay. Samples were incubated at 50°C for the complete dissolution of protein, and the same amount (40 µg) of protein were mixed with Bio-Rad Laemmli sample buffer at 1:1 (v/v), heated at 95°C for 5 min, and centrifuged at 12,000g for 5 min at 4°C. The subsequent supernatants were subjected to SDS-PAGE (12% acrylamide separating gel, 4% acrylamide stacking gel), and the proteins were electrotranslocated onto an Immun-Blot PVDF membrane (Bio-Rad Laboratories, Hercules, CA) presoaked in 100% methanol for 10 min. The membrane was then blocked with blocking buffer (5% nonfat milk, 10 mM Tris-HCl, 150 mM NaCl, and 0.05% Tween 20, pH 7.5) and then incubated with a TPH2-specific antibody (kind gift of Donald M. Kuhn, Wayne State University School of Medicine, Detroit, MI) at 1:1000 overnight at 4°C, and goat anti-rabbit IgG (Sigma-Aldrich) at 1:5000 for 2 h at room temperature, in blocking buffer. β-tubulin was blotted as a control of protein loading. Pierce ECL Western Blotting Substrate (Pierce, Rockford, IL) was used to detect the immunoreactive signals with an ECL-based LAS-3000 image system (Fujifilm Life Science, New Haven, CT). Densitometric analysis was carried out within linear range using ImageGauge (Fujifilm). Assays were performed on three independent occasions.

Measurement of the intracellular 5-HT level by ELISA

The 5-HT levels in the stable PC12 cells were measured by competitive enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions (IBL Immuno-Biological Laboratories, Hamburg, Germany). Briefly, the stable cells were lysed with Hepes-Triton Lysis Buffer with Glycerol (Boston Bioproducts, Worcester, MA). 5-HT in samples and controls were acylated with acetic anhydride in acetone, samples, controls, and standards were applied to 96-well microtitre plates coated with goat anti-rabbit IgG. Biotinylated 5-HT and rabbit antiserum to 5-HT were added to each well and incubated overnight at 4°C. Para-nitrophenylphosphate in a diethanolamine solution was used as a substrate following the application of alkaline phosphatase conjugated goat anti-biotin antibody. Samples were read at 405 nm on a Dynex MRX microplate reader (Dynex Technology, Chantilly, VA). The concentrations of 5-HT were determined using standards supplied by the manufacturer. Assays were performed in duplicate at three independent times.

Bioinformatics and data analysis

Secondary structures of the full-length *mTPH2* mRNA were predicted by the programs GeneBee (<http://www.genebee.msu.su/genebee.html>). Statistics were carried out using StatView 5.0 software (SAS Institute Inc., USA). Comparisons of the levels of *mTPH2* mRNA and protein, as well as the 5-HT production between the constructs were performed by an analysis of variance (ANOVA) or T test, and the repeated measures ANOVA was employed to compare the mRNA stability between the haplotypes.

Results

The mRNA levels of *mTPH2* in stable PC12 cell lines were measured by qRT-PCR. As shown in Fig. 2A, *mTPH2* mRNA was expressed in all of the stable PC12 cells transfected with pcDNA3.1/*mTPH2* constructs, but not in the control transfected with the empty pcDNA3.1. The relative mRNA levels in each cell line was calculated and shown in Fig. 2B. The cell line expressing the A-A haplotype had significantly higher mRNA levels than those expressing the other three haplotypes ($P < 0.05$).

Western blotting was then performed to detect the mTPH2 protein levels in the stable PC12 cells. As shown in Fig. 3, a single immunoreactive band of ~50 kDa size was observed for all stable PC12 cell lines except the control, and paralleling the mRNA level, the cell line expressing the A-A haplotype showed the highest protein expression. We also determined the 5-HT levels in the stable PC12 cells by ELISA, which also correlated to the protein level and enzymatic activity. As shown in Fig. 4, all the stable PC12 cells transfected with pcDNA3.1/mTPH2 constructs showed significantly higher 5-HT level than the control, with a signal-to-noise ratio of greater than 7:1, supporting the notion that PC12 cells could synthesize 5-HT if TPH2 was exogenously expressed. Again, the cell line expressing the A-A haplotype mTPH2 produced significantly higher 5-HT than the wild-type C-G haplotype, in good accordance with the observations of the mRNA and protein levels. The C-A and A-G haplotypes also tended to produce more 5-HT than the wild C-G haplotype, although the differences did not reach a statistical significance due to the large variance between independent experiments.

