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Authors
Carkaci-Salli, Nurgul
Salli, Ugur
Tekin, Izel
et al.

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Functional characterization of the S41Y (C2755A) polymorphism of tryptophan hydroxylase 2

Nurgul Carkaci-Salli[*], **Ugur Salli**[*], **Izel Tekin**[*], **Jeremy A. Hengst**[*], **Moe K. Zhao**[†], **T. Lee Gilman**[†], **Anne M. Andrews**[†], **Kent E. Vrana**[*]

[*]Department of Pharmacology, Penn State College of Medicine, Hershey, Pennsylvania, USA

[†]Semel Institute for Neuroscience & Human Behavior and Hatos Center for Neuropharmacology, University of California, Los Angeles, California, USA

Abstract

Human TPH2 (hTPH2) catalyzes the rate-limiting step in CNS serotonin biosynthesis. We characterized a single-nucleotide polymorphism (C2755A) in the *hTPH2* gene that substitutes tyrosine for serine at position 41 in the regulatory domain of the enzyme. This polymorphism is associated with bipolar disorder and peripartum depression in a Chinese population. Recombinant h TPH2 human proteins were expressed in bacteria and also stably expressed in PC12 cells. Following bacterial expression and purification, the tyrosine for serine substitution at position 41 (S41Y) polymorphic enzyme displayed increased V_{max} with unchanged K_m values. By contrast, enzyme stability was decreased *in vitro* from 32 min to 4 min (37°C) for the S41Y enzyme (as compared to the wild-type enzyme). The S41Y polymorphism decreased cyclic AMP-dependent protein kinase A-mediated phosphorylation ~ 50% relative to wild-type hTPH2, suggesting that the S41Y mutation may disrupt the post-translational regulation of this enzyme. Transfected PC12 cells expressed *hTPH2* mRNA, active protein, and synthesized and released serotonin. Paradoxically, while S41Y-transfected PC12 cells expressed higher levels of *hTPH2* than wild type, they synthesized less serotonin. These findings suggest a modified regulation of the S41Y gene variant leading to altered regulation and reduced neurotransmitter synthesis that may contribute to association of the polymorphism with bipolar disorder and depression.

Keywords

genetic polymorphism; neurotransmitter biosynthesis; PC12 cell; protein stability; serotonin; TPH2

TPH, tryptophan hydroxylase (TPH; EC 1.14.16.4), is a member of the pterin-dependent aromatic amino acid hydroxylase family, and is the initial and rate-limiting enzyme in the synthesis of serotonin. Serotonin (5-hydroxytryptamine, 5-HT) is a neurotransmitter present in the mammalian peripheral and central nervous systems. Imbalances in 5-HT signaling may contribute to neuropsychological disorders such as generalized anxiety disorder, depression, panic disorders, and obsessive compulsive disorder (Barnes and Sharp 1999;

Address correspondence and reprint requests to Kent E. Vrana, Department of Pharmacology H078, Penn State College of Medicine, PO Box 850, Hershey, PA 17033-0850, USA. kvrana@psu.edu.

Akimova *et al.* 2009; Berger *et al.* 2009). Moreover, many pharmaceutical agents target components of the 5-HT signaling pathway for the treatment of these disorders. (Berger *et al.* 2009; Altieri *et al.* 2012; Torrente *et al.* 2012). In mammals, two different TPH genes have been identified. TPH1 is mainly expressed in peripheral tissues (pineal gland, mast cells, GI tract), while TPH2 is expressed in specific regions of the CNS (primarily the raphe nucleus) (Walther and Bader 2003; Carkaci-Salli *et al.* 2011).

A number of studies have evaluated naturally occurring polymorphisms in *hTPH2* – the human TPH2 gene (Kulikov *et al.* 2005; Zhang *et al.* 2005; Sakowski *et al.* 2006; Winge *et al.* 2007; McKinney *et al.* 2009). Some of these *hTPH2* polymorphisms have been associated with unipolar or bipolar disorder (BPD) (Zhou *et al.* 2005a,b; Van Den Bogaert *et al.* 2006; Cichon *et al.* 2008; Grigoroiu-Serbanescu *et al.* 2012), depression (Zhou *et al.* 2005a), major depression (Zill *et al.* 2004a; Gao *et al.* 2012), suicidal behavior (Ke *et al.* 2006), and panic disorder (Maron *et al.* 2007; Preuss *et al.* 2013). One potentially clinically important coding region polymorphism is the C2755A (rs78162420) substitution first reported by Zhou *et al.* (2005a). This polymorphism is associated with BPD and peripartum depression in a population of Han Chinese (Allele Frequency: A = 0.033, C = 0.967) (Lin *et al.* 2007, 2009). This single-nucleotide substitution results in a serine-to-tyrosine substitution at amino acid position 41 (S41Y) in the regulatory domain of hTPH2.

The human TPH2 protein contains an additional 41 amino acids at the N terminus (including the S41 residue in question in this manuscript) that are not present in TPH1. The N-terminal regulatory domain of the hTPH proteins modulates enzyme activity and contains phosphorylation sites that may contribute to post-translational regulation (Vrana *et al.* 1994; Jiang *et al.* 2000; Kuhn *et al.* 2007). TPH2 is phosphorylated at two sites: Ser^{19} and Ser^{104}. It has been demonstrated that both Ser^{19} and Ser^{104} are phosphorylated by cyclic AMP-dependent protein kinase A (PKA). Ser^{19} is also phosphorylated by calmodulin-dependent protein kinase II (CaMKII) (McKinney *et al.* 2005; Kuhn *et al.* 2007; Murphy *et al.* 2008; Winge *et al.* 2008). Moreover, the initial one-third of the hTPH2 protein (the first 150 amino acids) can be removed without abolishing enzyme activity (Yang and Kaufman 1994; D'Sa *et al.* 1996; Kumer *et al.* 1997; Carkaci-Salli *et al.* 2006). Previous investigators have reported that the S41Y polymorphism increases the activity of the enzyme (McKinney *et al.* 2009). However, the enzyme kinetic factors underlying this effect have not been established, and the previous finding of increased enzyme activity (McKinney *et al.* 2009) is inconsistent with the reduced serotonin hypothesized to be associated with BPD and depression (Lin *et al.* 2007, 2009). Indeed, Lin *et al.* (2007) prepared transient transfections of the S41Y mutant in SH-SY5Y neuroblastoma cells and, while they did not quantify enzyme activity, they reported a 35% decrease in serotonin production in transfected cells.

In this study, we expressed wild-type or S41Y recombinant hTPH2 protein in bacteria and found that, while the maximal velocity (V_{max}) of the mutant enzyme was increased, the thermal stability of the purified mutant enzyme was decreased. We then created stably transfected PC12 cells to study hTPH2 in a eukaryotic cellular environment. We selected PC12 cells because they naturally produce monoamine neurotransmitters/hormones (i.e., epinephrine, norepinephrine) and, therefore, they have the native biochemical machinery – sans hTPH2 – needed to synthesize, package, and release serotonin [i.e., L-aromatic amino

acid decarboxylase, tetrohydrobiopterin (BH_4) biosynthesis, vesicular monoamine transporters, etc.]. While hTPH2 has been transiently expressed in PC12 cells (Zhang *et al.* 2004), it has not been stably transfected into eukaryotic cells. This study demonstrates that the non-synonymous single-nucleotide polymorphism (SNP) S41Y in hTPH2 affects enzyme activity, thermal stability, serotonin production, and PKA-mediated phosphorylation.

Methods and materials

Materials

Taq DNA polymerase was purchased from Fisher (Thermo Fisher, Pittsburgh, PA, USA). Oligonucleotides were synthesized by IDT (Integrated DNA Technology; Coralville, IA, USA). Restriction enzymes *Mlu*I and *Not*I were purchased from Promega, Madison, WI, USA. The Rapid DNA Ligation kit (Roche Applied Science, Indianapolis, IN, USA) was used for ligation reactions. PC12 cells (Tet-On) were obtained from Clontech (Mountain View, CA, USA). Lipofectamine, antibiotics, horse serum, medium, and other cell culture reagents were purchased from Invitrogen (Camarillo, CA, USA). Antibodies and immunoblot reagents were purchased from Sigma (St Louis, MO, USA).

