Biochemistry and Biophysics Reports 35 (2023) 101527

Contents lists available at ScienceDirect

Biochemistry and Biophysics Reports

journal homepage: www.elsevier.com/locate/bbrep

The A328 V/E (rs2887147) polymorphisms in human tryptophan hydroxylase 2 compromise enzyme activity

Nurgul Carkaci-Salli [a,1], Maria C. Bewley [b,1], Izel Tekin [a], John M. Flanagan [b], Kent E. Vrana [a,c,*]

[a] Departments of Pharmacology, Pennsylvania State University College of Medicine, Hershey, PA, 17033, USA
[b] Biochemistry and Molecular Biology, Pennsylvania State University College of Medicine, Hershey, PA, 17033, USA
[c] Pennsylvania State University College of Medicine, Hershey, PA, 17033, USA

ARTICLE INFO

Keywords:
Tryptophan hydroxylase
Serotonin (5-hydroxytryptamine 5-HT)
Single nucleotide polymorphism
Substrate inhibition
Enzyme activity

ABSTRACT

Human tryptophan hydroxylase 2 (hTPH2) is the rate-limiting enzyme for serotonin biosynthesis in the brain. A number of naturally-occurring single nucleotide polymorphisms (SNPs) have been reported for hTPH2. We investigated the activity and kinetic characteristics of the most common missense polymorphism rs2887147 (A328 V/E; 0.92% allelic frequency for the two different reported SNPs at the same site) using bacterially expressed hTPH2. The recombinant full-length enzyme A328E had no measurable enzyme activity, but A328V displayed decreased enzyme activity (V_{max}). A328V also displayed substrate inhibition and decreased stability compared to the wild-type enzyme. By contrast, in constructs lacking the N-terminal 150 amino acid regulatory domain, the A328V substitution had no effect; that is, there was no substrate inhibition, enzyme stabilities (for wild-type and A328V) were dramatically increased, and V_{max} values were not different (while the A328E variant remained inactive). These findings, in combination with molecular modeling, suggest that substitutions at A328 affect catalytic activity by altering the conformational freedom of the regulatory domain. The reduced activity and substrate inhibition resulting from these polymorphisms may ultimately reduce serotonin synthesis and contribute to behavioral perturbations, emotional stress, and eating disorders.

1. Introduction

The neurotransmitter serotonin [5-hydroxytryptamine (5-HT)] controls a broad range of biological functions including mood, body temperature, sleep, sexuality, and appetite. Reduced levels of serotonin signaling have been implicated in a range of disorders including depression, bipolar disorder, irritable bowel syndrome, and anxiety disorders [1–4]. There is an ongoing discussion as to whether tryptophan hydroxylase (TPH; rate-limiting enzyme in serotonin biosynthesis) polymorphisms may be linked to ADHD, and depression- and anxiety-linked personality traits [5,6].

Serotonin is synthesized in 2 steps from the amino acid tryptophan with tryptophan hydroxylase catalyzing the rate-limiting steps [7,8]. Until 2003, it was thought that only one isoform of TPH existed; however, deletion of this gene did not appreciably reduce the levels of 5-HT in the brain, leading to the discovery of TPH2, which is responsible for 5-HT synthesis in the brain throughout life [9,10]. TPH2 is also found within nerve terminals in the small intestine and ablation of this gene causes disturbances in colonic function and loss of GI motility [11–13]. Previous TPH2 knockout studies have shown that animals are grossly morphologically normal and fertile. On the other hand, their central 5-HT levels are dramatically reduced, with normal raphe serotonergic neuron formation [14]. The lack of serotonin in the central nervous system produces altered early postnatal growth, growth rates, heart rate and blood pressure, thermoregulation, autonomic control of sleep, survival, as well as aggressive behavior and maternal neglect [15,16]. In another study of the TPH2 knockout in zebrafish (Medaka), the homozygous knockout leads to widespread behavioral changes [17].

Abbreviations: (hTPH2), Human tryptophan hydroxylase 2; (SNPs), single nucleotide polymorphisms; (5-HT), 5-hydroxytryptamine; (A328V), alanine-to-valine mutation on residue 328; (A328E), alanine-to-glutamic acid mutation on residue 328; (NΔ150-hTPH2), regulatory domain removed human tryptophan hydroxylase 2; (IDT), Integrated DNA Technologies; (hPAH), human phenylalanine hydroxylase; (TyrA), prephenate dehydrogenase; (ANOVA), one-way analysis of variance; (PTD), prephenate dehydratase; (CNS), central nervous system; (hTH), human tyrosine hydroxylase.

* Corresponding author. Department of Pharmacology, R130, Penn State College of Medicine, PO Box 850, Hershey, PA, 17033-0850, USA.
E-mail address: kvrana@psu.edu (K.E. Vrana).

[1] The first two authors contributed equally.

https://doi.org/10.1016/j.bbrep.2023.101527
Received 20 June 2023; Received in revised form 3 August 2023; Accepted 3 August 2023

TPH2 belongs to a superfamily of aromatic amino acid hydroxylases (AAAH). These enzymes are iron- and tetrahydrobiopterin (BH_4)-dependent monooxygenases with a common catalytic mechanism [18]. All family members share a structurally homologous catalytic domain, an amino terminal regulatory domain of varying length and a C-terminal tetramerization domain that can be removed with retention of enzyme activity [19–22]. TPH2 contains a larger regulatory domain than TPH1 and residues 1–50 do not align with other hydroxylase family members. For hTPH2, the central catalytic domain (residues 151–466) is flanked by an amino terminal regulatory domain (1–150) and a C-terminal oligomerization tetramerization domain (residues 467–490) (Fig. 1A). Deletion of the regulatory domain results in protein that is active and maintains the same tetrameric quaternary structure as the full-length enzyme (Supplementary Fig. 2). In summary, the N-terminal domain of these enzymes is not essential for enzyme activity, but serves to regulate the activity. Similarly, the smaller C-terminal domain (~20 amino acids) supports tetramer formation to differing extents depending on the enzyme.

Polymorphisms in the TPH2 gene in humans and non-human primates have been linked to neurological and behavioral abnormalities [2, 23–30], although the results of some of these studies remain controversial. For this reason, it is important to understand the mechanisms whereby missense mutations in hTPH2 might affect production of serotonin. To date, hundreds non-synonymous single nucleotide polymorphisms (SNPs) have been reported within the coding region of hTPH2 (NCBI, dbSNP). The vast majority of these SNPs have not been functionally characterized, and are present at very low allelic frequency within the general population. Indeed, only 5 coding region SNPs (both synonymous and non-synonymous) have been documented with appreciable allelic frequency (≥0.005) and only 37 SNPs have been documented with allelic frequency ≥0.0001 (https://www.ncbi.nlm.nih.gov/snp/?term=TPH2).

