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REVIEW

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Tyrosine and tryptophan hydroxylases as therapeutic targets in human disease

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ABSTRACT

Introduction: The ancient and ubiquitous monoamine signalling molecules serotonin, dopamine, norepinephrine, and epinephrine are involved in multiple physiological functions. The aromatic amino acid hydroxylases tyrosine hydroxylase (TH), tryptophan hydroxylase 1 (TPH1), and tryptophan hydroxylase 2 (TPH2) catalyse the rate-limiting steps in the biosynthesis of these monoamines. Genetic variants of *TH, TPH1*, and *TPH2* genes are associated with neuropsychiatric disorders. The interest in these enzymes as therapeutic targets is increasing as new roles of these monoamines have been discovered, not only in brain function and disease, but also in development, cardiovascular function, energy and bone homeostasis, gastrointestinal motility, hemostasis, and liver function.

Areas covered: Physiological roles of TH, TPH1, and TPH2. Enzyme structures, catalytic and regulatory mechanisms, animal models, and associated diseases. Interactions with inhibitors, pharmacological chaperones, and regulatory proteins relevant for drug development.

Expert opinion: Established inhibitors of these enzymes mainly target their amino acid substrate binding site, while tetrahydrobiopterin analogues, iron chelators, and allosteric ligands are less studied. New insights into monoamine biology and 3D-structural information and new computational/experimental tools have triggered the development of a new generation of more selective inhibitors and pharmacological chaperones. The enzyme complexes with their regulatory 14–3–3 proteins are also emerging as therapeutic targets.

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Tyrosine hydroxylase; tryptophan hydroxylase; human disease; mental disorder; ADHD; 14-3-3; aromatic amino acid hydroxylase; dopamine; serotonin; osteoporosis

1. Introduction

All animals are dependent on catecholamine and serotonin signaling for many physiological functions, ranging from embryonic growth and development to neurotransmission, blood clotting and endocrine, kidney, and cardiovascular functions [1,2]. For many years, the enzymes, transporters, and receptors involved in the metabolism and signaling of these monoamine neurotransmitters and hormones have been primary therapeutic targets for many human diseases within neurology, cardiology, and psychiatry [3]. As new roles of monoamine signaling have been discovered in regulation of endocrine signaling, hemostasis, gastrointestinal function, weight, and bone homeostasis and liver function, the interest in pharmacological modification of these signaling pathways has increased [4]. Due to peripheral monoamines being unable to cross the blood–brain barrier, the central and peripheral serotonin and catecholamine systems can be separately targeted by different pharmacological agents [4].

New insights into the structure, regulation, and diverse roles of the aromatic amino acid hydroxylases (AAAHs): tyrosine hydroxylase (TH), tryptophan hydroxylase 1 (TPH1), and tryptophan hydroxylase 2 (TPH2) that synthesize these monoamines (Figure 1) have triggered a new interest in these enzymes as therapeutic targets.

2. AAAHs and human disease

TH, TPH1, and TPH2 belong to the AAAH family of enzymes that catalyze the hydroxylation of their respective aromatic amino acids in the presence of molecular oxygen, tetrahydrobiopterin (BH4), and iron [5,6]. In addition to TH, TPH1, and TPH2, phenylalanine hydroxylase (PAH) is also a member of this family and is responsible for the catalytic conversion of phenylalanine into tyrosine [5,7]. All the AAAHs occupy key regulatory positions in their metabolic pathways (Figure 1) [7–10].

Until recently, the interest in these enzymes has mainly been related to the roles of monoamines as neurotransmitters in the central nervous system (CNS) where they are involved in regulation of cognitive and motor functions, as regulators in the peripheral autonomic nervous system as well as 'stress hormones' [1,11,12]. Moreover, altered monoamine functions, triggered by dysfunction of the AAAHs, are associated with neurometabolic and neuropsychiatric disorders [1]. An example of this is phenylketonuria (PKU) which is associated with mutations in PAH and is characterized by the accumulation of phenylalanine and its degradation products. Untreated phenylketonuria has detrimental effects on brain development and function [13]. Different treatment strategies to target PAH deficiency and malfunction have recently been reviewed and will not be further discussed here [13,14].

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Article highlights

- Catecholamines and serotonin have multiple physiological functions. Altered functions of these monoamines have been implicated in many different disease states. The AAAHs: TH, TPH1, and TPH2 are key enzymes in the biosynthesis of these molecules. Here we describe how new insights into the structure, regulation and biological functions of TH, TPH1, and TPH2 have generated new interest in these enzymes as therapeutic targets.
- The monoamine signalling pathways and the involvement of TH, TPH1, and TPH2 are reviewed, as well as their protein structures and catalytic mechanisms.
- Various regulatory mechanisms of these AAAH's are presented, including feedback inhibition, phosphorylation, and regulation by 14-3-3 proteins.
- The structure, binding mechanism, and involvement of 14-3-3 proteins with AAAHs are described.
- Diseases associated with TH, TPH1, and TPH2 and animal models of disease are discussed, as well as past and current therapeutic approaches targeting these enzymes.
- Novel therapeutic approaches to treat AAAH associated disorders are suggested, including pharmacological chaperones targeting the mutant enzymes and compounds that can modulate their protein-protein interactions, notably with 14-3-3 proteins.

This box summarizes key points contained in the article.

2.1. TH

TH is the enzyme responsible for the rate limiting, catalytic conversion of tyrosine into L-DOPA, a precursor of dopamine, norepinephrine, and epinephrine [10] (Figure 1). At least four isoforms of human TH are generated by alternative splicing of a single gene: that is, TH1-4 [15]. TH is mainly expressed in the noradrenergic and dopaminergic neurons of the brain, chromaffin cells of the adrenal medulla, and sympathetic neurons [8,16]. In mice, and probably also humans, complete knockout of TH activity leads to cardiac failure [11,17]. This effect seems to be normalized by quite low residual TH activity, as also found in human patients with TH deficiency, where the major phenotype is motor dysfunction [1,18]. This is consistent with the important function of dopamine in the basal ganglia, which is necessary for the release of inhibition by the major GABAergic pathways that originate in the striatum [1,18]. In addition to their roles in motor systems, in the CNS, dopamine and norepinephrine are important neuromodulators involved in multiple functions, such as attention, working memory, learning, addiction, motivation, and emotions [1,12].