Bacterial expression of the hTPH2 S41Y variant

A previously reported hTPH2 bacterial expression construct [in pET28-tev; (Carkaci-Salli *et al.* 2006)] was used to express the S41Y polymorphism as a hexa-His-tagged recombinant protein. Mutagenesis was carried out using a QuikChange™ Site-Directed Mutagenesis kit (Stratagene, Kirkland, WA, USA) in a thermocycler under the following conditions: denaturation at 95°C, annealing at 55°C, and extension at 68°C for 20 cycles. Amplification products were transformed into BL21CodonPlus-(DE3)-RIL *E. coli* (Stratagene). Positive colonies were selected from kanamycin containing LB agar plates. The following primers were utilized to introduce the S41Y mutation into the *hTPH2* in pET28-tev: 5′-AATAAACCTAACTATGGCAAAAATGACGAC-3′ (forward) and 5′GTCGTCAT TTTTGCCATAGTTAGGTTTATTT-3′ (reverse). The *hTPH2* DNA sequences were determined in pET28-tev constructs using the forward T7 promoter primer (5′-TAATACGACTCACTATAGGG-3′) and the reverse T7 terminator primer (5′-TATGCTAGTTAT TGCTCAG-3′) to confirm desired substitutions and sequence integrity.

In vitro expression and protein purification

The recombinant constructs were expressed in BL21-CodonPlus (DE3)-RIL (*E. coli*) cells in ZYP-5052 autoinduction medium (Studier 2005; Carkaci-Salli *et al.* 2006) plus 50 μM L-tryptophan. Following induction, *E. coli* cells were harvested and lysed with benzonase and r-lysozyme (Novagen, EMD Biosciences, Inc., Madison, WI, USA) in the presence of protease inhibitor cocktail (Complete EDTA-free, Roche Applied Science). The lysate was then centrifuged at high speed (40 000 *g*) to obtain soluble proteins. The clarified lysate was subjected to a rapid one-step metal chelate affinity chromatography procedure using a 5 mL or 1 mL prepacked HiTrap nickel column and an Akta FPLC (Fast protein liquid chromatography, Amersham Pharmacia Biotech, Piscataway, NJ, USA) workstation at 4°C. Proteins were then eluted stepwise with phosphate buffer containing 50, 300, and 500 mM

imidazole and 1 mM dithiothreitol. The recombinant hTPH2 protein was eluted by the buffer containing 300 mM imidazole (Thermo Fisher) and used for further experiments.

Enzyme activity assay

Exogenously expressed hTPH2 and endogenous rat PC12 tyrosine hydroxylase activities were assayed using radioenzymatic [^{3}H]-H_2O release assays as previously described by Beevers *et al.* (Beevers *et al.* 1983), Reinhard, and colleagues (Reinhard *et al.* 1986), and modified by Vrana *et al.* (1993). Activity values derived from each assay were either normalized to the amount of total protein present in the homogenates, as determined by the Bradford protein assay (Bio-Rad, Berkeley, CA, USA), or to the amount of specific hTPH2 protein measured by western immunoblotting. Michaelis–Menten kinetic analyses were conducted by varying the concentration of one substrate [tryptophan or BH_4 (Sigma)] at a fixed concentration of the other substrate (50 μM BH_4 or 50 μM tryptophan, respectively), and a fixed and ambient concentration of oxygen. Data were analyzed by curve fitting to the Michaelis–Menten equation with GraphPad Prism 5 (GraphPad Software, San Diego, CA, USA) to determine kinetic constants (K_m, V_{max}).

SDS–PAGE and western immunoblot analysis

Proteins were resolved on 4–12% NuPAGE Bis/Tris gels (Invitrogen, Carlsbad, CA, USA). Gels were stained with Coomassie Blue (Pierce, Rockford, IL, USA) to visualize total protein and to assess purity. In addition, selected gels were transferred to polyvinylidene fluoride membranes (Immobilon P; Millipore, Billerica, MA, USA) in NuPAGE Transfer buffer. hTPH2 was detected by probing with a mouse anti-TPH monoclonal antibody (diluted 1 : 3000; Sigma) that recognizes TPH1 and TPH2 and cross-reacts with TH. Tyrosine hydroxylase (TH) is an endogenous enzyme related to TPH that is natively expressed in PC12 cells and is responsible for the synthesis of catecholamines in this cell line. A mouse anti-TH monoclonal antibody (diluted 1 : 3000; Sigma) was used to quantify TH levels as an internal control. Moreover, an antibody targeting a house-keeping gene (mouse anti-actin antibody diluted 1 : 3000; Sigma) was also used to normalize protein abundance. Donkey anti-mouse IgG secondary antibodies conjugated with horseradish peroxidase (1 : 3000; Sigma) were used to visualize the protein–antibody complexes followed by enhanced chemiluminescence detection (Amersham). Bands corresponding to hTPH2, the endogenous rat TH, and actin were quantified using the Image Quant program. hTPH2 and rat TH western blot densities were normalized with respect to actin densities.

Enzyme stability *in vitro*

TPH enzymes are notoriously unstable *in vitro* (Hasegawa and Nakamura 2010). To assess the thermal stability of the recombinant proteins, purified wild-type hTPH2 and S41Y enzymes (purified from bacteria or present in PC12 cell homogenates) were incubated at 37°C or 4°C for varying periods of time. Enzyme activity was then determined as described above, and analyzed as a function of activity decay (on a logarithmic plot) to calculate $t_{1/2}$ values.

Molecular cloning of hTPH2 into pTRE2hyg for eukaryotic cell expression

Full-length, wild-type *hTPH2* or the S41Y mutant were introduced via *Mlu*I and *Not*I restriction sites into multiple cloning sites of the pTRE2hyg plasmid (Clontech). First, N-terminal *Mlu*I and C-terminal *Not*I restriction sequences were inserted into the wild-type *hTPH2* gene in such a way as to create intact, full-length, non-fusion-tagged recombinant proteins. This was accomplished using the following primers: 5′CTTTACTTCCAGGGCCATACGCGTATGCAGCCAGCAATGATG3′ (forward) and 5′CATCATTGCTGGCTGCATACGCGTATGGCCCTGGAAGTAAAG3′ (reverse) for *Mlu*I; and 5′CAATATCTGGGGATTTGAGCGGCCGCGGATCCGAATTCGAGCTC3′ (forward) and 5′GAGCTCGAATTCGGATCCGCGGCCGCTCAAATCCCCAGATATTG3′ (reverse) for *Not*I. The Rapid DNA Ligation Kit (Roche) was used for ligation reactions. Recombinant plasmid DNA was transfected into XLBlue II cells (Stratagene) and colonies were selected on ampicillin plus chloramphenicol LB agar plates. To create the S41Y substitution in the pTRE2- *hTPH2* construct, the same primers that had been used above to create the polymorphism in the pET28tev vector were employed. The pTRE2hyg-*hTPH2* construct sequences were confirmed with sequencing primers designed against the vector sequence: 5′-TCGTTTAGTGAACCGTCAGATCGC-3′ (forward) and 5′-ACTCACCCTGAAGTTCTCAGCTCT-3′ (reverse).