The rs2887147 SNP, that encodes an alanine-to-glutamic acid (A328E) or alanine-to-valine (A328V) substitution at position 328 within the catalytic domain of hTPH2, has been documented in the NCBI databases by seven different study groups (https://www.ncbi.nlm.nih.gov/projects/SNP/snp_ss.cgi?subsnp_id = ss4079267; accessed on April 1, 2023). In general, these variants, while rare, are the most common missense polymorphisms in human TPH2. The allelic frequencies are 0.92% for A328V and 0.0008% for A328E. Previously, the A328V SNP was partially characterized and the authors concluded that A328V represented a loss of function mutation that could not be explained in terms of reduced solubility or expression [31]. In the present study, we performed detailed kinetic analysis on this variant in the presence and absence of the regulatory domain.

2. Methods

2.1. Enzyme polymorphisms

To determine the functional consequences of the naturally occurring polymorphisms (A328E, A328V), they were constructed in hexahistidine-tagged wild type enzyme using the primers described in

Table 1
Nucleotide sequences of oligonucleotide primers used for cloning, sequencing, and site directed mutagenesis of hTPH2.

Primer name	Primer sequence (5′ to 3′)
T7 promoter - sense	5′-AATACGACTCACTATAGG-3′
T7 terminator - antisense	5′-GCTAGTTATTGCTCAGCGG-3′
A328E - sense	5′-CACTACTTGAGGATCCT-3′
A328E - antisense	5′-AGGATCCTCAAGTAGTG-3′
A328V - sense	5′-CACTACTTGTGGATCCT-3′
A328V - antisense	5′-AGGATCCACAAGTAGTG-3′

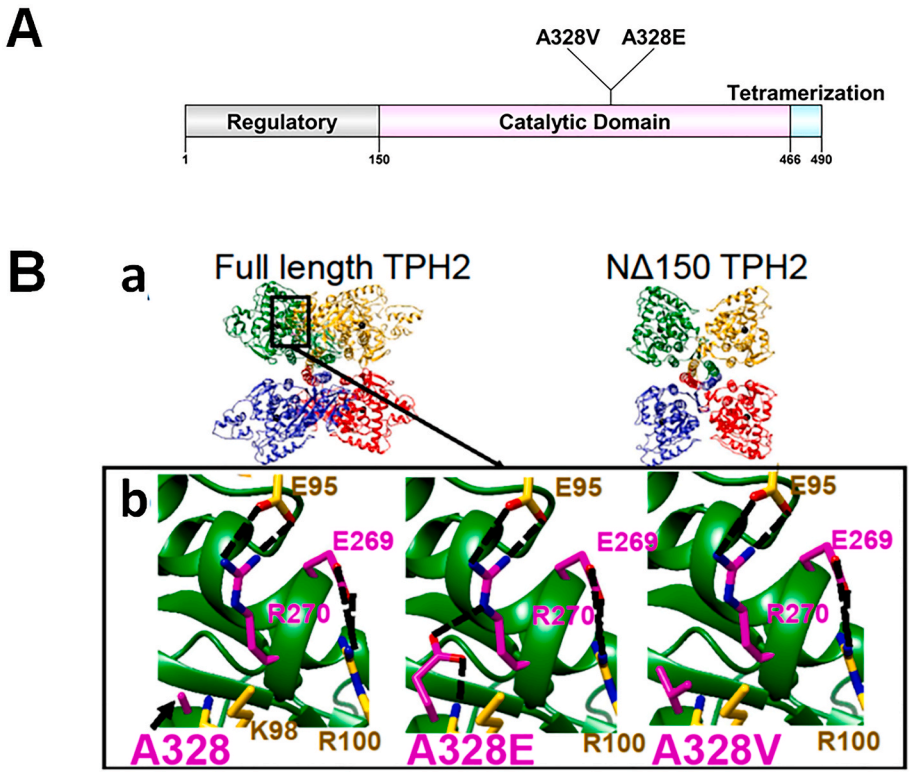

Fig. 1. A. Schematic diagram of hTPH2 domain organization showing the location of the A328 E/V polymorphisms. The amino terminal regulatory domain (amino acids 1–150 of hTPH2), the catalytic domain (residues 151 through 465) and the leucine zipper-based tetramerization domain (residues 466–490) are shown as blocks. **B.** A328 is located at the binding interface of the ACT domain from an adjacent molecule. Upper Panels – Ribbon diagram of full length TPH2 model (left) and NΔ150 TPH2 (right). Domains are colored individually (1, green; 2, gold; 3, red; 4, blue) and the iron atoms are shown as black spheres. Analogous to phenylalanine hydroxylase, residues 1–149 are domain-swapped with an adjacent molecule. Lower Panels – Alanine 328 is located in a depression on the surface of the catalytic domain and, in the absence of the ACT domain (NΔ150-TPH2), would be surface-exposed. Left panel: In the full-length protein, it is predicted to form interdomain salt-bridging interactions as labelled. Right panel: Substitution at 328 with a valine residue can be easily accommodated and should have little predicted effect on catalysis. Center panel: A glutamic acid residue at position 328, however, would add charge neutralization interactions that might stabilize the ACT domain in this position, impacting any conformational changes necessary for function. Selected side chains from molecules 1 and 2 are shown in stick representation and colored magenta and yellow, respectively; oxygen and nitrogen atoms are colored red and blue, respectively. Putative hydrogen bonds are shown as black dashed lines. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Table 1 (using the QuikChange Site Directed Mutagenesis Kit (Stratagene, La Jolla, CA)). Also, A328E and A328V mutations were created in the NΔ150 catalytic domain lacking the N-terminal regulatory domain [21]. All primers were obtained from IDT (Integrated DNA Technologies, Inc., Coralville, IA). PCR conditions were: denaturation at 95 °C, annealing at 55 °C, and extension at 68 °C for 20 cycles.

2.2. Expression and purification of hTPH2

Full length hTPH2 and variants were expressed in the bacterial pET28 TEV recombinant vector. Altered recombinant proteins were expressed in BL21 (DE3) pRIL (*E. coli*) cells in ZYP-5052 auto-induction media and proteins partially purified on HiTrap nickel columns as previously described [21,32].

2.3. Enzyme activity assay

A radio-enzymatic ^3H_2O release assay was used to measure hTPH2 activity as previously described [21,33,34]. Activity values derived from each assay were normalized to specific hTPH2 protein as determined by Western blot analysis (ImageQuant TL v2005). Michaelis-Menten models of enzyme kinetic analyses were conducted by varying the substrate concentrations of tryptophan in the range 3–400 μM and BH_4 in the range 0.5–200 μM separately, each at a fixed concentration of the other substrate (50 μM BH_4 and 50 μM tryptophan, respectively), and at ambient saturating concentrations of oxygen. The resulting data were converted to specific enzyme activities (correcting for varying radioactive amino acid concentrations, as necessary) and analyzed by Prism (GraphPad Prism 5 Software, San Diego, CA) to determine kinetic constants (K_M, V_{max}, K_i [substrate inhibition]).

2.4. Enzyme stability

Purified wild type hTPH2 and variants were incubated at 37 °C and aliquots were removed and placed on ice every 5 min from initiation to 30 min. Enzyme activity was then determined as described above and analyzed as a function of activity decay (on a logarithmic plot) with substrate concentrations of 50 μM BH_4 and 50 μM tryptophan. Given the previously documented stability of the NΔ150-hTPH2 construct (the enzyme lacking the amino terminal regulatory domain [21]), these enzymes were analyzed over the course of 12 h.