2.2. TPH1 and TPH2

The TPHs are responsible for the catalytic conversion of L-tryptophan into 5-hydroxy-L-tryptophan which is a precursor of serotonin and melatonin (Figure 1) [4]. Serotonin is a neurotransmitter and a hormone in several physiological functions, such as sleep, pain, appetite, sexual behavior, and mood [1,2,4]. Melatonin is associated with consolidation of sleep and other functions [19]. Human TPH1 and TPH2 are encoded by two different genes located on chromosomes 11 and 12, respectively, and the two human TPH proteins have an overall sequence identity of 71% [20]. Although they have identical catalytic mechanisms and similar substrate specificities, they have different phosphorylation sites, different patterns of expression, and are involved in distinct physiological processes [20]. TPH1 is mainly expressed in the enterochromaffin cells of gastrointestinal tract, but also in adrenal glands, kidney, and the pineal gland, where melatonin is synthesized. Most of the circulating serotonin is derived from TPH1 but is transported into platelets where it is stored in dense granules. In contrast, TPH2 is found in serotonergic neurons originating

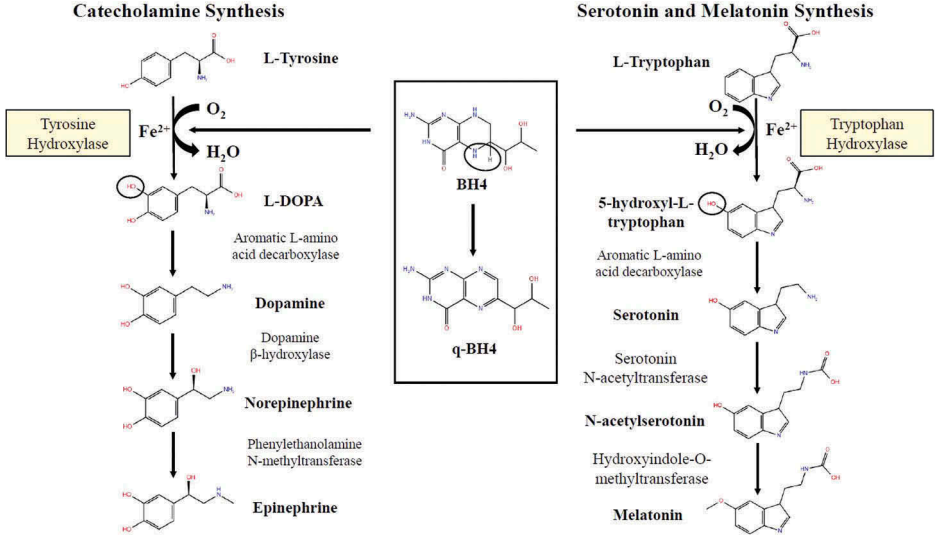

Figure 1. Reaction pathway of the catecholamine synthesis (left) and serotonin and melatonin synthesis (right) pathways. In the catecholamine synthesis, L-tyrosine is converted to L-DOPA by tyrosine hydroxylase in the presence of BH4, O_2, and Fe^{2+}. L-DOPA is further converted into dopamine by aromatic L-amino acid decarboxylase. Dopamine is converted to norepinephrine by dopamine β-hydroxylase. Finally, norephinephrine is converted to epinephrine by phenylethanolamine N-methyltransferase. In the serotonin and melatonin synthesis pathway, L-tryptophan is converted to 5-hydroxyl-L-tryptophan by tryptophan hydroxylase in the presence of BH4, O_2, and Fe^{2+}. 5-hydroxyl-tryptophan is converted to serotonin by aromatic L-amino acid decarboxylase. Following this, serotonin is converted to N-acetylserotonin by serotonin N-acetyltransferase. Finally, N-acetylserotonin is converted to melatonin by hydroxyindole-O-methyltransferase. In both pathways, BH4 is converted to q-BH4 in the process of the reaction.

from the raphe nuclei in the brain with widespread projections to many cortical areas [4].

2.3. AAAH structure and catalysis

The AAAHs are tetrameric enzymes of identical subunits, but PAH can also be dimeric [5,21]. All four enzymes present a 3-domain organization including N-terminal regulatory, catalytic, and C-terminal oligomerization domains (Figure 2) [22]. The AAAHs display high-sequence identity. The 293-residue long catalytic domains, which are largely α-helical, exhibit approximately 65% sequence identity across the four human enzymes. Several partial AAAH structures, mainly of the catalytic domains, have been published or deposited in the Protein Data Bank (Figure 2). In addition, the crystal structure of full-length rat PAH (PDB: 5DEN) [23], as well as the solution structures of the regulatory domain (PDB: 2MDA) [24], and full-length TH has recently been published [25]. As expected from the high-sequence identity, the superimposition of human AAAH structures also shows high 3D structural homology, notably for the catalytic domains (Figure 3(a)) [22,26]. The geometric arrangement of the domains may vary. The structure of full-length tetrameric PAH shows that the ACT regulatory domains are separated from each other (Figure 3(b)) and probably dimerize upon activation of the enzyme by L-Phe [23]. In contrast, the regulatory domains of TH dimerize also in the resting state, in the absence of substrate, as observed both by NMR [24] and SAXS [25]. Another peculiarity of TH is its 43-residue unstructured N-terminal that imposes an elongated conformation to TH and has an important regulatory role, hosting the phosphorylation sites and interacting with binding partners [25,27].