Stable introduction of pTRE2hygTPH2 constructs into PC12 Tet-On cells

The hTPH2 and S41Y eukaryotic expression constructs, described above, were transfected into PC12 Tet-On cells (PC12 Tet-On; Clontech) using Lipofectamine (Invitrogen). The PC12 Tet-On cell is a rat pheochromocytoma (PC12) cell line that has been stably transfected with the pTet-On plasmid as detailed previously (Nagase *et al.* 2005). Subsequently, cells were selected for stable integration. Briefly, 24 h following transfection, media were replaced with fresh media containing 50 μg/mL hygromycin (Roche) (an optimal concentration determined by separate titration experiments) to select cells that harbor the hTPH2 constructs. Media were replaced after 48 h and every 3 days thereafter. Once hygromycin-selected cells reached 80% confluency, expression of hTPH2 constructs was examined by western blot analysis and enzyme activity assays following exposure to 2 μg/mL doxycycline (DOX) for 48 h. Selected cells (stably transfected) were maintained in Dulbecco's modified Eagle's medium as described above.

RT-qPCR analysis of gene expression

Total RNA from stably transfected PC12-*hTPH2* and PC12-S41Y cells was isolated using the Qiagen RNeasy Plus Mini Kit. cDNAs were synthesized using the Omniscript reverse transcriptase kit (Qiagen, Germantown, MD, USA). qPCR was performed on a 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA, USA) using primers for rat β-*Actin* (Rn00667869), rat *TPH1* (Rn01476869), rat *TPH2* (Rn00598017), and human *TPH2* (Hs00998776) genes. Relative quantities were calculated using ABI SDS 2.2.2 RQ software and the $2^{-\Delta\Delta Ct}$ analysis method (Livak and Schmittgen 2001; Bowyer *et al.* 2007; VanGuilder *et al.* 2008) with *ß-actin* (Rn00667869) as the endogenous control gene. These studies were conducted in the Functional Genomics Core Facility of the PSU Division of Shared Research Resources.

Cellular stability analysis of wild-type and mutant S41Y hTPH2 in PC12 cells

Upon reaching 80% confluency, *hTPH2*-PC12 cells and S41Y-*hTPH2*- PC12 cells were cultured with 2 μg/mL DOX for 48 h to induce expression of their constructs. At the end of the induction period, cells were rinsed twice with phosphate-buffered saline (to remove inducing doxycyline) and treated with freshly prepared cyclohexamide (50 μg/mL) to block further protein synthesis. Cells from independent cultures were then harvested at 0, 1, 2, 4, and 8 h. At the end of the each interval, cells were rinsed with phosphate-buffered saline and collected with a rubber cell scraper and centrifuged at 300 *g* for 5 min at 4°C. Following centrifugation, cell pellets were frozen immediately on dry ice.

Measurement of 5-HT and dopamine levels in hTPH2 producing PC12 Tet-On cell

Once hTPH2 expression was established in the stably transfected Tet-On PC12 cells, serotonin and dopamine (DA) levels were determined in PC12 cells expressing wild-type or S41Y *hTPH2*. Media were removed from cultures containing ~2 × 10^6 cells (passage #19) and cells were scraped into 250 μL of 0.4 M $HClO_4$ containing 5 μM 5-hydroxy-*N*-ω-methyltryptamine as the internal standard. Cells were disrupted by sonication and centrifuged at 7200 *g* at 4°C for 10 min. The supernatants were stored at −70°C until analysis by high-performance liquid chromatography (HPLC)using electrochemical detection at +270 mV with a graphite working electrode (Eicom WE-3G; Eicom USA, San Diego, CA, USA) versus Ag/AgCl as described previously (Luellen *et al.* 2003). After thawing, supernatants were centrifuged at 7200 *g* at 4°C and 20-μL aliquots were injected onto a 100 × 3.2 mm 3 μm C18 Spherisorb column (Thompson Instrument Co., Chantilly, VA, USA). The mobile phase consisted of 0.1 M monochloroacetic acid (pH 2.6), 1.4 mM 1-octanesulfonic acid, 10 μM ethylenediamine-tetraacetic acid, 8% (v/v) acetonitrile, and 0.01% (v/v) triethylamine. Characteristic retention times of DA and serotonin were 6 min and 16 min, respectively. Peak areas were analyzed using PowerChrom (Eicom WE-3G; Eicom USA) and quantified against standards of known concentration. Serotonin levels are corrected for low levels of endogenous serotonin (~ 5 pmol 5-HT/million cells) in the untransfected PC12 cell line used to produce the wild-type and S41Y hTPH2 expressing lines. In addition to measuring stored serotonin, the ability of cells to release serotonin was investigated following K^+-induced depolarization (50 mM).

In vitro cyclic AMP-dependent protein kinase A phosphorylation assays

PKA-mediated phosphorylation of WT and the S41Y mutant was conducted as follows. Briefly, equal quantities of partially purified recombinant hTPH2 proteins (purification described above) were combined with 2500 units of the catalytic subunit of PKA (NEB; Ipswitch, MA, USA) in reaction buffer containing 50 mM Tris-HCl, pH 7.5, and 10 mM $MgCl_2$. Reactions were initiated by the addition of 25 μM ATP containing 2 μCi of [γ-^{32}P]-ATP (Perkin Elmer, Waltham, MA, USA), as a tracer, and were incubated for 15 min at 37°C. Reactions were terminated by the addition of sodium dodecyl sulfate–polyacrylamide gel electrophoresis gel loading buffer and were incubated at 95°C for 5 min. Phosphorylated proteins were resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis gel electrophoresis, transferred to polyvinylidene difluoride membrane, and visualized by autoradiography on X-ray film sandwiched between two intensifying screens. For detection

of total TPH proteins, polyvinylidene difluoride membranes were subsequently analyzed by western blot analysis using anti-TPH antibodies. The time scale differences between ^{32}P autoradiography (4 h) and western blot chemiluminescence (≤ 1 min) ensured that the signals did not interfere with each other (autoradiography was conducted first, then chemiluminescence). Quantitation of phosphorylated and total TPH proteins was conducted using Image-Quant. Similar experiments were performed with AMP kinase (NEB) and CaMKII (NEB) using conditions recommended by the manufacturers.

Statistical analysis

All statistical analyses were conducted using GraphPad-5 Prism and one-way ANOVA followed by unpaired Student's *t*-tests. Statistical significance was associated with values of $*p < 0.05$, $**p < 0.01$, or $***p < 0.001$.

Results

Bacterial expression of recombinant wild-type hTPH2 and S41Y coding region mutant constructs

The serine-to-tyrosine S41Y coding region mutant was constructed in the pET28-tev vector and expressed in *E. coli* as described previously (Carkaci-Salli *et al.* 2006). Although recombinant hTPH2, when expressed in bacteria, is largely packaged in insoluble inclusion bodies (Carkaci-Salli *et al.* 2006), sufficient soluble active protein can be purified by exploiting the hexa-histidine tag incorporated into the amino terminus of the enzyme. Purified material was then used for enzymatic characterization.

Enzymatic activity and kinetic properties

Both the affinity-purified wild-type and S41Y mutant forms of the bacterially expressed enzymes demonstrated robust activity. Enzyme activities were normalized to the amount of immunoreactive protein. The S41Y mutant is at least as active as WT, (S41Y 131 ± 6% compared to wild-type hTPH2 100 ± 3%, $n = 3$ for each enzyme; $p < 0.0025$).This is consistent with the apparent increase in activity reported by Haavik and colleagues (McKinney *et al.* 2009). To explore the kinetic basis underlying this phenomenon, we performed steady-state kinetic activity at varying concentrations of one substrate (tryptophan or tetrahydrobiopterin), while holding the other substrate concentration constant (Table 1). To summarize, there were no differences in Michaelis constants (K_Ms) for either BH_4 or tryptophan. By contrast, there was an increase in the V_{max} of the S41Y mutant when assayed with varying concentrations of either substrate. The V_{max} values were increased by either 80% or 42% (depending on the specific fixed concentrations of BH_4 or tryptophan, respectively).