2.5. SDS-PAGE and structural analysis

Purified recombinant proteins were subjected to SDS-PAGE in 4–12% NuPAGE Bis/Tris gels and analyzed by Western blot to confirm molecular weights and to measure the abundance of the proteins as described previously [21]. Partially purified wild type hTPH2 and A328V (both full length and NΔ150 TPH2) were subjected to size-exclusion chromatography using a Superdex 200 10/300 GL column (Amersham Bioscience) in 25 mM HEPES (pH 7.5), containing 100 mM NaCl, 1 mM DTT, and 0.1 mM EDTA as previously described [21].

2.6. Homology modeling

To generate a model of the tetrameric NΔ150 TPH2, the unpublished structure of the catalytic domain of TPH2 determined to 2.6 Å resolution was employed (4V06.pdb) by applying a rotation to the dimer across a crystallographic dyad. To generate a model of the full-length construct, homology modeling was performed. TPH2 belongs to the superfamily of pterin-dependent aromatic amino acid hydroxylases and the structures of other family members have been determined, providing a range of templates on which to model full length hTPH2. The crystal structure of human phenylalanine hydroxylase (hPAH) in an auto-inhibited conformation (5DEN.pdb) was found to be the best template for residues 41–490 (56% identity; GA341 = 1; dope = −0.9). A weak model (GA341 = 0.86, Zdope = 0.31) for the regulatory domain (residues 3–139) was also generated based on the structure of L-phenylalanine-inhibited prephenate dehydratase (2QMX residues 130–275; identity 23%). This dimeric protein has an N-terminal PTD and a C-terminal ACT domain (the name originates from three of the proteins in the domain family: aspartokinase, chorismate mutase, and TyrA (prephenate dehydrogenase)). Since the two homology models contained ~100 amino acids that overlap, superposition of equivalent residues in each model allowed a full-length homology model to be constructed. Superposition between this model and the structures of other members of this protein family show excellent agreement within the catalytic domains. By contrast, the relative tilt of the terminal helix varied significantly resulting in larger RMSD between the monomers comprising the tetramer. Therefore, in order to simplify modeling, the entire structure of PAH in its auto-inhibited state was used as a template for the tetrameric full length TPH2 model presented here.

2.7. Statistical analysis

The hTPH2 activity determinations were analyzed using one-way analysis of variance (ANOVA), followed by an unpaired Student's t-test (no data points were excluded for statistical analysis). Statistical significance was associated with values of $p < 0.05$. GraphPad-5 Prism software was used for kinetic constant determinations and substrate inhibition calculations.

3. Results

3.1. Expression efficiency and solubility of full length and NΔ150-hTPH2 expressed in E. coli

The full-length wild type and variant hTPH2 proteins containing an N-terminal hexa-histidine affinity tag expressed well in the auto-induction media (a representative Coomassie stained gel is presented Fig. 2A). However, based on the total expression and quantification of Coomassie-stained SDS-PAGE gels, only about 6% of the enzyme is recovered in a soluble form (data not shown) consistent with previous reports [21]. These proteins were therefore quantified based on the densities determined by Western blot analysis (a representative Western blot in presented in Fig. 2B). By contrast, removal of 150 amino acids from the amino terminus (NΔ150 WT, NΔ150 A328V and NΔ150 A328E) resulted in approximately 70% of the protein being soluble consistent with our previous report [21]. Electrophoresis demonstrated that wild type and A328V and A328E hTPH2 (in both full length and NΔ150 forms) displayed identical molecular weights on denaturing SDS-PAGE (Fig. 2). Size exclusion chromatography demonstrated that they all migrated as tetramers (Supplementary Fig. 1 and Supplementary Fig. 2). Note that insufficient amounts of the inactive full-length A328E could be purified for size exclusion chromatography. Furthermore, as demonstrated by these gel filtration profiles, while the enzymes are extensively purified, they are not homogeneous.

3.2. Enzyme activity

Initial enzyme activity assays were performed using 50 μM BH_4 and 50 μM L-tryptophan. In the context of the full-length protein, the A328V variant had reduced activity (60% reduction in activity compared to wild type), whereas the A328E variant had no measurable activity (Fig. 3A). This observation was similar when the regulatory domain was removed; although, NΔ150-A328E had a very small, albeit reproducible, activity (2% of wild type; Fig. 3B).

3.3. Thermal stability of the wild type and variant proteins

The stabilities of the hTPH2 and A328V variant proteins, expressed from the full length and NΔ150-hTPH2 constructs, were determined at

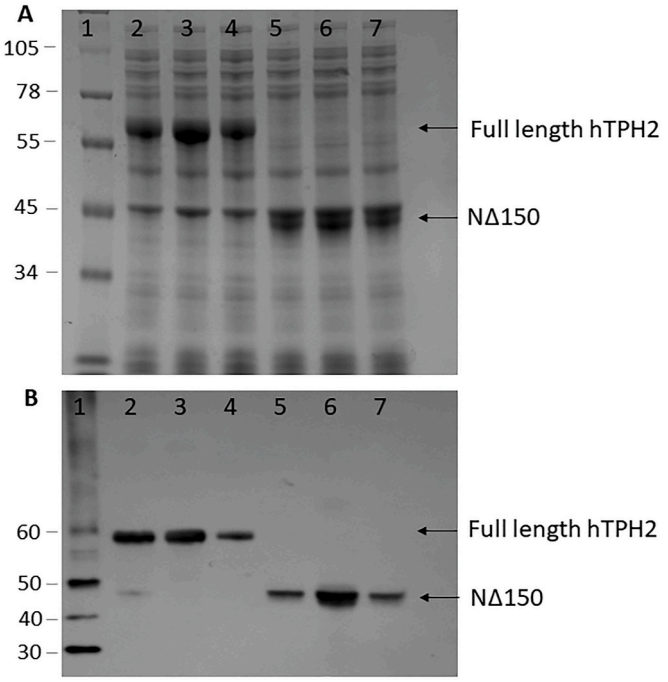

Fig. 2. A. Representative Coomassie staining of total proteins expressed in autoinduction media [32] in *E. coli* BL21 (DE3) pRIL. Lane 1 – Molecular weight markers; High speed supernatants of TPH expressed in *E. coli* BL21: Lane 2 – Wild-type hTPH2; Lane 3 – A328E; Lane-4 A328V, Lane 5 – Wild Type NΔ150; Lane 6 – NΔ150-A328E; Lane 7 – NΔ150-A328V. **B.** Representative Western blot of the partially purified TPH: Lane 1 – Molecular weight markers; Lane 2 – Wild Type hTPH2; Lane 3 – A328E; Lane 4 – A328V; Lane 5 – Wild Type NΔ150; Lane 6 – NΔ150-A328E; Lane 7 – NΔ150-A328V.

37 °C. A328E had insufficient activity (in either construct) to accurately measure stability, the half-life of the A328V variant was reduced by 50% with respect to the wild type hTPH2 enzyme (Fig. 4). However, just as previously reported in Carkaci-Salli et al. [21], the NΔ150 constructs were significantly stabilized and there was no difference between NΔ150 wild type and NΔ150 A328V (Fig. 4; note differences in scale).