The AAAHs hydroxylate their respective amino acid substrates, using nonheme Fe^{2+} and tetrahydrobiopterin (BH4) as cofactors and dioxygen as additional substrate (Figure 1). A highly reactive Fe^{4+}=O hydroxylating intermediate – formed by the active site iron, oxygen, and BH4 – catalyzes the hydroxylation of the substrate [7]. In the reaction, molecular oxygen is catalytically cleaved and each of the oxygen atoms is incorporated into BH4 and the aromatic ring of the substrate. The hydroxylated cofactor (BH4–4a-carbinolamine) is regenerated back to BH4 by the consecutive action of pterin 4a-carbinolamine dehydratase and dihydropteridine reductase [5,7].

For PAH, crystal structures are available for the enzyme with bound BH4 and substrate analogs (norleucine and thienylalanine), and the active site of PAH, as a representative of the AAAH's active sites, is shown in Figure 4. The catalytic iron binds to conserved His290, His285, and Glu330 (numeration in

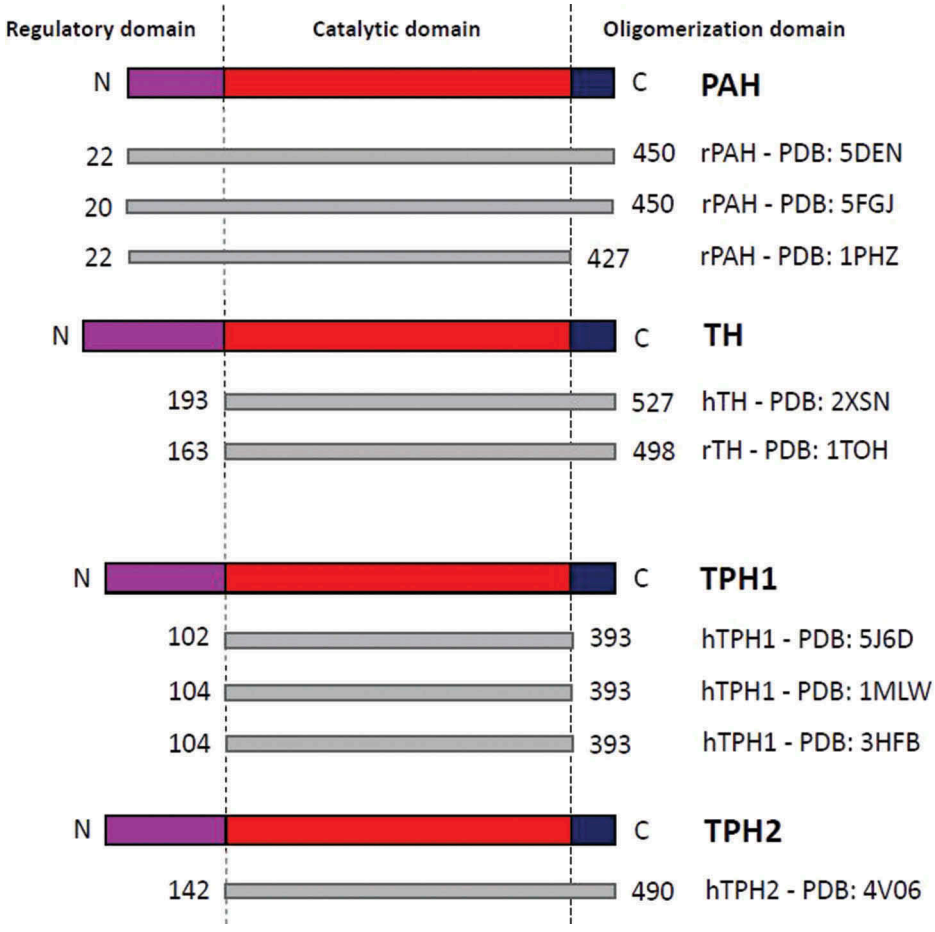

Figure 2. Schematic representation of the AAAH domains, regulatory (purple), catalytic (red) and oligomerization (blue) domains. The relative sizes of the known crystal structures are indicated as well as the PDB code. The AAAH proteins are labelled with the species (r: rat; h: human). The presented structures were selected based on top hits of BLAST runs with the PDB database. The query sequence was the sequence of the *Homo sapiens* AAAH available on Uniprot. Full color available online.

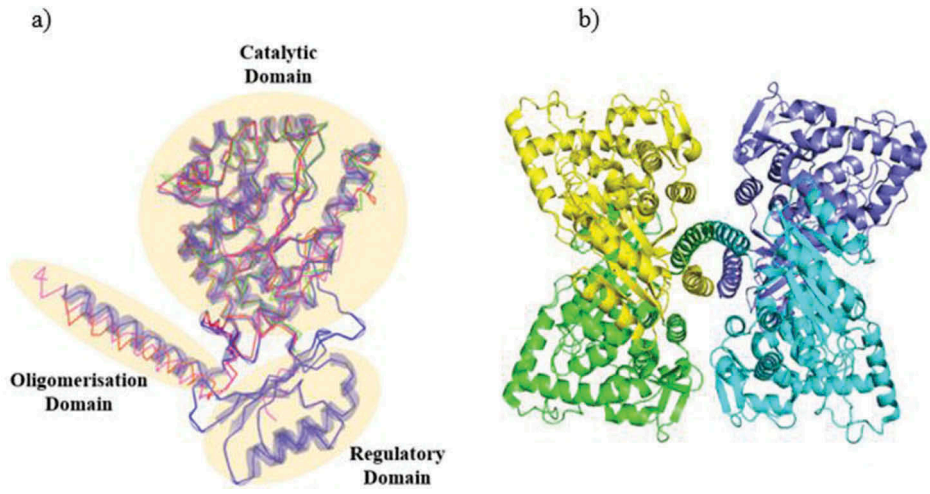

Figure 3. The domain organization and the tetrameric structure of the AAAHs. a) Superimposition of one subunit of each of the AAAHs, PAH, TH, TPH1, and TPH2. Blue: PAH (PDB: 5DEN), red: TH (PDB: 2XSN), green: TPH1 (PDB: 1MLW) and magenta: TPH2 (PDB: 4V06). The domains are highlighted and labelled. b) Tetrameric form of PAH (PDB: 5DEN). Each monomer of the tetrameric complex is represented in different colours. Full color available online.