The S41Y mutant displays reduced thermal stability *in vitro* at physiological temperature

The TPH enzymes are generally thermally unstable (Hasegawa and Nakamura 2010). A number of reports suggest that particular polymorphisms decrease stability further (Zhang *et al.* 2006; Winge *et al.* 2007). Inactivation time-course experiments were conducted for the affinity-purified wild-type hTPH2 and S41Y mutant enzymes at both 37°C and at 4°C (Fig. 1). Activity was measured following varying periods of incubation at 37°C over the course

of 90 min. The S41Y half-life for loss of enzyme activity at 37°C was markedly reduced (4.1 min; $n = 5$) compared to WT (32 min; $n = 5$) (Fig. 1a). However, at 4°C. Here, the half-lives were about the same 13 ± 2 h and 15 ± 1 h ($n = 3$) for wild-type and S41Y enzymes, respectively (Fig. 1b).

Doxycycline-induced expression of hTPH2 in PC12 cells

We created stably transfected PC12 cells expressing wild-type and S41Y hTPH2 enzymes to investigate enzyme activity in a eukaryotic cellular environment (at 37°C). Wild-type and S41Y constructs were created using a Tet-On pTRE-hyg expression vector (in this case as non-fusion, native proteins). This vector drives expression of the recombinant proteins via a cytomegalovirus (CMV) promoter controlled by a tetracycline operator. Vectors were stably transfected into PC12 Tet-On cells. These cells express the tetracycline transcription factor to permit DOX-mediated upregulation of tryptophan hydroxylase expression. As demonstrated in Fig. 2a, while there is a low basal level of hTPH2 expression in PC12-*hTPH2* cells, TPH activity is induced 5-fold following 48 h of induction with DOX. There is no detectable hTPH2 activity in non-transfected Tet-On PC12 cells (data not shown). The increase in DOX-stimulated hTPH2 activity is mediated by an increase in hTPH2 protein (Fig. 2a). Even so, PC12 cells express seven times greater endogenous rat TH activity than induced hTPH2 activity. Moreover, the induction of hTPH2 did not affect endogenous expression of rat TH protein or activity (Fig. 2b).

Comparison of wild-type and S41Y hTPH2 in PC12 cells

Detailed comparisons were then made between wild-type PC12-*hTPH2* and PC12-S41Y cell lines (Fig. 3). No morphological differences were observed in these cells, nor were their growth rates significantly different. Tet-On PC12 cells exhibited basal levels of S41Y expression that was induced following 48 h of DOX stimulation (Fig. 3a). Unlike bacterially expressed recombinant hTPH2 molecules, PC12 cell-expressed hTPH2 and S41Y proteins were completely soluble (Fig. 3b). Increased amounts of S41Y protein compared to wild-type hTPH2 resulted from increased mRNA levels (Fig. 3c) and produced comparable differences in hTPH2 activity (Fig. 3d). It is unknown whether these expression variations result from increased numbers of incorporated gene copy numbers or reflect the sites of incorporation. However, these are not clonal lines so there is cell-to-cell variation present within cell populations. Importantly, when hTPH2 enzyme activity levels are corrected for amounts of immuno-reactive protein (for example, Fig. 3a), there remains a 42% increase in the specific activity for S41Y compared to wild-type hTPH2 (Fig. 3e), similar to the bacterially expressed enzymes. In transfected PC12 cells, both hTPH2 enzyme isoforms were highly stabilized suggesting that cellular components (chaperones, post-translational modifications or binding partners) protect these enzymes from inactivation. However, in spite of having greater hTPH2 enzyme activity, S41Y-expressing cells synthesized less serotonin.

PC12-hTPH2 and PC12-S41Y cells synthesize and release serotonin

In spite of increased levels of S41Y mRNA, protein, and hTPH2 activity, stably transfected PC12-S41Y cells contained 33% less 5-HT than PC12-*hTPH2* cells (Fig. 4). Upon K^+-induced cellular depolarization, both sets of transfected cells released the majority of their 5-

HT stores into the medium. The expression of hTPH2 had no effect on the cellular content of DA (one of the endogenous catecholamines; data not shown). Examination of biogenic amines in the media produced variable and inconsistent results; therefore, studies focused on the cellular content of serotonin and DA. However, this also made it impossible to measure basal release of biogenic amines from the cells.

PC12 cell constituents stabilize hTPH2 and S41Y at 37°C

Stability of the recombinant hTPH2 enzymes was examined within the PC12 cell environment. Following 48-h induction with DOX, this tetracycline analog was removed from cell medium and replaced with cycloheximide (to block *de novo* protein synthesis). Cells were harvested at varying periods following termination of protein synthesis and hTPH2 activity and immunoreactive protein levels were assessed (Fig. 5a). The half-life for loss of enzyme activity for the two enzymes was comparable:11.9 ± 1.1 h for wild-type hTPH2 and 12.7 ± 1.6 h for S41Y ($n = 5$ for each determination). Decreases in enzyme activity were not mediated by losses of protein as assessed by immunoblot analysis (Fig. 5c). Interestingly, both enzymes demonstrated reduced stability at 37°C *in vitro* following preparation of PC12 homogenates (41.4 ± 1.5 min and 34.6 ± 1.7 min, for hTPH2 and S41Y, respectively), but the stability of the polymorphic enzyme was modestly lower (but statistically significant; $p < 0.05$; Fig. 5b).

In vitro cyclic AMP-dependent protein kinase A phosphorylation assays

Phosphorylation site predictions were performed on the hTPH2 WT and S41Y protein sequences with the program (http://scansite.mit.edu/motifscan_seq.phtml), and multiple predicted phosphorylation sites were identified. One such sequence, Y41 is a candidate for adenosine monophosphate-activated kinase phosphorylation. However, we were unable to detect any TPH2 phosphorylation when we performed *in vitro* phosphorylation assays of either hTPH2 WT or S41Y proteins with adenosine monophosphate-activated kinase (data not shown). In contrast, as shown in Fig. 6, we did observe a decrease in the PKA-mediated control phosphorylation of the S41Y mutant, relative to hTPH2 WT protein. Comparison of the stoichiometric ratios of PKA phosphorylated hTPH2 WT and S41Y suggest that the S41Y mutation results in 50% reduction of PKA phosphorylation. We have assayed the changes in enzyme activities upon phosphorylation. Unfortunately, the phosphorylation conditions required for PKA activity caused a dramatic decrease in bacterially expressed TPH2 activity levels. The resulting values were too low for us to reliably detect changes in the activity (data not shown). In independent experiments, we confirmed that hTPH2 is a substrate for CaMKII as previously reported by Kuhn and colleagues (Kuhn *et al.* 2007), but that the S41Y polymorphism did not alter that phosphorylation (data not shown).

Discussion

There is evidence that polymorphisms in the gene that codes for the rate-limiting enzyme in the synthesis of serotonin in the CNS (*hTPH2*) are associated with neuropsychiatric disorders (Zill *et al.* 2004a,b; Zhou *et al.* 2005b; Ke *et al.* 2006; Van Den Bogaert *et al.* 2006; Maron *et al.* 2007). The S41Y SNP (rs78162420) was first described in 2005 by Goldman and colleagues (Zhou *et al.* 2005b) and has been associated with peripartum depression and

bipolar disorder in a population of Han Chinese (Lin *et al.* 2007, 2009). In agreement with previous findings by Haavik and co-workers, we find that S41Y has a higher intrinsic specific activity compared to wild-type hTPH2 (McKinney *et al.* 2009). Here, we establish that this greater activity is mediated by increased V_{max} for S41Y and not via a change in the Michaelis constants of the enzyme for its cosubstrates. This finding is noteworthy because S41Y resides within the N-terminal regulatory domain [near the known phosphorylation site at serine-19 (Winge *et al.* 2008)]. Since the polymorphism is not in the catalytic domain, we must infer that it alters either the overall structure of the protein, or more likely, the regulatory domain/catalytic domain interaction.