3.4. Kinetic parameters

The kinetic properties of the wild type and A328V variant hTPH2 enzymes were investigated in the context of the full length and NΔ150-hTPH2 (Table 2). In the full-length enzyme, the K_M values for tryptophan and BH_4 for the A328V variant were similar to the wild type enzyme; however, in initial screening assays, the activity at sub-saturating substrate concentrations was reduced (Fig. 3A). In agreement with this, during Michaelis-Menten analysis, the maximal velocity obtained under varying BH_4 concentrations was found to be reduced approximately 6-fold (Table 2, top panel) and with varying tryptophan concentrations, the V_{max} was reduced over 3-fold. Within the enzyme lacking the amino terminal regulatory domain (NΔ150) these effects were abolished and none of the kinetic constants were statistically different (Table 2, bottom panel). Significant tryptophan substrate inhibition was detected for the A328V variant enzyme within the context of full-length enzyme (Fig. 5B; K_i ~300 μM, data not shown), but not observed in the construct lacking the N-terminal 150 amino acid regulatory domain (Fig. 5D). No appreciable substrate inhibition was observed for either of the wild-type enzymes (Fig. 5A and C). Moreover, no substrate inhibition was evident for tetrahydrobiopterin (see Fig. 6).

3.5. Homology modeling

In our homology models, alanine 328 resides within a hydrophobic

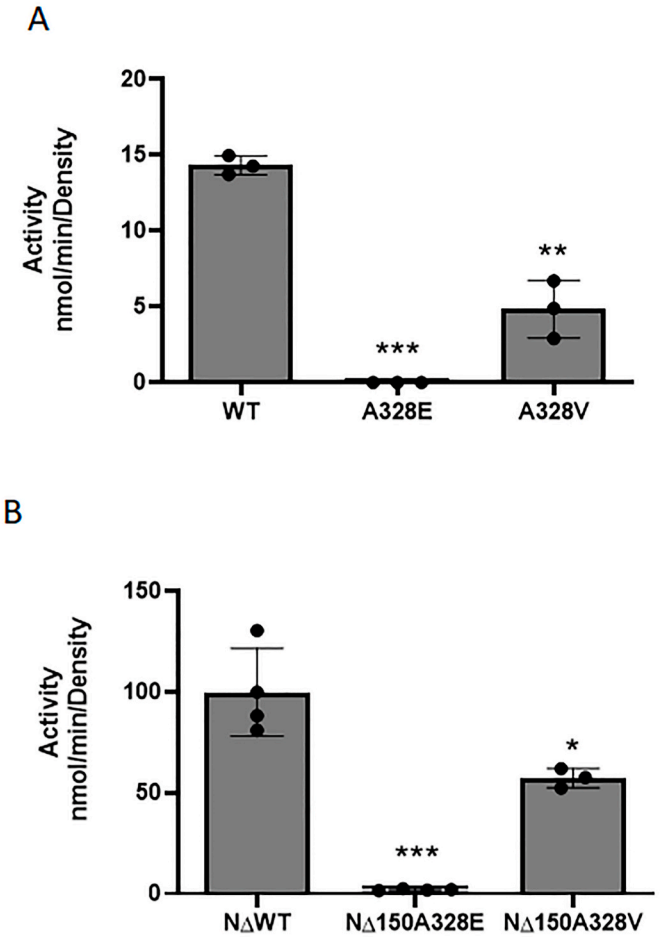

Fig. 3. A. Enzyme activity of full-length wild type hTPH2 (WT), A328E and A328V enzymes assayed under sub-saturating conditions of 50 μM tetrahydrobiopterin (BH_4) and 50 μM tryptophan. **B.** Activity of the variant enzymes in the context of the NΔ150-hTPH2 catalytic domain. Data are presented as mean ± SEM (n = 3 independent cell culture preparations, protein purifications, and enzyme activity assays). *p < 0.05; **p < 0.01; ***p < 0.001.

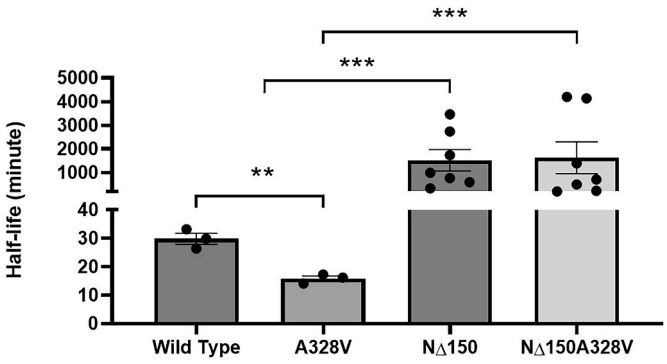

Fig. 4. Thermal stability of full-length WT hTPH2 and polymorphic A328V at 37 °C. Data are presented as mean ± SEM (n = 3 independent cell culture preparations, protein purifications, and enzyme activity assays) for both full-length and NΔ150-hTPH2 forms of the enzyme. **p < 0.01; ***p < 0.001.

depression on the surface of the catalytic domain. For the NΔ150 hTPH2 construct, substitution with valine can be accommodated without altering the conformation of the domain and we would predict little effect on catalysis (Fig. 1B). Substitution with a glutamic acid residue could result in a new interaction with the guanidine group of R270. In

Table 2
Table of kinetic constants. Full length and NΔ150 analyzed separately. (± Standard Error).

	$K_{m,\ L\text{-}Trp}$ (μM)	$V_{max,\ L\text{-}Trp}$ (nmol/min/density)	$K_{m,\ BH4}$ (μM)	$V_{max,\ BH4}$ (nmol/min/density)
Wild type TPH2	39.6 ± 5.7	17 ± 4.7	27.6 ± 4.5	27.6 ± 3.7
A328V	28.5 ± 7.4	5.1 ± 1.8	18.3 ± 5.6	4.7 ± 0.2
	p = 0.140 n = 4	p = 0.027 n = 4	p = 0.139 n = 3–4	p = 0.0003 n = 3–4
NΔ150-hTPH2	57.6 ± 14.3	5.3 ± 0.4	2.7 ± 1.4	4.5 ± 0.2
NΔ150-A328V	36.2 ± 1.1	7.3 ± 3.4	1.99 ± 0.4	3.6 ± 0.6
	p = 0.283	p = 0.294	p = 0.326	p = 0.089
	n = 3	n = 3	n = 3	n = 3

the model of the full-length tetramer, alanine 328 is occluded by the ACT domain of a neighboring subunit, although it does not interact with it directly. The equivalent residue in hPAH is a serine, which could be incorporated without steric or chemical clashes. In phenylalanine hydroxylase, the ACT domain undergoes a conformational change from auto-inhibited to active which involves movement towards the oligomerization motif, possibly forming a dimer with the adjacent ACT domain. Based upon this reasoning, introduction of a valine at position 328 should also be accommodated with relative ease; however, it would change the surface character of the region, perhaps hindering the movement of the regulatory domain. Substitution with a glutamic acid residue would allow electrostatic interaction with arginine 270 and lysine 98 from an adjacent regulatory domain, perhaps favoring the auto-inhibited conformation (consistent with the inactive variant enzyme we observe; Fig. 3). However, it is noteworthy that the surface exposed NΔ150 A328E hTPH2 is also found to be largely inactive (Fig. 3B).