As shown in Fig. 5, both the nonsynonymous SNPs are predictive of alteration in the secondary structure of the mTPH2 mRNA, especially for the G223A substitution, which leads to a totally different mRNA structure regardless of the allele at the other locus (C-G vs C-A and A-G vs A-A). The effect of C74A substitution on mRNA structure seems to depend on the allele at the G223A locus, with considerable and minor changes in mRNA structure being observed for 223G (C-G vs A-G) and 223A (C-A vs A-A) alleles, respectively. Accordingly, the free energy of the mTPH2 mRNA was predicted to be affected by the G223A and C74A substitutions. In accordance with the highest mRNA level observed in the cell line expressing the A-A haplotype, predicted mRNA structure of this haplotype has the lowest free energy (-422.6 kcal/mol), suggesting it might be more stable than other haplotypes. We then compared the mRNA stability between the four haplotypes in stable PC12 cells, and found that the degradation rate of the wild-type C-G haplotype mRNA was significantly higher than A-A and C-A mRNAs ($P < 0.01$ for both C-G vs A-A and C-G vs C-A), and it tended to (but not significantly by statistics) be higher than A-G mRNA (shown in Fig. 6A), suggesting that the G223A and C74A SNPs (especially the former) is capable of stabilizing the mTPH2 mRNA in vitro. A similar pattern of mRNA degradation for the four haplotypes was also observed in HEK-293 cells transiently transfected with the four haplotypes (Fig. 6B), indicating that the relative mRNA stability of the four haplotypes is independent of the cell line and transfection (stable or transient).

Discussion

TPH2 genetic variance has been widely investigated and is reportedly associated with numerous personality traits and psychiatric disorders. Recent studies of our lab and other groups have revealed that specific polymorphisms in the 5'- and 3'-regulatory regions of *TPH2* have a significant effect on gene expression (Chen et al., 2008; Scheuch et al., 2007; Lin et al., 2007; Chen et al., 2006). In addition, the functional significance of a certain number of nonsynonymous polymorphisms of *TPH2* have been identified in human (S41Y and P206S) (Lin et al., 2007; Cichon et al., 2008), chimpanzee (Q468R) (Hong et al., 2007), and mouse (P447R) (Zhang et al., 2004). In the present study, we demonstrated that stable transfection of rhesus monkey *TPH2* mutated with the nonsynonymous SNPs rendered differential mRNA, protein, 5-HT expression and mRNA stability in stable PC12 cell lines.

Because nonsynonymous coding SNPs lead to AA substitutions, mechanisms regarding the stability and/or function of the protein products are usually firstly considered. However, coding SNPs (both nonsynonymous and synonymous) can also influence the transcriptional process and mRNA stability, and can thereby exert a profound effect on gene expression. For example, the human catecho-O-methyltransferase (COMT) haplotypes divergent in two synonymous and one nonsynonymous position modulate protein expression by altering mRNA secondary

structure (Nackley et al., 2006). It has also been reported that mRNA expression was differentiated by nonsynonymous SNPs in human genes coding OPRM1, XPD/ERCC2, transient receptor potential vanilloid 1 (TRPV1), and cystatin A (CSTA), as well as synonymous SNPs in human genes coding dopamine receptor D2 (DRD2), corneodesmosin (CDSN) multidrug resistance polypeptide 1 (MDR1) (Zhang et al., 2005b; Wolfe et al., 2007; Xu et al., 2007; Vasilopoulos et al., 2007; Duan et al., 2003; Capon et al., 2004; Wang et al., 2005). In particular, the nonsynonymous C344T in CSTA and synonymous SNPs in DRD2, CDSN and MDR1 lead to altered mRNA stability. Similarly, our present study shows that nonsynonymous SNPs in rhesus monkey *TPH2* can affect mRNA stability. As the nonsynonymous SNPs in the human (P206S) (Cichon et al., 2007), chimpanzee (Q468R) (Hong et al., 2007), and mouse (P447R) (Zhang et al., 2004) were reported to affect protein function (enzyme activity and/or binding affinity), our findings provide an additional mechanism by which nonsynonymous SNPs of *TPH2* exert their effects on biological functions leading to alteration in behavioral traits and disease susceptibility. Not all coding SNPs identified in human *TPH2* have been examined for their functional significance, among which the V328A is predictive of a considerable increase (7.3 kcal/mol) in free energy of *TPH2* mRNA (data not shown). Accordingly, it will be interesting if V328A or other coding SNPs can affect mRNA stability.