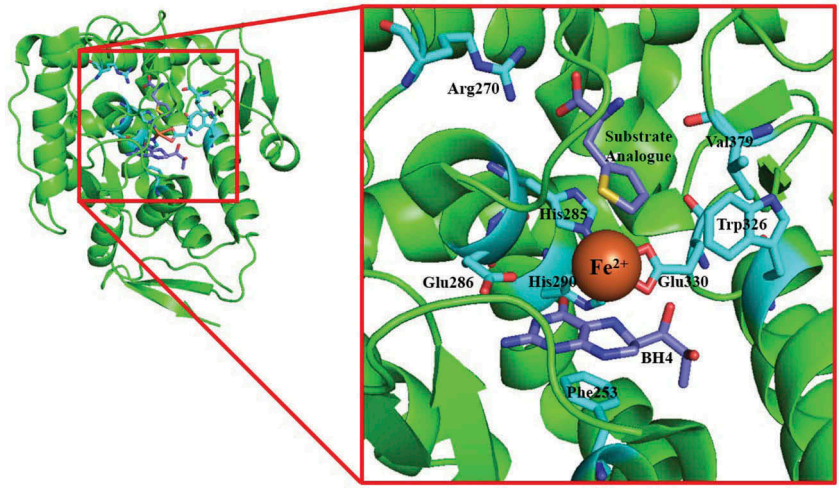

Figure 4. Crystal structure of PAH (PDB: 1MMK) cocrystalized with tetrahydrobiopterin (BH4) and substrate analogue thienylalanine. The active site is magnified to show the Fe^{2+} which interacts with His285, His290, and Glu330. The substrate analogue binds at the amino acid substrate binding site of the AAAHs.

human PAH), which form a 2-His-1-carboxylate facial triad. The cofactor binds close to the iron through conserved interactions within the AAAHs, that is, π-stacking with Phe254 and electrostatic/hydrogen bonding with Glu286 (Figure 4) [26]. The dihydroxypropyl side chain at C6 of BH4 also establishes specific hydrogen bonding interactions with non-conserved residues, which determine the specific configuration of this side chain for BH4 bound to each AAAH. These differences have been discussed in the context of hydroxylase-specific cofactor analogs and inhibitors [26]. X-ray crystallography has also provided the structure of TPH1 complexed with the cofactor (PDB: 1MLW) [28] natural substrate L-Trp (PDB: 3E2T) [29], which is in agreement with the binding of substrate analogs to PAH (Figure 4). The amino acid substrate binds opposite to the cofactor, at the other side of the iron, interacting with conserved Arg270 and His285 (in PAH), whereas Val379/Asp425/Ile366 and Trp326/Trp372/Phe313 (numeration in human PAH/TH/TPH1) contribute to the specificity of the AAAHs for their corresponding amino acid substrates [26].

2.4. Regulation of TH and TPH

As the biosynthetic pathways for dopamine and serotonin share several of the same pathway components (Figure 1), including the vesicular monoamine transporter, similar mechanisms for regulation could be expected. One major homeostatic or adaptive regulation of these monoamines is by controlling the cellular or even subcellular activity of TH and the TPHs. This makes the hydroxylases attractive targets for modulating monoamine levels and function. There are, however, some notable differences, such as end product feedback inhibition for homeostatic regulation of catecholamines and serotonin. Whereas TH is feedback inhibited and stabilized by catecholamines binding to the active site, serotonin lacks the catechol structure with the two adjacent hydroxyl groups that coordinate to the active site iron atoms and does not display such inhibition [5,30–32]. On the other hand, all AAAHs are inhibited by catechol binding [5,26,32], although the physiological relevance for the inhibition of PAH and the TPHs is unclear.

In the CNS, homeostatic regulation by cellular autoreceptors is found at dopaminergic, noradrenergic, and serotonergic neurons. The autoreceptors not only lower the excitation and secretion from these cells, but also target the hydroxylases by altering signal-mediated phosphorylation of the enzymes, leading to lowered cellular activity [33]. In addition to the cAMP/protein kinase A (PKA)-signaling pathway that is modulated by autoreceptors [33], several other pathways are found to regulate the phosphorylation status and activities of these enzymes. Thus, multiple phosphorylation sites are described, most of them residing in the N-terminal regulatory domain [5,32].

2.4.1. Regulation of TH by phosphorylation

Mammalian TH has four phosphorylation sites in its regulatory domain, corresponding to Ser8/Thr8, Ser19, Ser31, and Ser40 in the murine/bovine or human TH1 sequences. Phosphorylation of Ser40 is best understood, as it directly activates TH by reversing binding of catecholamine inhibitors and by increasing the affinity for BH4 [30,34]. Ser40 is targeted by cyclic nucleotide-activated kinases, as well as kinases downstream of MAPK1/2 such as MAPK-activated protein kinase 1 (MAPKAP-K1 or p90 Ribosomal S6 Kinase) and mitogen- and stress-activated kinase 1 (MSK1) that is also activated by p38 MAPKs [35,36]. The MAPK1/2 on the other hand phosphorylates TH primarily at Ser31, a site that is also targeted by cyclin-dependent kinase 5, and phosphorylation of this site is shown to activate TH about twofold *in situ* [37]. On the other hand, phosphorylation of Ser19 on TH does not directly alter its activity but allows for binding of the regulatory 14-3-3 proteins that can lead to an activation and stabilization of TH [38,39]. Major kinases that target Ser19 are the Ca^{2+}/calmodulin-dependent protein kinase II (CaM-KII) and the stress-activated kinase MAPK-activated protein kinase 2 (MAPKAP-K2) downstream of p38 MAPK [36,40]. The Ser40 site of TH seems to be under strict control in catecholamine producing cells and tissues, with a general low phosphorylation stoichiometry (3–5%/subunit), whereas the phosphorylation levels of Ser19 and Ser31 are higher (about 10–30%/subunit) [41].