Unexpectedly, we found that the S41Y SNP produces a reduction in the thermal stability of the already labile wild-type hTPH2. Specifically, following purification, the wild-type enzyme has a half-life of 32 min at 37°C *in vitro*, while S41Y has a $t_{½}$ of only 4 min. These results also suggest that the change in one amino acid in the regulatory domain has widespread consequences on the global structure of the enzyme. Such a reduction in stability, at 37°C, of the rate-limiting enzyme in the biosynthesis of serotonin would be expected to lead to reduced levels of this neurotransmitter *in vivo* (as shown in Fig. 4) and so might contribute to neuropsychiatric symptoms.

PC12 cells were originally derived from a rat pheochromocytoma and have several advantageous features for the present experiments. First, they are a primary resource for studying the regulation of an enzyme closely related to tryptophan hydroxylase – tyrosine hydroxylase – which is responsible for catecholamine biosynthesis (DA, norepinephrine, and epinephrine). Therefore, the cells possess all of the machinery for expression and vesicular packaging of biogenic amines. Second, rat and human *TPH2* genes are highly conserved and vary at only 31 positions of the 490 (and several of those are homologous substitutions). Therefore, rat *TPH2* binding partners might be expected to provide appropriate regulation of hTPH2. Finally, stably transfected PC12-*hTPH2* cells natively synthesize tyrosine hydroxylase, which is studied here as a positive control for characterizing hTPH2 regulation.

The stably transfected PC12-*hTPH2* and PC12-S41Y cells express *hTPH2* mRNA, protein, and demonstrate hTPH2 enzyme activity. An immediate benefit of this eukaryotic system is the expression of recombinant enzymes in a soluble state (Fig. 3b) – in contrast to bacterial expression (Carkaci-Salli *et al.* 2006). When corrected for variable amounts of immunoreactive protein, PC12 cells, expressing the S41Y polymorphic enzyme, possess higher intrinsic maximal hTPH2 activity than PC12 cells expressing wild-type hTPH2 (Fig. 3e). However, while PC12-S41Y cells have significantly more protein and activity, they produce less serotonin. This is in agreement with the findings of Lin and co-workers (Lin *et al.* 2007). We hypothesized that this would be due to reduced enzyme stability but, paradoxically, find that PC12 cell-expressed hTPH2 and S41Y exhibit comparable stabilities when *de novo* protein synthesis is inhibited. The enzymes are significantly stabilized with half-lives on the order of 12 h. These findings argue for the presence of binding chaperones and/or a favorable redox environment for stabilizing the enzyme within intact cells. For instance, Haavik and colleagues have extensively characterized the interaction of human TPH with the chaperone protein 14-3-3 (McKinney *et al.* 2005). When we prepared cell homogenates, however, stability is reduced to levels comparable to those seen with purified

bacterially expressed enzymes (30–40 min; Fig. 5b). Surprisingly, the S41Y recombinant protein is similar to wild-type – suggesting that stabilizing binding partners remain associated with the polymorphic (and inherently less stable) mutant enzyme.

The reason that the higher TPH activity of the S41Y variant produces less serotonin is not clear. Although we lack three-dimensional structural information about the regulatory domain of hTPH2, it is possible that residues of this domain may interact with the catalytic domain of hTPH2 to control substrate access. Mutation of these residues, such as S41Y, may open the access and increase the enzyme activity under *in vitro* conditions as we observe here. Consistent with this, we have previously reported that deletion of the regulatory domain increases the activity and solubility of the catalytic domain of this enzyme (Carkaci-Salli *et al.* 2006). However, when examining post-translational phosphorylation of recombinant hTPH2, we found that PKA-mediated modification is decreased by 50% (Fig. 6). The S41Y polymorphism is located in the regulatory domain of hTPH2 in proximity to one of two potential PKA phosphorylation sites (Ser^{19}). One intriguing possibility is that the S41Y polymorphism induces a conformational change within the regulatory domain such that the PKA phosphorylation on Ser^{19} is masked. Alternatively, the polymorphism may affect the other, less well-characterized phosphorylation site (Ser^{104}). The loss of phosphorylation by PKA in the S41Y mutant may disrupt post-translational regulation of TPH2 activity and decrease serotonin production, even though S41Y expression is higher than WT TPH2.

These studies provide important insights into the potential role of this SNP in bipolar disorder and peripartum depression as described by Lin *et al.* (2007). Notably, in spite of the fact that the S41Y enzyme has higher activity *in vitro*, it synthesizes less serotonin in intact cells. This strongly suggests that post-translational regulatory mechanisms are suppressing the steady-state activities of the polymorphic enzyme. In addition to altered enzyme phosphorylation, increased end-product feedback inhibition, or decreased activator protein binding (e.g., 14-3-3 activator interaction) may occur. The polymorphism may also disrupt 14-3-3 binding and the stabilization of the enzyme that is observed in the case of TH (Nakashima *et al.* 2007). Alternatively, the sensitivity of the enzyme to feedback inhibition by the end-product neurotransmitter serotonin may be altered by this SNP in the regulatory domain. In any event, the observation that cells that express more S41Y hTPH2 protein and activity, yet produce less serotonin, provides a biological link between the enzyme polymorphism and its genetic association with BPD and/or depression. The key finding, in this regard, is that even though PC12 cells make more of the polymorphic hTPH2 (with higher intrinsic activity), the cells produce less serotonin. Therefore, patients harboring this polymorphism are also expected to produce less serotonin. In summary, this work provides important mechanistic support for previous association studies and suggests that the S41Y polymorphism (rs78162420) disrupts serotonin synthesis contributing to depression and bipolar disorder (Lin *et al.* 2007, 2009). The availability of the stably transfected cell line that is analogous, in many respects, to the natural biogenic amine synthesizing environment of the serotonergic cell, will provide an important new platform for investigating the identity of binding partners/chaperones and regulatory mechanisms.

Acknowledgments and conflict of interest disclosure

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All experiments were conducted in compliance with the ARRIVE guidelines. The authors have no conflicts of interest to declare.

Abbreviations used:

5-HT	5-hydroxytryptamine
BH_4	tetrohydrobiopterin
BPD	bipolar disorder
DA	dopamine
DOX	doxycycline
H	cellular homogenates
hTPH2	human TPH2 gene
hTPH2	tryptophan hydroxylase 2 protein
K_Ms	Michaelis constants
S41Y	tyrosine for serine substitution at position 41
S	high speed supernatants
SNP	single-nucleotide polymorphism
TH	tyrosine hydroxylase
V_{max}	maximal velocity
WT	wild type