While both mRNA and protein levels reflect the gene expression of *TPH2*, the level of intracellular 5-HT is determined by both gene expression and protein function of *TPH2*. In our present study, all *TPH2*-transfected cell lines expressed *TPH2* mRNA and protein, and produced 5-HT. While a G1463A (R441H) variant in *hTPH2*, although its natural existence has not yet been replicated, causes ~80% loss of enzymatic function determined by the 5-HT level in stable PC12 cells (Zhang et al., 2005a), our current work failed to find loss-of-function coding SNPs in rhesus monkey *TPH2*. However, the cell lines transfected with different *mTPH2* haplotypes varied in expression level. As the *mTPH2* mRNA and protein expression parallel each other, the variations in *mTPH2* protein level and 5-HT production are apparently due to haplotype-dependent mRNA levels; however, the frequency of chromosomal integration in each stable cell line might differ between the haplotypes even under the same concentration of G418 for selection, and this difference may constitute a confounding factor affecting the interpretation of gene expression between the haplotypes. It is apparent, however, that the C-A and A-G haplotypes showed similar mRNA stability with A-A haplotype but lower mRNA level than A-A haplotype in stable PC12 cells, perhaps due to the different mRNA transcription between the haplotypes. Thus, the possibility that *mTPH2* nonsynonymous SNPs affect protein function can not be excluded. Actually, the C-A and A-G haplotypes tended to produce more 5-HT than the wild C-G haplotype, although the differences did not reach a statistical significance due to the large variance between independent experiments. The resultant amino acid substitutions caused by the C74A and G223A are present in the N-terminal of *TPH2*, and the latter locates in the ACT domain that is highly conservative for both *TPH1* and *TPH2*. *In silico* analysis gives conflicting speculation regarding the likelihood of the two nonsynonymous SNPs to cause a functional impact on the protein. For example, evolutionary analysis by using Panther Classification System (<http://www.pantherdb.org/tools/csnpscoreForm.jsp>) showed both SNPs are unlikely to exert a profound effect on protein function, while the effect of G75S on protein function was predicted to be “benign” by the PolyPhen Program (<http://genetics.bwh.harvard.edu/pph/>). Meanwhile, G75S is predictive of altering protein secondary structure using several programs (data not shown).

Though effects of the nonsynonymous SNPs of *mTPH2* on protein function is yet to be resolved, this study provides convincing evidence for the effect of *mTPH2* nonsynonymous SNPs on mRNA stability. As the stability of a particular mRNA is controlled by specific interactions between its structural elements and RNA-binding proteins (Guhaniyogi and

Brewer, 2001), the free energy is an important but not the sole factor affecting mRNA stability. Thus, it is not surprising that the A-G mRNA showed a similar free energy with C-G mRNA but a higher stability than the latter. Since both C74A and G223A are predicted to change mRNA secondary structure, it is tempting that they might hamper specific RNA-protein interaction involved in mRNA degradation and thereby stabilize the mRNA. Nevertheless, the mechanism(s) by which the two nonsynonymous SNPs affect mRNA stability are yet to be verified by further study. Since genetically-determined TPH2 activity has been associated with an increasing number of behavioral traits, drug responsivity (in both human and mouse model), and disease susceptibility, it could be speculated that the two nonsynonymous SNPs of *mTPH2* could have pathophysiological significance by elevating the mTPH2 mRNA level and 5-HT synthesis. Regardless of the linkage disequilibrium (LD) between the two SNPs, the frequencies of the mutant haplotypes C-A, A-G and A-A in rhesus monkeys were estimated to be 0.053, 0.018 and 0.001, respectively, making it possible that the *mTPH2* coding region haplotypes account in part for the variation in TPH2 expression across the population. Because these two SNPs are in linkage with specific polymorphisms in the 5'- and 3'-regulatory regions of *mTPH2* (Chen et al., 2006), their effect on gene expression should be considered when we investigate the *in vivo* functional significance of specific polymorphisms in *mTPH2* 5'- and 3'-regulatory regions.

In summary, this study has demonstrated that *mTPH2* coding region haplotypes affect mRNA stability. Taken together with our previous studies, our present findings reveal an additional mechanism by which nonsynonymous SNPs affect TPH2 function, and advanced our understanding of *TPH2* gene expression regulation. Moreover, the polymorphisms in *mTPH2* as well as those reported in our previous studies make it feasible for us to develop rhesus monkey models for the genotype-phenotype correlation relevant to *TPH2* genetic variance in humans.

Acknowledgements

The authors would like to thank Mrs. Hong Yang for the excellent technician support. We also thank Dr. Bin Jia at HMS/NEPRC for the helpful suggestion and kind help for this study. We are grateful to Mrs. Jennifer Carter for the administrative support.

This study was supported by AA016194 (GMM), DA016606 (GMM), DA021180 (GMM), and RR00168.