2.4.2. Regulation of TPH by phosphorylation

The Ser19 site of TPH2, which is lacking in TPH1, is homologous to the Ser19-TH site and is phosphorylated by both CaM-KII and PKA [20,42]. PKA additionally phosphorylates TPH2 at Ser104, but the modest activation seems to arise from the Ser19 site, as does the association with 14-3-3 proteins [43]. Binding of 14-3-3 proteins further activates TPH2 and stabilizes the enzyme [44]. In addition, the bound 14-3-3 inhibits dephosphorylation of TPH2 and thereby prolongs the activation signal. Although TPH1 lacks the Ser19 site, it still binds to 14-3-3 proteins in response to phosphorylation by PKA and CaM-KII through its Ser58 site [45,46]. More potent activation by 14-3-3 is reported for TPHs purified from native sources than for TPHs expressed in bacteria [44].

2.4.3. 14-3-3 proteins

The 14-3-3 proteins are a highly conserved family of acidic proteins encoded by multiple genes in all eukaryotic species, including plants and mammals (13 genes in Arabidopsis and 7 in mammals, respectively) [47]. Binding assays have revealed more than 100 cellular binding partners, although the physiological role of the interaction is known only for a minority of these proteins. Remarkably, out of all these binding partners, TH and TPH were the first proteins to be identified to interact with 14-3-3 [48].

14-3-3 binding is in most cases dependent on Ser/Thr phosphorylation of the target protein. The most common binding site is a serine residue flanked by an arginine and proline residue [47,49]. There are three binding motifs of 14-3-3, called 'modes I–III': mode I: RSXpSXP, mode II: RXY/FXpSXP, and mode III: pS/pT X_{1-2}–CO_2H (X is not Pro) [47]. These binding modes are considered to be optimal, but due to the large number of binding partners, this is not absolute [49]. Binding of 14-3-3 can structurally alter its binding partner or its interaction with other molecules, which can give rise to a number of different regulatory effects. These properties, along with the many binding partners, have placed 14-3-3 proteins as important players in cell cycle and signal regulation, metabolism and cytoskeletal control, trafficking, apoptosis, and gene transcription [47,49].

In mammals, there are seven isoforms of 14-3-3: beta, gamma, epsilon, sigma, zeta, eta, and tau. The sequence identity between the seven isoforms is around 46%. Comparison between structures available on PDB also shows a high structural similarity [49]. As 14-3-3 is involved in such essential cellular pathways, 14-3-3 is found in various tissue types throughout the body. Different isoforms of 14-3-3 are expressed at different levels between tissue types, but all isoforms are highly expressed in brain [47,49].

14-3-3 proteins form dimers and depending on the isoform can form homo- or heterodimers. A single 14-3-3 monomer is made up of nine antiparallel α-helices (Figure 5). A single monomer has a large, 'C' shaped, amphipathic, ligand binding groove which is formed by H3, H5, H7, and H9 α-helices. The 14-3-3 dimer forms a 'W' shape with each of the ligand bindings grooves making up the inner surface of the dimer. The dimerization interface of a dimer is between H1 and H2 of one monomer and H3 and H4 of the other. The dimerized 14-3-3 reveals a large possibility of flexibility between the individual subunits, which probably contributes to the promiscuous binding capacity of 14-3-3s. Despite this flexibility, 14-3-3 maintains its shape in the apo form and possibly acts as a 'molecular anvil,' forming a rigid scaffold when binding to a ligand protein [49].

2.4.4. Structural considerations of the 14-3-3–TH complex

Currently, there are many structures available for the mammalian 14-3-3 isoforms, both as apo proteins and in complex with ligand proteins and peptides [47,49]. Thus far, the crystal structure of TH and TPHs including their N-terminal domain has not yet been solved. This limits our understanding of the hydroxylase-14-3-3 protein complex formation, which requires phosphorylation of Ser19 in both TH and TPH2 for binding to 14-3-3 [27,43]. However, the crystal structure of the Ser19 phosphorylated N-terminal peptide region of TH (pNT; residues 1–43) bound to 14-3-3 has been solved (PDB: 4J6S). Here, the pNT region adopts an extended conformation that binds

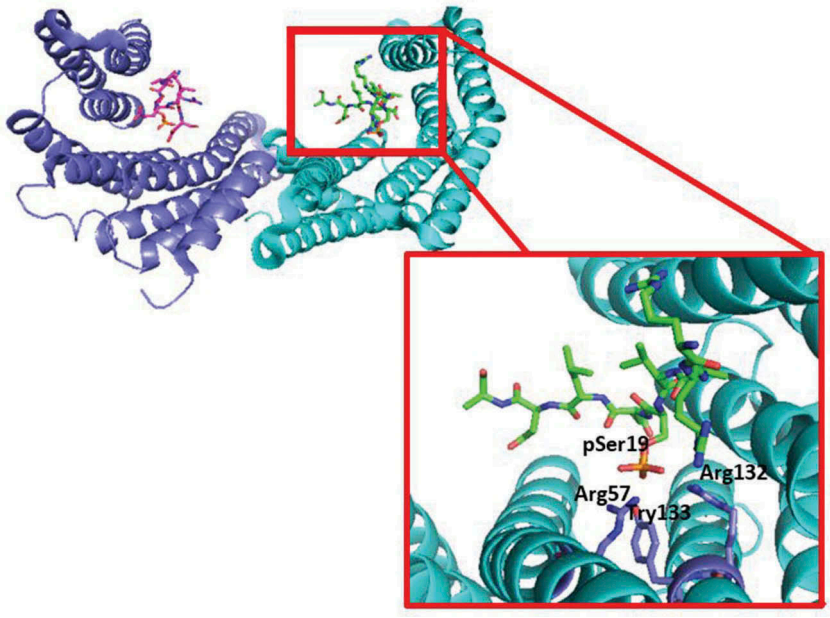

Figure 5. The dimeric structure of 14–3-3γ in complex with the Ser19 phosphorylated N-terminal region of TH peptide (pNT-TH; 1–43 residues) (PDB: 4J6S). The 14–3-3γ monomers are represented as blue and cyan. The pNT-TH peptides are coloured magenta and green. The box on the right presents the binding mode of pNT-TH with 14–3-3γ Phosphorylated Ser19 interacts with Arg57, Arg132, and Tyr133 (14–3-3γ numericals), and these residues are represented in blue. pNT-TH is represented in green. Full color available online.