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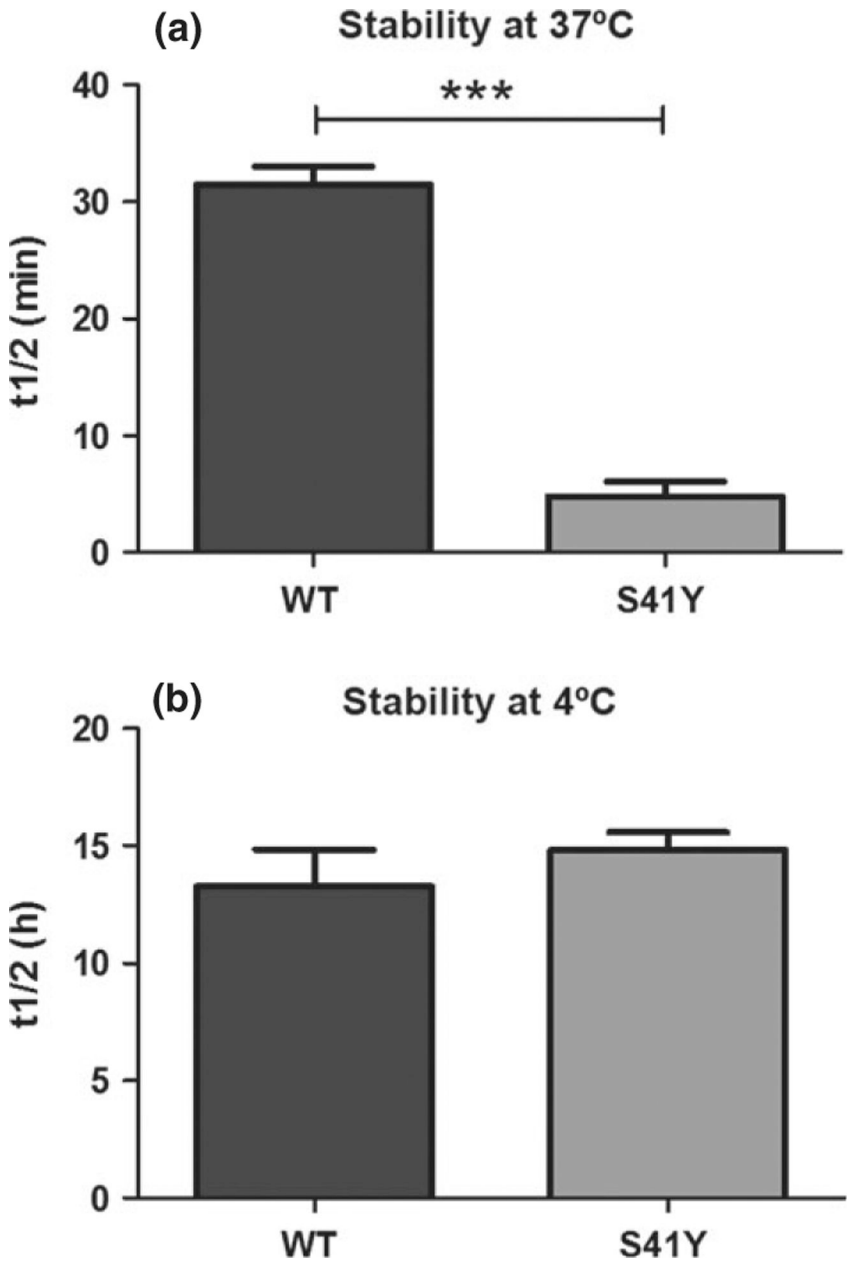

Fig. 1.

The tyrosine for serine substitution at position 41 (S41Y) mutation is associated with reduced thermal stability. (a) Purified recombinant enzymes were incubated for varying periods of time at 37°C and then assayed for remaining enzyme activity (expressed as percentages of starting enzyme activity) of wild-type (WT) hTPH2 and S41Y. (b) A similar stability assay was conducted at 4°C, resulting in stabilization of hTPH2 proteins. All data are expressed as mean ± SEM ($n = 5$ for 37°C; $n = 3$ for 4°C). ***$p < 0.001$.

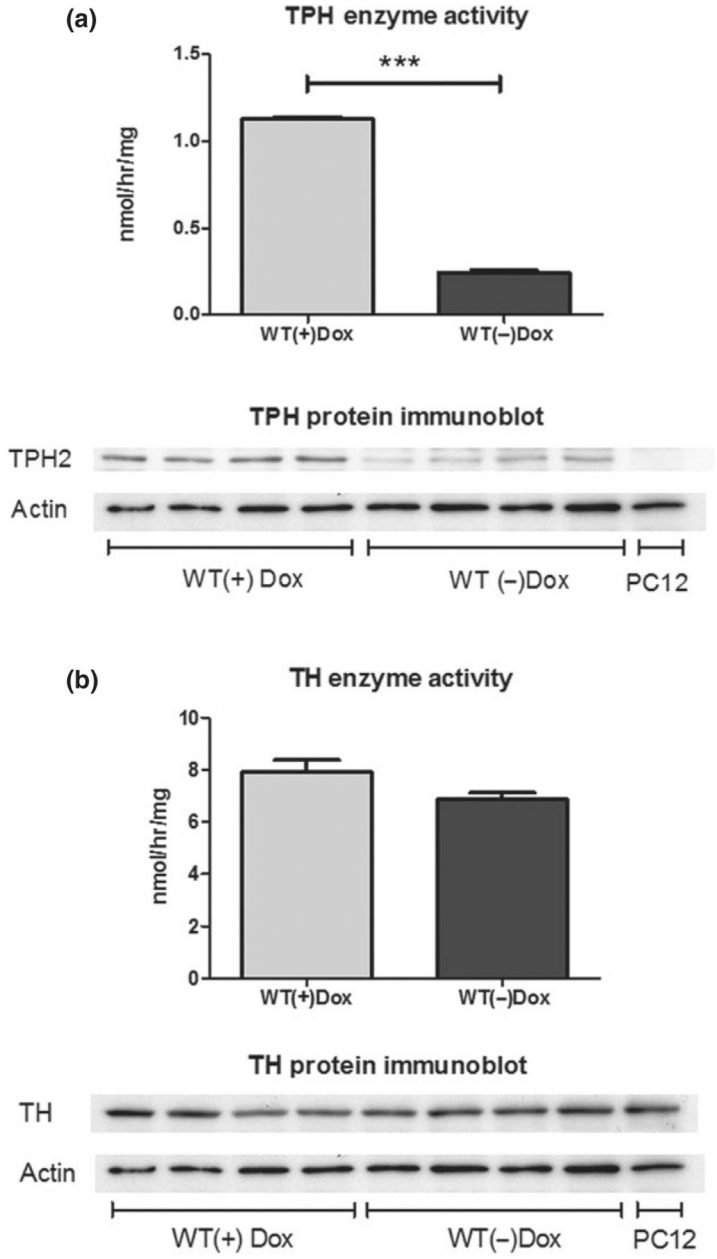

Fig. 2.

Stable expression of hTPH2 in rat PC12 cells. WT hTPH2 was stably transfected into Tet-On PC12 cells (rat pheochromocytoma cells stably expressing the tetracycline-regulated activator) in the pTRE2hyg plasmid vector. Following extended antibiotic selection, cells were challenged with doxycycline (DOX) for 48 h. (a) hTPH2 activity was determined in cells with and without DOX ($n = 3$) and hTPH2 immunoreactivity was visualized by western blotting [hTPH2 was detected by probing with a mouse anti-TPH monoclonal antibody (diluted 1 : 3000; Sigma)]. The empty PC12 cell lane demonstrates that there is no detectable hTPH2 protein expressed in non-transfected cells. (b) As a negative control, the effects of DOX treatment were evaluated on the endogenous PC12 cell tyrosine hydroxylase (rat TH) activity and protein expression [a mouse anti-TH monoclonal antibody (diluted 1 :

3000; Sigma) was used to quantify TH levels]. All data are expressed as mean ± SEM (n = 4). ***$p < 0.001$.

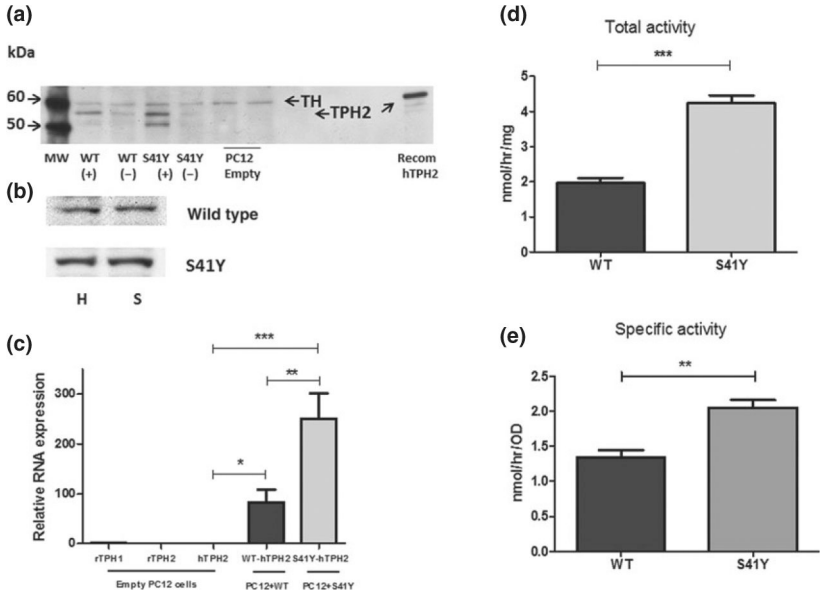

Fig. 3.