4. Discussion

The serotonin field was surprised by the discovery, in 2003, of a novel gene responsible for TPH activity in the central nervous system (TPH2; [9,10]). In the time since the initial description of TPH2, work has moved forward on the characterization of hTPH2 polymorphisms in mental health and disease. To date, there have been hundreds coding region polymorphisms reported by NCBI dbSNP (https://www.ncbi.nlm.nih.gov/snp/?term=TPH2);

accessed on April 1, 2023). Of these, 37 have been reported by the 1000 Genomes Project, and seven of them are statistically associated with human disorders. Moreover, nine coding region polymorphisms reported in hTPH2 and their activity and solubility have been characterized [31]. The A328 V/E SNPs have 0.92% allelic frequencies in the general population, but there has been no disease association reported.

In the present report, we have investigated the enzymatic consequences of these changes in hTPH2 (A328V and A328E) and the effects of the regulatory domain on their respective phenotypes. We found that introduction of the valine substitution produced a marked 60% reduction in activity, while introduction of the A328E SNP results in complete loss of activity. The A328V activity results for gross enzyme activity (Fig. 3) were similar to those reported by McKinney and coworkers [31]. Under maximal velocity characterizations (Michaelis-Menten analysis), however, reductions in V_{max} were more pronounced (Table 2). Moreover, A328V displays enhanced substrate inhibition (Fig. 5; K_i ~300 μM). Additional characterization indicates that this polymorphism results in a measurable decrease in enzyme stability (Fig. 4). Studies on tyrosine hydroxylase, phenylalanine hydroxylase and tryptophan hydroxylase have shown a strong correlation between loss of enzyme function and thermostability and solubility [31].

Examination of the NCBI website indicates that A328V has an allelic frequency in the general population of 0.92%. While rare, this is still the most prevalent missense polymorphism in the enzyme and homozygotes would be predicted to be present at a frequency of 1:12,000 individuals. One must therefore consider the marked reduction in activity observed for the enzyme with this polymorphism with the continued presence of this variant in the general population given the importance of serotonin. First, we note that this SNP does retain activity. Second, *in vitro* and *in vivo* studies using neurotoxins, SSRIs, or genetically driven changes in

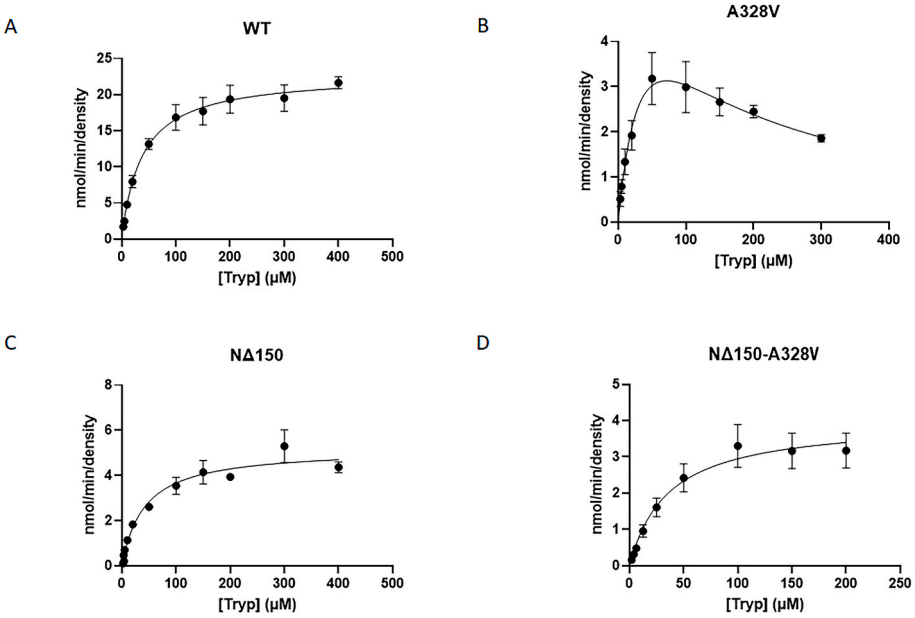

Fig. 5. Tryptophan substrate inhibition of wild type hTPH2 and A328V variant enzymes both as full-length enzymes (**A** & **B**) and NΔ150 enzymes (**C** & **D**). Tryptophan was varied at a constant concentration of BH_4 (50 μM). Each curve represents the average of three independent assays performed on independent enzyme purifications. Data are presented as mean ± SEM.

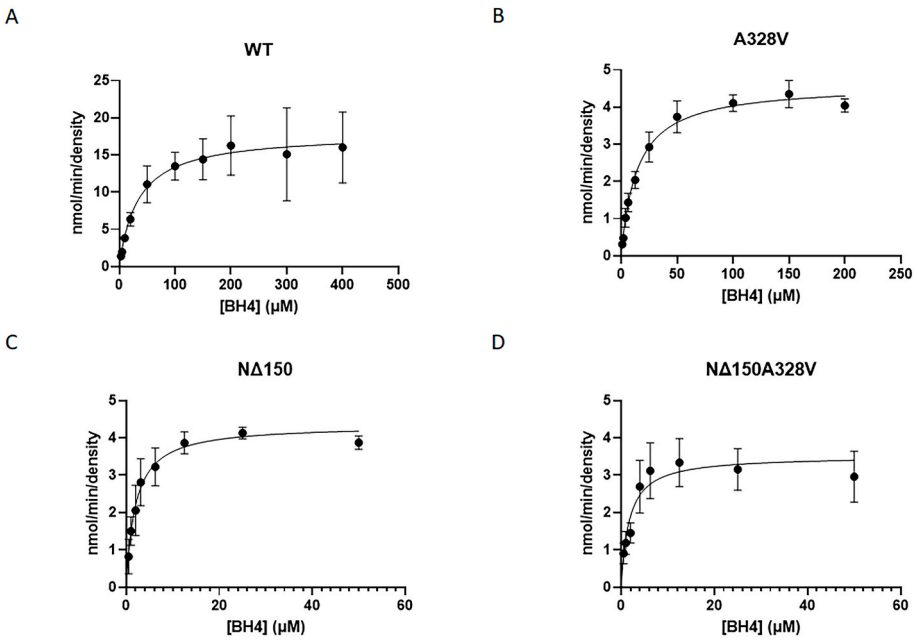

Fig. 6. BH4 kinetics for wild type hTPH2 and A328V variant enzymes both as full-length enzymes (**A** & **B**) and NΔ150 enzymes (**C** & **D**). Tetrahydrobiopterin was varied at a constant concentration of Tryptophan (50 μM). Each curve represents the average of three independent assays performed on independent enzyme purifications. Data are presented as mean ± SEM.

neurotransmitter synthesis [35–37] show that 5-HT deficiencies produce viable animals with altered brain functions. Third, it is well-known, for genetic knockouts of the related tyrosine hydroxylase, that heterozygote animals ($TH^{+/-}$) fully compensate and produce normal levels of catecholamines [38]. We therefore anticipate a similar compensation in hTPH2 A328V heterozygotes. Previous research has shown that the loss of one gene copy in heterozygous $TPH2^{+/-}$ mice results in just a 13% drop in 5-HT level in the brain [39], in spite of a 50% reduction in TPH2 mRNA in the total brain. A drop of 22% in 5-HT was found in another investigation utilizing TPH2 heterozygous mutant mice [40].