to the phosphopeptide binding 'triad' involving two arginines and one tyrosine within the main amphipathic groove (Figure 5). The complex structure reveals that pNT-TH binds to 14-3-3 in a similar fashion as to other known peptides e.g. 1QJA, 1A38, 1A37, 2B05 [50,51]. As TPH has a high-sequence identity with TH and binds to 14-3-3 via a similarly phosphorylated serine, this 14-3-3–pNT-TH complex may be used as a model for the 14-3-3–TPH complex [43] (Figure 5).

Although Ser19 phosphorylation of TH is a determinant for complex formation with 14-3-3, the interactions involved in full length TH are probably more extensive than for pNT-TH. Thus, the affinity constant measured between 14-3-3γ and Ser19 phosphorylated TH is two orders of magnitude higher that the affinity measured for the pNT-TH [27,52]. As different mammalian 14-3-3 dimers have similar affinity toward Ser19 phosphorylated TH, the interaction surfaces may mainly be comprised of conserved surface regions on 14-3-3, close to the binding groove [38,49]. As found in the complexes of 14-3-3 with $PMA2H^+$-ATPase (PDB: 2O98) and 14-3-3 with serotonin *N*-acetyltransferase (AANAT) (PDB: 1IB1), both proteins have a flexible loop which binds to the phosphopeptide binding triad of 14-3-3 [53,54]. The maximal stoichiometry of the TH:14-3-3 complex was found to be two 14-3-3 dimers for one TH tetramer [52]. Although TH with low phosphorylation stoichiometry (0.15 phosphate/subunit) at Ser19 showed very similar binding kinetics to 14-3-3 as that of fully phosphorylated TH, further experimental evidence is needed to conclude on the possible binding of one or two phospho-Ser19 residues to the same 14-3-3 dimer [52]. Furthermore, this result also points to the importance of other TH regions than phospho-Ser19 determining the binding affinity, justifying efforts to solve the structure of the TH:14-3-3 complex with the major brain isoforms.

3. Diseases associated to mutations and dysfunction of the AAAH

Most of the drugs targeting monoamine signaling, including some inhibitors of AAAHs that are marketed today, are based on serendipitous discoveries made 50–100 years ago. This was before the advent of modern structural biology, the omics revolution, high-throughput screening, and computational methods. Recently, it has been shown that with the use of modern drug discovery methods, it is possible to develop novel and innovative drugs that target AAAHs.

As shown in Table 1, many different human symptoms or diseases have been attributed to decreased or elevated levels of catecholamines or serotonin. With the exception of the Mendelian forms of DOPA responsive dystonia and/or infantile parkinsonism, all these diseases are complex conditions with a pathophysiology involving many genetic and environmental factors. Still, promising results have been obtained by drugs that modulate AAAH functions and indirectly alter monoamine levels.

3.1. TH in human physiology, diseases, and mouse models of THD

Tyrosine hydroxylase deficiency (THD; OMIM *191290) produces a varied clinical picture, mainly as a DOPA-responsive dystonia and/or infantile parkinsonism [1]. This rare autosomal recessive neurometabolic disorder is caused by mutations in the TH gene, largely missense mutations, but a few variants in the promoter region have also been reported. THD is diagnosed in infancy with symptoms ranging from mild Parkinson's disease-like symptoms to severe encephalopathy. Based on clinical and biochemical features, THD can be

Table 1. Examples of human conditions that may be targeted by inhibitors or stimulators of TH or TPH activities.

	Inhibition	Compound(s)	Stimulation	Compound(s)
TH	Essential hypertension	Pyratrione [55] Pyridazine compounds [56] U-5021 [57] GYKI 11473 [58]	THD (DOPA-responsive dystonia/infantile parkinsonism)	L-DOPA supplementation [18]
	Pheochromocytoma symptoms (hypertension)	Alpha-methyl-tyrosine [59]	Parkinson's disease	L-DOPA supplementation [60]
			Depression	Tyrosine [61]
TPH1	Osteoporosis	LP533401 [62,63]	Irritable bowel syndrome/ constipation [64]	Tryptophan
	Carcinoid syndrome symptoms	Telotristat-etiprate [65] PCPA [66]		
	Irritable bowel syndrome	LX-1031 [67,68] LP-615819 [69]		
	Obesity	LP533401 [70]		
	Ulcerative colitis	LX-1031 [71] LP920540 [71]		
	Pulmonary arterial hypertension [72–74]	LP533401 [74] Gene therapy [75]		
TPH2	Anxiety [76]		Depression	Tryptophan [61], BH4

This list includes past and present treatment possibilities. Along with the examples of human conditions that may be targeted, potential pharmacological treatment options that have been proposed are also presented.