Comparison of WT hTPH2 and tyrosine for serine substitution at position 41 (S41Y) expression in PC12 cells. Stable transfectants of tetracycline-regulated WT hTPH2 and S41Y PC12 cells were evaluated. (a) doxycycline (DOX)-stimulated expression of hTPH2. The same number of cells was lysed to examine expression efficiency. A total of 15-μg protein were loaded into each well. Immunoblots were probed with a TPH antibody that detects the exogenous hTPH2 and endogenous rat TH (this antibody cross-reacts with both enzymes and TH bands in the figure therefore serve as an internal control). Empty PC12 cell protein and recombinant hTPH2 were loaded as controls (the size of the recombinant protein is 63 kDa as a result of a 26-amino acid fusion segment). Immunoblots were probed with a TPH antibody that detects the exogenous hTPH2 and endogenous rat TH (the antibody cross-reacts with both enzymes). Note that DOX did not affect rat TH expression and that, while the TPH antibody recognizes both rat TH and hTPH2, it does not do so equally, so that relative expression comparisons cannot be made. (+) Indicates treatment with DOX; (−) indicates cells without DOX treatment. (b) Unlike bacterially expressed hTPH2, enzyme expressed in PC12 cells was soluble as determined from high speed supernatants (S) of cellular homogenates (H). (c) Higher S41Y expression levels are due to increased mRNA levels as determined by RT-PCR. Rat TPH1 = rTPH1; rat TPH2 = rTPH2. (d) Higher amounts of S41Y mRNA and immunoprotein are reflected in increases enzyme activity. (e) Following correction for specific amounts of hTPH2 proteins, the S41Y mutant exhibited higher inherent activity (similar to Table 1 for bacterially expressed proteins). All data are expressed as mean ± SEM ($n = 3$). ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$.

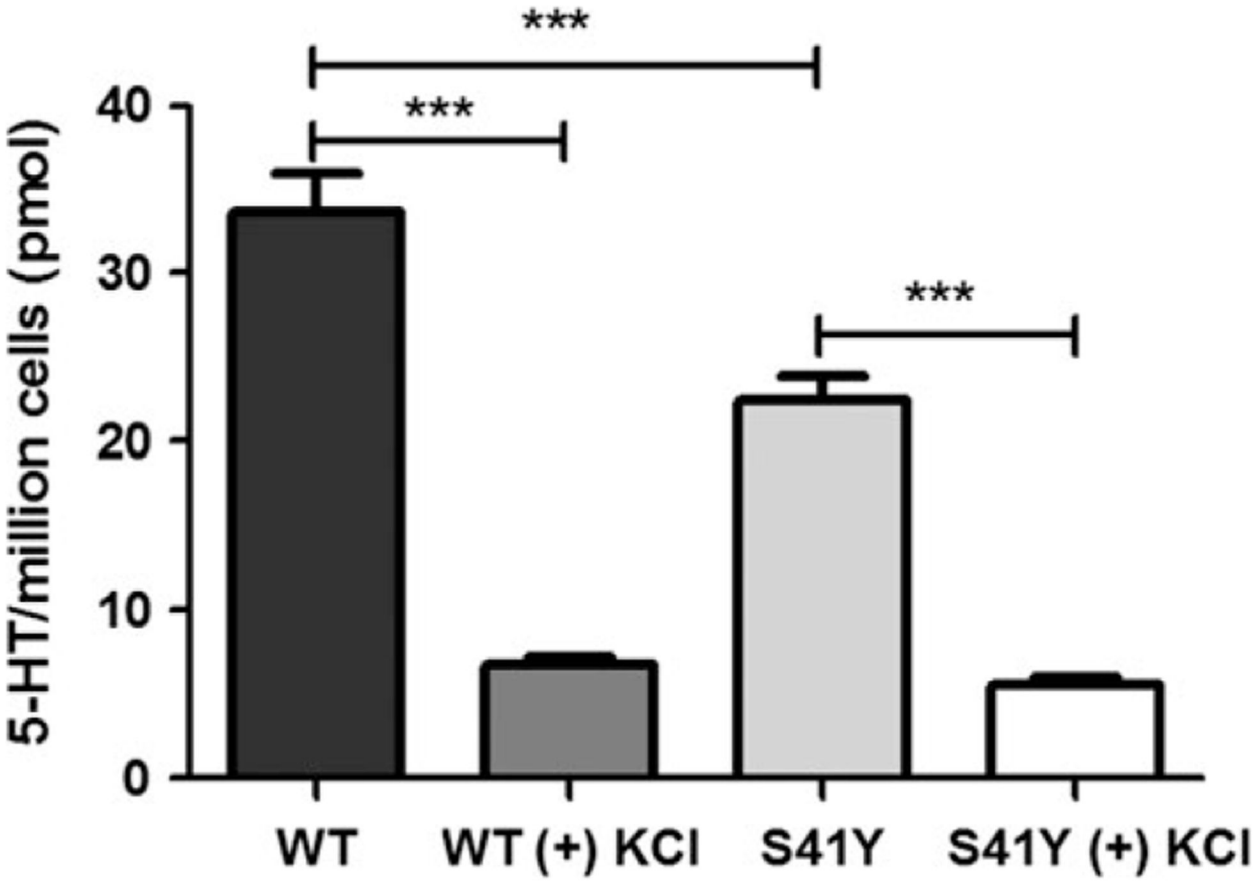

Fig. 4.

PC12 cells stably transfected with recombinant hTPH2 synthesize serotonin and release it in response to depolarization. Cellular levels of 5-hydroxytryptamine (5-HT) (serotonin) were determined in WT *hTPH2*- and tyrosine for serine substitution at position 41 (S41Y)-transfected PC12 cells (following 48 h of doxycycline (DOX) induction) before and after potassium-induced depolarization. All data are expressed as mean ± SEM ($n = 3$). ***$p < 0.001$.