The hTPH2 A328E polymorphism is much rarer in the population (allele frequency of 0.0008%), perhaps because it abolishes enzyme activity. Indeed, it appears to represent a single heterozygote individual of the 60,706 humans tested. In spite of this low frequency, however, it provides important insights into hTPH2 function given that it eliminates enzyme activity.

We also detected substrate inhibition by tryptophan in the A328V variant enzyme compared to wild type enzyme and, after deletion of the regulatory domain, substrate inhibition was eliminated (Fig. 5). Previous reports [41–43] indicate that the wild-type hTPH2 shows very weak substrate inhibition; however, in our hands there is little or no substrate inhibition (Fig. 5). Our kinetic data show that the K_M for BH_4 utilization is unaffected by this polymorphism, while V_{max} was reduced by 80%. Similarly, there was no difference in K_M for tryptophan, but the V_{max} was reduced by 70%. In the absence of the regulatory domain, the enzyme kinetics are the same between A328V and the wild type protein indicating that the active site itself is unperturbed by this substitution as suggested previously for A328V [31].

Our homology model, based on the auto-inhibited structure of PAH, provides one explanation. The regulatory sequence contains an N-terminal PTD domain and a C-terminal ACT domain followed by a canonical catalytic domain and a C-terminal oligomerization domain that likely undergo a complex set of conformational changes to the active form, by analogy to PAH. The current structures for family members show that the active site loop (residues 160–180) are in an open conformation or partially disordered unless both substrates/analogs are present. An ordered ACT domain is observed in only 3 structures of PAH (5DEN [44], 5FGJ, and 5EGQ [45]) despite being present in the crystal of TPH1 with an active site inhibitor (5L01.PDB). Interestingly, the tetramerization domain was also disordered in this construct, lending evidence to the idea that the regulatory and tetramerization domains communicate. The templates automatically selected for residues 51–490, based on sequence homology, were unremarkable. However, we were intrigued by the identification of the regulatory domain of PDT (prephenate dehydratase) as a template for the first 150 amino acids (Fig. 1A). This enzyme is responsible for synthesis of phenylpyruvate – a precursor in the synthesis of phenylalanine, tyrosine, and tryptophan. PDT is allosterically regulated by a phenylalanine binding site in the regulatory domain that controls transition from an open to a closed form. The sequence of the binding site is conserved (IESRXSR; residues 64 through 100 in hTPH2) between hPAH, hTPH2 and PTD and it has been suggested that that this domain may have been incorporated as a small module with the potential to provide allosteric regulation via transmission of finely tuned conformational changes [46]. This would be consistent with the related enzyme phenylalanine hydroxylase that is thought to be regulated by phenylalanine and phosphorylation acting in concert to activate the enzyme through a combination of intrasteric and possibly allosteric mechanisms. It is interesting then that TPH2 is also stabilized by phenylalanine [47]. In addition, the PTD and ACT domains homo-oligomerize to form an activated protein. A similar dimerization of the ACT domain has been proposed based on conformational changes observed in small angle x-ray scattering profiles of PAH.

The homology model and present data permit new insight into this enzyme and the A328 E/V polymorphisms. In the case of A328V, this region is a highly divergent site within the catalytic domains of the hydroxylases (residues 326–331 in hTPH2). Moreover, in hTPH2, this region begins with a conserved proline (P325 in hTPH2; P279 in hTPH1; P338 in human tyrosine hydroxylase (hTH); P292 in hPAH and, in hTPH2, the residues are followed by a proline (P330). Therefore, this is likely to be an area of constrained and potentially important structure/function relationships. A328V and A328E are located at the dimer interface between their own catalytic domain and residues 75–78 and 95–98 in the regulatory domain of an adjacent enzyme subunit. They pack against L245, F264 and R270 of their own monomer and K98 of the adjacent monomer. The Nζ of K98 makes a hydrogen bond with the carbonyl oxygen atom of A328. It forms part of the putative amino acid

binding pocket that has been implicated in allosteric regulation in other family members [46].

Addition of a bulkier side chain at this position would change the shape of this pocket, either impacting the putative amino acid binding at the regulatory site, or affecting the conformational changes necessary for activation. Moreover, substitution of a larger and negatively charged residue A328E could easily produce misfolding and disrupt enzyme function. This also provides an explanation for why there is such sequence variability. If indeed there is amino acid binding dictated by this region, the different sequences in each protein would shape the pockets in unique ways.

In addition to its potential physiological significance in brain serotonin synthesis, the introduction of substrate inhibition into TPH2 in the A328V variant provides novel mechanistic insight. *In vivo*, at low concentrations of tryptophan and high or saturating concentrations of BH_4, the substitution would have only modest impact on enzyme activity. However, tryptophan-substrate inhibition would become pronounced if BH_4 levels were low and/or tryptophan levels high. This may account, in part, for the lack of pronounced phenotypes for this substitution. Unlike wild type TPH2, TPH1 displays strong tryptophan substrate inhibition and the mechanism has been studied in some detail [48]. Tryptophan hydroxide formation occurs via a partially ordered Ping-Pong reaction in which BH_4 must bind prior to tryptophan for catalysis to occur [49]. A328 lies in a region that was previously shown to bind an allosteric inhibitor of TPH1, NVS-TPH120. This inhibitor acts via a mixed mode mechanism that results in increased affinity for tryptophan, decreased affinity for BH_4 and dramatically reduced tryptophan hydroxylase activity both *in vitro* and *in vivo* [50]. The authors indicate that it binds in a cavity terminated at one end by A328 that is structurally conserved in both TPH1 and 2. Based upon the overall high sequence conservation between TPH1 and TPH2 in their catalytic domains, the presence of allosteric regulatory site near A328 in TPH2 suggests an explanation for the effect of the valine substitution. In this model, the substitution results in repacking of the region surrounding the bulkier valine residue stabilizing a conformation similar to that promoted by NVS-TPH120. This conformation would have a higher affinity for tryptophan, as is seen with the inhibitor bound to TPH1, and thus promote the formation of the dead-end TPH2-tryptophan complex. This conformational switch may also be aided by the presence of the N-terminal regulatory domain, since the effects are greater in full length vs NΔ150 TPH2. Unfortunately, due to the limited published information on NVS-TPH120, a more detailed analysis of how this compound and the A328V substitution affect catalysis is difficult, but these observations further highlight the potential significance of this region in developing compounds that selectively target the two enzymes.