broadly classified into type A and type B. Type A THD is a progressive hypokinetic-rigid syndrome with dystonia with symptoms often appearing within the first year of life. Type B THD is a complex encephalopathy with onset within the first 3 months of life [18]. To date, data from approximately 70 patients have been recorded (according to www.biopku.org/pnddb) and certain genotype–phenotype correlations have been proven [77]. The most recurrent mutation in the *TH* gene (ca. 30% of all mutant alleles) is p.R233H (p.R202H in the TH1 isoform), largely associated with type B THD, showing a substantial reduction of dopamine levels and often a bad response to L-DOPA treatment [18]. Whereas the lack of viability of *Th*-knockout mice was established early [11,17], it is still unclear what is the threshold activity of TH needed for sufficient striatal dopamine levels, and how this relates to missense mutations affecting TH stability versus TH activity. Recent development of THD models using knock-in technology has shown the loss of function due to protein misfolding and instability. Thus, the *Th*-knock-in mouse with the mutation p.R203H, equivalent to the human TH-p.R233H (TH1-p.R202H), showed normal survival but gradual loss of total brain TH-immunoreactivity and central catecholamines that is not caused by loss of dopaminergic neurones. Whereas the *substantia nigra* presented almost normal levels of TH, the enzyme was distinctly absent in the striatum [78]. Both the loss of TH and its mislocalization in the nigrostriatal pathway were explained by the conformational instability of the mutant, further aggravated by a deficient stabilization by catecholamines that would result in defective transport to striatal axonal terminals. The p.R203H mice suffered from hypotension, hypokinesia, reduced motor coordination, wide-based gate, and catalepsy, with a marked diurnal fluctuation of the motor defects. All together these symptoms, which did not improve with standard L-DOPA treatment, point to this mouse as a suitable clinical model of Type B THD.

The *Th*-knock-in mouse with the mutation p.Q382K, equivalent to the human TH-p.Q412K (TH1-p.Q381Q), has also been characterized, rather showing a recapitulation of the core features of Type A THD, with a dystonia that is L-DOPA responsive [79]. In addition to the kinetic defect of the mutation [80], a conformational destabilization and misfolding was also noted [77] and associated to a marked decrease of TH in brain regions containing axonal terminals [79]. These authors also characterized defects in the microstructure of striatal synapses and found specific abnormal receptor responses in the p.Q382K mouse. Furthermore, the diurnal fluctuation in the dystonia was so striking that these mutant mice showed normal motor performance right after the sleep period [78,79]. Although THD is not accompanied by neurodegeneration, it shares several traits with Parkinson's disease, where degeneration of dopaminergic neurons in the midbrain results in loss of striatal dopamine and the characteristic motor phenotype [81].

Multiple sources of evidence indicate that other neuropsychiatric disorders, such as schizophrenia, depression, bipolar disorder, autism, and ADHD, either directly or indirectly, are associated with altered monoamine functions, notably dopamine [1]. Most of the current medications for these conditions either increase or decrease the effective levels of dopamine, norepinephrine, and epinephrine in monoaminergic synapses. For example, the dopamine and norepinephrine transporter blockers methylphenidate, amphetamine, and atomoxetine are used to treat ADHD symptoms [82], whereas as L-DOPA-responsive dystonia, Parkinson's disease is mainly treated by supplying L-DOPA, the product of TH [18,60] (Figure 1). An alternative treatment aiming to stimulate dopamine synthesis in such conditions could be stimulation or stabilization of the TH enzyme. As discussed below, such treatment options might include pharmacological chaperones (PCs) or small molecule modulation of protein–protein interactions (PPIs). The presently available THD mice models not only represent robust models of the human patients that carry the corresponding mutations and contribute to our understanding of pathophysiological mechanisms of THD, but are also valuable for the development of these alternative therapeutic approaches. Furthermore, they seem suitable for the study of treatment strategies based on circadian regulation of TH activity and/or restorative properties of sleep, which have so far been the subject of little research.

3.2. Synthetic TH inhibitors and substrates

Rapidly following the discovery of TH as the rate-limiting enzyme in catecholamine synthesis, a range of different natural and synthetic compounds was tested as alternative substrates and inhibitors of this enzyme (reviewed in Refs. [5,6]). Although many of the substances were shown to reduce catecholamine production in experimental systems, few compounds have been subject to detailed mechanistic studies or clinical trials [5,6,55,59]. In particular, the substrate analogs alpha-methyl-tyrosine (AMPT) and 3-iodo-L-tyrosine were shown to be potent TH inhibitors [5,6,83]. Due to the rapid degradation 3-iodo-L-tyrosine *in vivo*, it has not been subjected to detailed pharmacological studies. However, AMPT has been used in clinical trials in humans to treat hypertension in patients with pheochromocytoma but has been less effective in the treatment of essential hypertension in humans [59,84]. More recently, the tyrosine analog 3-fluoro-L-α-methyl-tyrosine has also been shown to be effective in imaging of malignant tumors, due to its high affinity to the L-type amino acid transporter [85].

A range of synthetic analogs of the BH4 co-substrate has also been synthesized and shown to support enzymatic activity for all the AAAHs [5,6,86,87]. As some of the BH4 analogs containing hydrophobic substituents in 6-position of the pyrazine moiety have higher affinities for the AAAHs than the natural cofactor 6R-BH4, they have been suggested to be effective in stimulating monoamine production *in vivo* [86,87]. However, due to their limited solubility and poor blood–brain barrier penetration, the therapeutic value of these compounds has so far been limited. The structural basis for these interactions with TH, TPH1, TPH2, and PAH has previously been reviewed [26].

In addition to compounds targeting the amino acid and BH4 binding sites, iron chelators, and structural analogs of the catecholamine feedback inhibitors have been tested as TH inhibitors. As the natural catecholamines are nonspecific inhibitors of the all the AAAHs, search for more potent and selective catechol analogs has been conducted [34]. These studies showed that dopamine agonists such as SKF-38393 and apomorphine are potent inhibitors of human TH and possibly can contribute to the clinical effects of such compounds [34]. Tetrahydroisoquinolines are catecholamine derivatives formed in the nigrostriatal system of the human brain. It has been reported that the levels of these compounds are increased in the cerebrospinal fluid of patients with Parkinson's disease, but the clinical significance of this finding is unclear. These catechol type of TH inhibitors include *N*-methyl-norsalsolinol and salsolinol that are hypothesized to contribute to the decreased production of catecholamines found in Parkinson's disease [88,89].