Abbreviations

TPH2	tryptophan hydroxylase-2
5-HT	serotonin
ADHD	attention deficit hyperactivity disorder
SSRI	serotonin selective reuptake inhibitor
SNP	single nucleotide polymorphism
AA	amino acid
LD	

	linkage disequilibrium
OPRM1	mu opioid receptor
ELISA	enzyme-linked immunosorbent assay
TRPV1	transient receptor potential vanilloid 1
CSTA	cystatin A
DRD2	dopamine receptor D2
CDSN	corneodesmosin
MDR1	Multidrug resistance polypeptide 1

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		(C74A) P25H	V36L	S41Y	R55C	(G223A) G75S	Accession No.
TPH2							
HUMAN	MQPAMMMFSSKYWARRGFS	LD	SA	VP	EEHQL	LGSS	STLNKPN
CHIMPANZEE	MQPAMMMFSSKYWARRGFS	LD	SA	VP	EEHQL	LGSS	STLNKPN
MACACA	MQPAMMMFSSKYWARRGFS	LD	SA	VP	EEHQL	LGSS	STLNKPN
RAT	MQPAMMMFSSKYWARRGL	SL	DS	AV	PEEHQ	IL	GG
MOUSE	MQPAMMMFSSKYWARRGL	SL	DS	AV	PEEHQ	IL	GG
DOG	MQPAMMMFSSKYWARRGL	SL	DS	AV	PEEHQ	IL	GG
CHICKEN	MQPAMMMFSSKYWARRGL	SL	DS	AV	PEEHQ	IL	GG
HORSE	MQPAMMMFSSKYWARRGL	SL	DS	AV	PEEHQ	IL	GG
CATTLE	MQPAMMMFSSKYWARRGL	SL	DS	AV	PEEHQ	IL	GG
ZEBRAFISH	MQPAMMMFSSKYWARRGL	SL	DS	AV	PEEHQ	IL	GG
TORAFUGU	MQPAMMMFSSKYWARRGL	SL	DS	AV	PEEHQ	IL	GG
TPH1							
HUMAN	M	---	---	---	---	---	---
CHIMPANZEE	M	---	---	---	---	---	---
MACACA	M	---	---	---	---	---	---
RAT	M	---	---	---	---	---	---
MOUSE	M	---	---	---	---	---	---
DOG	M	---	---	---	---	---	---
CHICKEN	M	---	---	---	---	---	---
HORSE	M	---	---	---	---	---	---
CATTLE	M	---	---	---	---	---	---
ZEBRAFISH	M	---	---	---	---	---	---
TORAFUGU	M	---	---	---	---	---	---

Fig. 1.

Alignment of the proximal *N*-terminal of the two TPH isoforms (TPH2 and TPH1) of 11 species. Amino acid (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. * -100% 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).

Fig. 2. Quantitative real-time PCR amplification curves for the cDNA derived from stable PC12 cells (A) and the relative mTPH2 mRNA levels (%) of the pcDNA3.1/mTPH2 constructs with different haplotypes of mTPH2 coding region (B). The cross-points (Cp) of the amplifications are given in parentheses following the haplotypes, and the standard curve for mRNA quantification is shown as an inset. Data of mRNA levels are shown as Means $\pm$ SE. * P <0.05 compared with other three haplotypes.

Fig. 3. Comparison of the TPH2 protein expression between mTPH2 haplotypes. (A) Western blot using the indicated antibodies; (B) Quantification of TPH2 levels (n=3). was used as loading control. Data are shown as Mean $\pm$ SE. * P <0.05 compared with other haplotypes;

Fig. 4. Comparison of the 5-HT level in stable PC12 cells between *mTPH2* haplotypes. Data are shown as Means $\pm$ SE. CTL-control. * P <0.05 compared with the C-G haplotype; ** P <0.01 compared with the four *mTPH2* haplotypes.

Fig. 5. The predicted mRNA secondary structures (partial) of the four *mTPH2* coding region haplotypes. Only part of the structure containing both nonsynonymous SNPs is shown, while the truncated part of mRNA structure (not shown) exhibits no difference between the four haplotypes. The free energy (kcal/mol) of the full-length mRNA is shown in parentheses. The minor differences between C-A and A-A haplotypes are indicated by arrows.

Fig. 6. Comparison of the mRNA degradation rate between the four *mTPH2* haplotypes in stable PC12 (A) and transient HEK-293 cells (B). The mRNA levels were expressed relative to the values at 0 hr (non-treatment) for each haplotype. Data are shown as Means $\pm$ SE.