Our findings suggest that these polymorphisms exert their effects through the conformational freedom of the regulatory domain. Reductions in catalytic activity of the full-length protein, as well as substrate inhibition as a result of the polymorphisms, may change the biological function of hTPH2 significantly, resulting in a reduction in the synthesis of serotonin. The variation in levels of serotonin, related to these polymorphisms, may contribute to behavioral perturbations, emotional stress, and eating disorders.

Funding

This work was supported by R01GM-38931 (KV) and the PSU Elliot S. Vesell endowment (KV).

Declaration of competing interest

The authors declare no financial competing interests.

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrep.2023.101527.

References

[1] N.M. Barnes, T. Sharp, A review of central 5-HT receptors and their function, Neuropharmacology 38 (1999) 1083–1152.
[2] Y.M. Lin, S.C. Chao, T.M. Chen, T.J. Lai, J.S. Chen, H.S. Sun, Association of functional polymorphisms of the human tryptophan hydroxylase 2 gene with risk for bipolar disorder in Han Chinese, Arch. Gen. Psychiatr. 64 (2007) 1015–1024.
[3] E. Akimova, R. Lanzenberger, S. Kasper, The serotonin-1A receptor in anxiety disorders, Biol. Psychiatr. 66 (2009) 627–635.
[4] M. Berger, J.A. Gray, B.L. Roth, The expanded biology of serotonin, Annu. Rev. Med. 60 (2009) 355–366.
[5] K. Sheehan, N. Lowe, A. Kirley, C. Mullins, M. Fitzgerald, M. Gill, Z. Hawi, Tryptophan hydroxylase 2 (TPH2) gene variants associated with ADHD, Mol. Psychiatr. 10 (2005) 944–949.
[6] S.V. Faraone, E. Mick, Molecular genetics of attention deficit hyperactivity disorder, Psychiatr. Clin. 33 (2010) 159–180.
[7] M.H. Fernstrom, J.D. Fernstrom, Brain tryptophan concentrations and serotonin synthesis remain responsive to food consumption after the ingestion of sequential meals, Am. J. Clin. Nutr. 61 (1995) 312–319.
[8] S.M. Mockus, K.E. Vrana, Advances in the molecular characterization of tryptophan hydroxylase, J. Mol. Neurosci. 10 (1998) 163–179.
[9] D.J. Walther, J.U. Peter, S. Bashammakh, H. Hortnagl, M. Voits, H. Fink, M. Bader, Synthesis of serotonin by a second tryptophan hydroxylase isoform, Science 299 (2003) 76.
[10] D.J. Walther, M. Bader, A unique central tryptophan hydroxylase isoform, Biochem. Pharmacol. 66 (2003) 1673–1680.
[11] L. Gutknecht, C. Kriegebaum, J. Waider, A. Schmitt, K.P. Lesch, Spatio-temporal expression of tryptophan hydroxylase isoforms in murine and human brain: convergent data from Tph2 knockout mice, Eur. Neuropsychopharmacol 19 (2009) 266–282.
[12] M.D. Gershon, 5-Hydroxytryptamine (serotonin) in the gastrointestinal tract, Curr. Opin. Endocrinol. Diabetes Obes. 20 (2013) 14–21.
[13] D. Coyle, J.M. Murphy, B. Doyle, A.M. O'Donnell, J. Gillick, P. Puri, Altered tryptophan hydroxylase 2 expression in enteric serotonergic nerves in Hirschsprung's-associated enterocolitis, World J. Gastroenterol. 22 (2016) 4662–4672.
[14] L. Gutknecht, J. Waider, S. Kraft, C. Kriegebaum, B. Holtmann, A. Reif, A. Schmitt, K.P. Lesch, Deficiency of brain 5-HT synthesis but serotonergic neuron formation in Tph2 knockout mice, J. Neural. Transm. 115 (2008) 1127–1132.
[15] N. Alenina, D. Kikic, M. Todiras, V. Mosienko, F. Qadri, R. Plehm, P. Boye, L. Vilianovitch, R. Sohr, K. Tenner, H. Hortnagl, M. Bader, Growth retardation and altered autonomic control in mice lacking brain serotonin, Proc. Natl. Acad. Sci. U. S. A. 106 (2009) 10332–10337.
[16] Z. Li, H.Y. Yang, Y. Wang, M.L. Zhang, X.R. Liu, Q. Xiong, L.N. Zhang, Y. Jin, L. S. Mou, Y. Liu, R.F. Li, Y. Rao, Y.F. Dai, Generation of tryptophan hydroxylase 2 gene knockout pigs by CRISPR/Cas9-mediated gene targeting, J. Biomed. Res. 31 (2017) 445–452.
[17] S. Ansai, H. Hosokawa, S. Maegawa, K. Naruse, Y. Washio, K. Sato, M. Kinoshita, Deficiency of Serotonin in Raphe Neurons and Altered Behavioral Responses in Tryptophan Hydroxylase 2-Knockout Medaka (Oryzias latipes), 2017. Zebrafish.
[18] P.F. Fitzpatrick, Mechanism of aromatic amino acid hydroxylation, Biochemistry 42 (2003) 14083–14091.
[19] S.C. Daubner, D.L. Lohse, P.F. Fitzpatrick, Expression and characterization of catalytic and regulatory domains of rat tyrosine hydroxylase, Protein Sci. 2 (1993) 1452–1460.
[20] H. Erlandsen, F. Fusetti, A. Martinez, E. Hough, T. Flatmark, R.C. Stevens, Crystal structure of the catalytic domain of human phenylalanine hydroxylase reveals the structural basis for phenylketonuria, Nat. Struct. Biol. 4 (1997) 995–1000.
[21] N. Carkaci-Salli, J.M. Flanagan, M.K. Martz, U. Salli, D.J. Walther, M. Bader, K. E. Vrana, Functional domains of human tryptophan hydroxylase 2 (hTPH2), J. Biol. Chem. 281 (2006) 28105–28112.
[22] X. Liu, K.E. Vrana, Leucine zippers and coiled-coils in the aromatic amino acid hydroxylases, Neurochem. Int. 18 (1991) 27–31.
[23] P. Zill, T.C. Baghai, P. Zwanzger, C. Schule, D. Eser, R. Rupprecht, H.J. Moller, B. Bondy, M. Ackenheil, SNP and haplotype analysis of a novel tryptophan hydroxylase isoform (TPH2) gene provide evidence for association with major depression, Mol. Psychiatr. 9 (2004) 1030–1036.
[24] X. Zhang, R.R. Gainetdinov, J.M. Beaulieu, T.D. Sotnikova, L.H. Burch, R. B. Williams, D.A. Schwartz, K.R. Krishnan, M.G. Caron, Loss-of-function mutation in tryptophan hydroxylase-2 identified in unipolar major depression, Neuron 45 (2005) 11–16.
[25] A.V. Kulikov, D.V. Osipova, V.S. Naumenko, N.K. Popova, Association between Tph2 gene polymorphism, brain tryptophan hydroxylase activity and aggressiveness in mouse strains, Gene Brain Behav. 4 (2005) 482–485.
[26] S. Walitza, T.J. Renner, A. Dempfle, K. Konrad, C. Wewetzer, A. Halbach, B. Herpertz-Dahlmann, H. Remschmidt, J. Smidt, M. Linder, L. Flierl, U. Knolker, S. Friedel, H. Schafer, C. Gross, J. Hebebrand, A. Warnke, K.P. Lesch, Transmission disequilibrium of polymorphic variants in the tryptophan hydroxylase-2 gene in attention-deficit/hyperactivity disorder, Mol. Psychiatr. 10 (2005) 1126–1132.