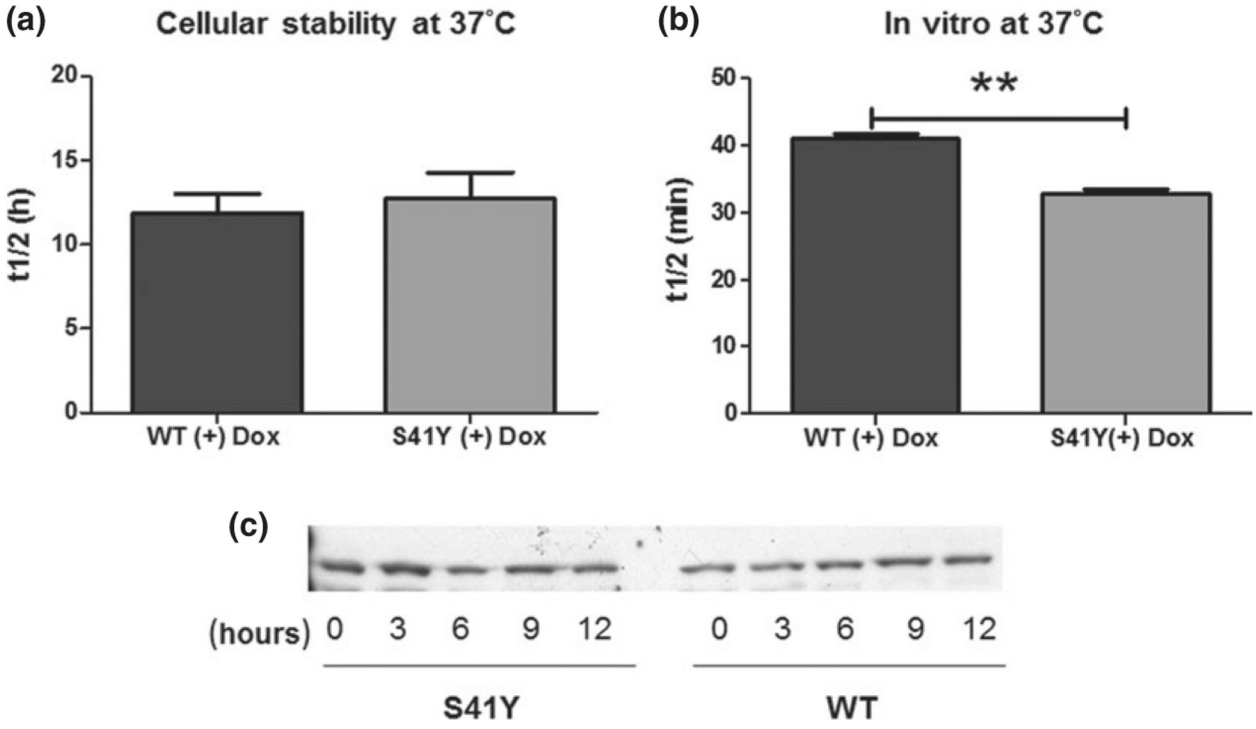

Fig. 5.

WT hTPH2 and tyrosine for serine substitution at position 41 (S41Y) enzyme stability in PC12 cells and cellular homogenates are comparable. WT and S41Y hTPH2 expression were induced in PC12 cells with 48 h of doxycycline (DOX) treatment. (a) Following DOX induction, protein synthesis was terminated by removing this tetracycline analog (to stop induction of hTPH2 expression) and adding cycloheximide (to block *de novo* protein synthesis). Cell samples were harvested at the designated time points. Enzyme activities are calculated as percentages of starting values. All data are expressed as mean ± SEM ($n = 5$) of the $t_{1/2}$ for loss of enzyme activity. (b) Following enzyme induction, cell homogenates were prepared and incubated *in vitro* at 37°C for the specified times. Note the different time scale (min instead of h). All data are expressed as mean ± SEM ($n = 3$) of the $t_{1/2}$ for loss of enzyme activity. $^{**}p < 0.01$ (c) Western blot analysis of recombinant hTPH2 levels at the times noted in (a).

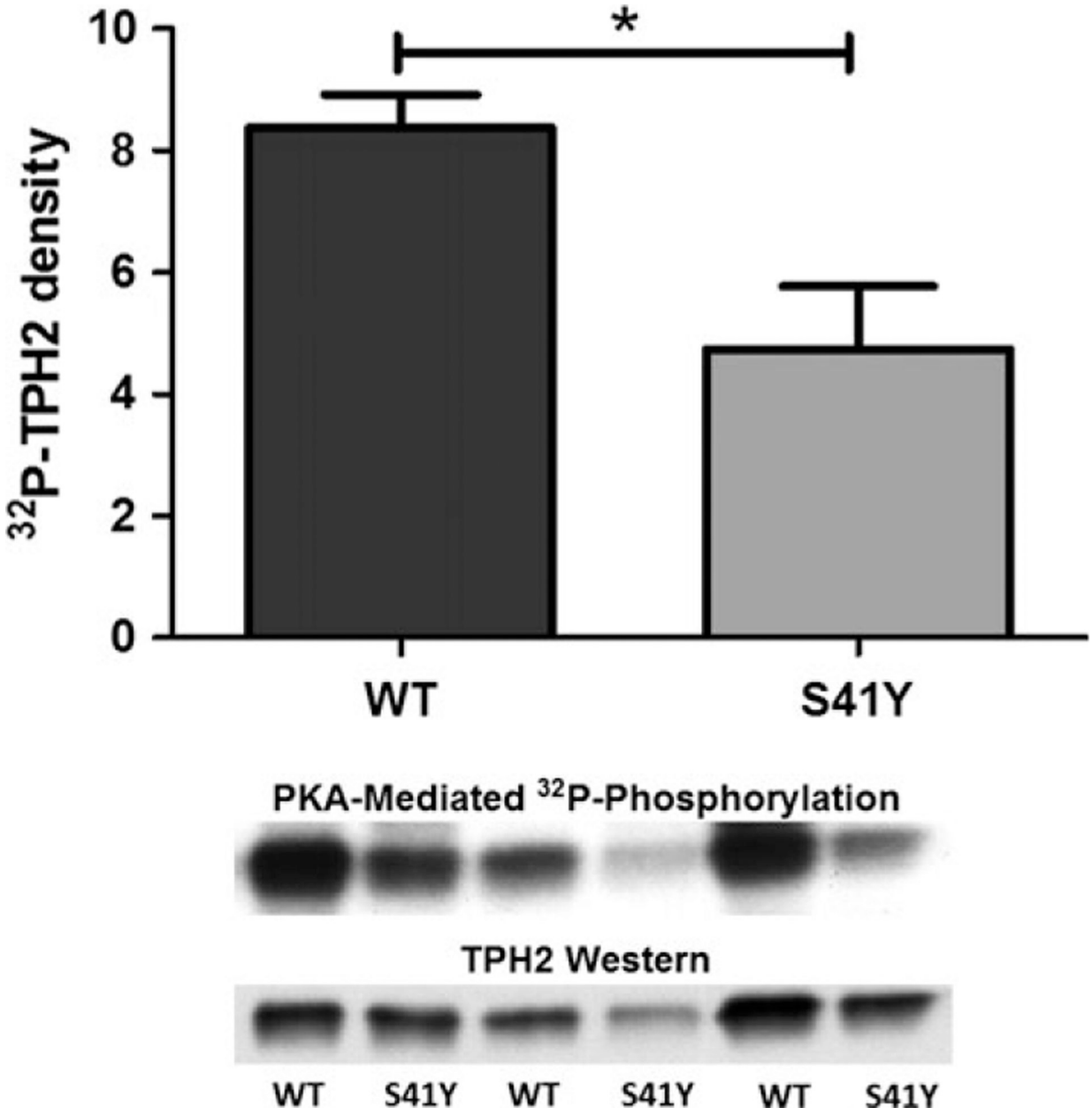

Fig. 6.

Protein kinase A (PKA)-mediated phosphorylation of the tyrosine for serine substitution at position 41 (S41Y) mutant and the WT protein of hTPH2. PKA phosphorylation was assessed by autoradiography using [γ-^{32}P]-ATP following resolution of phosphorylated proteins by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) gel electrophoresis. Total TPH proteins were detected by western blot analysis using anti-TPH antibodies. Quantitation of phosphorylated and total TPH protein was conducted using Image-Quant. The results for three independent protein preparations analyzed on the same blot ($n = 3$; $^*p < 0.05$) are illustrated in this figure and identical findings were obtained in five additional experiments.

Table 1

Steady-state kinetic parameters for the recombinant wild-type and S41Y variant hTPH2 isolated from bacteria

	$K_{m,\ L\text{-}Trp}$ (μM)	$V_{max,\ L\text{-}Trp}$ (nmol/h/OD)	$K_{m,\ BH4}$ (μM)	$V_{max,\ BH4}$ (nmol/h/OD)
Wild-type hTPH2	30.2 ± 11.3 $n = 3$	114.0 ± 16.0 $n = 3$	25.9 ± 7.9 $n = 5$	106.2 ± 12.6 $n = 5$
S41Y	46.9 ± 10.8 $n = 3$	205.3 ± 12.3 $n = 3$ **	21.3 ± 6.1 $n = 6$	150.4 ± 13.9 $n = 6$ *

** $p < 0.01$;

* $p < 0.05$.

Michaelis–Menten constants (K_M) and maximal velocities (V_{max}) were determined at varying concentrations of one substrate while the concentration of the other substrate remained fixed. V_{max} values were standardized to amounts of immunoreactive hTPH2 protein (optical density) and are expressed as nmol/h/OD.