[27] R. Mossner, S. Walitza, F. Geller, A. Scherag, L. Gutknecht, C. Jacob, L. Bogusch, H. Remschmidt, M. Simons, B. Herpertz-Dahlmann, C. Fleischhaker, E. Schulz, A. Warnke, A. Hinney, C. Wewetzer, K.P. Lesch, Transmission disequilibrium of polymorphic variants in the tryptophan hydroxylase-2 gene in children and adolescents with obsessive-compulsive disorder, Int. J. Neuropsychopharmacol. 9 (2006) 437–442.
[28] V.A. Lopez, S. Detera-Wadleigh, I. Cardona, L. Kassem, F.J. McMahon, Nested association between genetic variation in tryptophan hydroxylase II, bipolar affective disorder, and suicide attempts, Biol. Psychiatr. 61 (2007) 181–186.
[29] G.L. Chen, M.A. Novak, J.S. Meyer, B.J. Kelly, E.J. Vallender, G.M. Miller, The effect of rearing experience and TPH2 genotype on HPA axis function and aggression in rhesus monkeys: a retrospective analysis, Horm. Behav. 57 (2010) 184–191.
[30] K.W. Hong, A. Weiss, N. Morimura, T. Udono, I. Hayasaka, T. Humle, Y. Murayama, S. Ito, M. Inoue-Murayama, Polymorphism of the tryptophan hydroxylase 2 (TPH2) gene is associated with chimpanzee neuroticism, PLoS One 6 (2011), e22144.
[31] J.A. McKinney, B. Turel, I. Winge, P.M. Knappskog, J. Haavik, Functional properties of missense variants of human tryptophan hydroxylase 2, Hum. Mutat. 30 (2009) 787–794.
[32] F.W. Studier, Protein production by auto-induction in high density shaking cultures, Protein Expr. Purif. 41 (2005) 207–234.
[33] J.F. Reinhard Jr., G.K. Smith, C.A. Nichol, A rapid and sensitive assay for tyrosine-3-monooxygenase based upon the release of 3H2O and adsorption of [3H]-tyrosine by charcoal, Life Sci. 39 (1986) 2185–2189.
[34] S.L. Vrana, S.I. Dworkin, K.E. Vrana, Radioenzymatic assay for tryptophan hydroxylase: [3H]H2O release assessed by charcoal adsorption, J. Neurosci. Methods 48 (1993) 123–129.
[35] P. Gaspar, O. Cases, L. Maroteaux, The developmental role of serotonin: news from mouse molecular genetics, Nat. Rev. Neurosci. 4 (2003) 1002–1012.
[36] E.A. Daubert, B.G. Condron, Serotonin: a regulator of neuronal morphology and circuitry, Trends Neurosci. 33 (2010) 424–434.
[37] K.L. Simpson, K.J. Weaver, E. de Villers-Sidani, J.Y. Lu, Z. Cai, Y. Pang, F. Rodriguez-Porcel, I.A. Paul, M. Merzenich, R.C. Lin, Perinatal antidepressant exposure alters cortical network function in rodents, Proc. Natl. Acad. Sci. U. S. A. 108 (2011) 18465–18470.
[38] K. Kobayashi, S. Morita, H. Sawada, T. Mizuguchi, K. Yamada, I. Nagatsu, T. Hata, Y. Watanabe, K. Fujita, T. Nagatsu, Targeted disruption of the tyrosine hydroxylase locus results in severe catecholamine depletion and perinatal lethality in mice, J. Biol. Chem. 270 (1995) 27235–27243.
[39] V. Mosienko, S. Matthes, N. Hirth, D. Beis, M. Flinders, M. Bader, A.C. Hansson, N. Alenina, Adaptive changes in serotonin metabolism preserve normal behavior in mice with reduced TPH2 activity, Neuropharmacology 85 (2014) 73–80.
[40] L. Gutknecht, N. Araragi, S. Merker, J. Waider, F.M. Sommerlandt, B. Mlinar, G. Baccini, U. Mayer, F. Proft, M. Hamon, A.G. Schmitt, R. Corradetti, L. Lanfumey, K.P. Lesch, Impacts of brain serotonin deficiency following Tph2 inactivation on development and raphe neuron serotonergic specification, PLoS One 7 (2012), e43157.
[41] P.A. Friedman, Kappelma Ah, S. Kaufman, Partial purification and characterization of tryptophan hydroxylase from rabbit hindbrain, J. Biol. Chem. 247 (1972) 4165 (&).
[42] J. McKinney, P.M. Knappskog, J. Haavik, Different properties of the central and peripheral forms of human tryptophan hydroxylase, J. Neurochem. 92 (2005) 311–320.
[43] M.C. Reed, A. Lieb, H.F. Nijhout, The biological significance of substrate inhibition: a mechanism with diverse functions, Bioessays 32 (2010) 422–429.
[44] E.C. Arturo, K. Gupta, A. Heroux, L. Stith, P.J. Cross, E.J. Parker, P.J. Loll, E. K. Jaffe, First structure of full-length mammalian phenylalanine hydroxylase reveals the architecture of an autoinhibited tetramer, Proc. Natl. Acad. Sci. U. S. A. 113 (2016) 2394–2399.
[45] S.P. Meisburger, A.B. Taylor, C.A. Khan, S. Zhang, P.F. Fitzpatrick, N. Ando, Domain movements upon activation of phenylalanine hydroxylase characterized by crystallography and chromatography-coupled small-angle X-ray scattering, J. Am. Chem. Soc. 138 (2016) 6506–6516.
[46] J.S. Liberles, M. Thorolfsson, A. Martinez, Allosteric mechanisms in ACT domain containing enzymes involved in amino acid metabolism, Amino Acids 28 (2005) 1–12.
[47] S. Ogawa, H. Ichinose, Effect of metals and phenylalanine on the activity of human tryptophan hydroxylase-2: comparison with that on tyrosine hydroxylase activity, Neurosci. Lett. 401 (2006) 261–265.
[48] K.D. Tidemand, G.H. Peters, P. Harris, E. Stensgaard, H.E.M. Christensen, Isoform-specific substrate inhibition mechanism of human tryptophan hydroxylase, Biochemistry 56 (2017) 6155–6164.
[49] K.M. Roberts, P.F. Fitzpatrick, Mechanisms of tryptophan and tyrosine hydroxylase, IUBMB Life 65 (2013) 350–357.
[50] K. Waloen, R. Kleppe, A. Martinez, J. Haavik, Tyrosine and tryptophan hydroxylases as therapeutic targets in human disease, Expert Opin. Ther. Targets 21 (2017) 167–180.