An early indication that the AAAHs were iron containing enzymes came from the inhibitory effects of metal ion chelators [31]. Several low-molecular chelators bind to the active site iron and some of these also effectively extract the iron from the enzyme [90]. Some of these inhibitory chelators also inhibit the AAAHs *in vivo* and have been proposed as agents to explore their physiological functions, although their therapeutic potential probably is limited, due to their lack of specificity [91].

3.3. TPH1 and TPH2 in human physiology, disease, and mouse models of TPH1 and TPH2

The majority of peripheral serotonin, which is produced by TPH1, is found in the intestine where it is involved in gastrointestinal motility and homeostasis [92]. Circulating serotonin is mainly stored in platelet dense granules and is involved in regulation of platelet aggregation [93] not only through receptor binding but also by protein serotonylation that activate alpha-granule release [90]. Released serotonin increases vasoconstriction and has been implicated in vascular remodeling, leading to pulmonary arterial hypertension [72]. In addition, it has been suggested that inhibitors of TPH1 may be used to treat or prevent thrombosis [94].

Further investigations of the *Tph1*-knockout mouse have indicated a role of peripheral serotonin in bone density remodeling through stimulation of osteoclastogenesis, leading to lowered bone density [95]. Pharmacologic inhibition of TPH1 confirms this observation [62]. Interestingly, *Tph1*-knockout mice show protection from obesity and insulin resistance from high-fat diet, suggesting a role for serotonin in brown adipose tissue thermogenesis and identifying TPH1 as a possible target for treatment of obesity and metabolic dysfunction [70]. Carcinoid syndrome is a condition that is associated with elevated peripheral serotonin, gastrointestinal symptoms, and cardiac vascular disease [65]. Inhibition of TPH1 is an approach to target some of the symptoms associated with this syndrome.

In contrast to *Th* knockouts that are not viable, mice harboring the combined deletion of both *Tph1* and *Tph2* genes are viable but display behavioral changes compatible with a role of serotonin in brain function [96]. *Tph2*−/− mice have impaired early postnatal growth as well as many physiological differences, the most notable being an altered autonomic sleep pattern. In addition, these mice have several behavioral differences including decreased anxiety, increased aggression, and maternal neglect [76,97].

In humans, several disorders have been reported to be related to dysregulation of the serotonin system such as depression, schizophrenia, and ADHD [98]. Serotonin dysregulation has also been proposed to play a role in autism [99]. Variants in TPH2 have also been associated with major depression, bipolar disorder, suicidal behavior, personality disorder, and ADHD [100]. However, in large genome wide association studies, common variants in TH, TPH1, or TPH2 have not shown convincing associations with any known human phenotypes tested so far. A recent comprehensive analysis of variants in TH, TPH1, and TPH2, as well as the 14-3-3 genes (*YWHAs*), across several psychiatric disorders has shown modest effects for the hydroxylase genes, but significant association of 14-3-3 epsilon (*YWHAE*) with schizophrenia [100].

As for TH, many rare coding variants, including complete loss of function variants, have been described for TPH1 and TPH2. Unlike for TH, humans homozygous for complete loss of function variants of TPH1 and TPH2 have not been described. However, many heterozygous missense variants are known and have been reported to be associated with various psychiatric phenotypes [101,102]. Intriguingly, maternal loss of function variants in TPH1 are associated with increased

psychiatric symptoms in the offspring, as also found in mouse *TPH1*-knockout studies [103,104]. In addition, mutations in TPH1 as a peripheral enzyme have been reported to be involved in gastrointestinal disorders [32]. TPH1 inhibitors and *TPH1* knockouts have been shown to be effective in treating pulmonary arterial hypertension in mouse models [75] and have been suggested for treatment in humans [72–75].

3.4. Synthetic TPH1 and TPH2 inhibitors and substrates

Since the AAAHs were discovered around 1960, research on these enzymes has been conducted in parallel. The same classes of synthetic substrates and inhibitors have been tested for all enzymes, mainly targeting the amino acid binding site, cofactor binding, and the active site iron, as mentioned for TH. A number of patents have recently been filed for novel pharmacological compounds to inhibit TPH and its use to treat serotonin associated disorders [105–108].

The substrate analog *p*-chlorophenylalanine (fenclonine, PCPA) has a modest affinity for these enzymes *in vitro* ($IC_{50} \approx 250$ μM) [69]. However, *in vivo* it is a potent and irreversible inhibitor of TPH (and PAH) that increases phenylalanine levels and rapidly depletes serotonin from the nervous system [109]. In contrast, it has little effect on catecholamine levels. This compound has been frequently used in animal studies to deplete serotonin levels and in limited human studies to treat symptoms of carcinoid syndrome. However, due to unacceptable side effects, including depression, it has been abandoned for this indication in humans [66]. Still, much of what we know about the physiological roles of serotonin is based on animal experiments with this compound during the past 50 years.

The mechanism of action of *p*-chlorophenylalanine has been debated. It has been suggested that the halogenated amino acid is a suicide substrate that is covalently incorporated into the protein structures of PAH and TPH, but not TH [110]. However, as low amounts of radiolabelled *p*-chlorophenylalanine were recovered from immunoprecipitated TPH enzyme, an alternative hypothesis has been proposed, namely that a metabolic derivative of *p*-chlorophenylalanine is responsible for the irreversible inhibition. Different levels of the postulated converting enzyme might explain why *p*-chlorophenylalanine is not equally effective in depleting serotonin levels in all serotonin producing tissues [111].

More recently, several new more selective and reversible inhibitors of TPH1 and TPH2 have been synthesized and tested in different systems [69,112,113]. Due to their similar active site structures, these compounds inhibit purified TPH1 and TPH2 to a similar extent [69]. However, due to the different tissue distribution of the enzymes and the low blood–brain barrier permeability of some of these relatively bulky compounds, they mainly inhibit TPH1 *in vivo* [69]. Such TPH1 inhibitors have shown promising effects in treatment of osteoporosis in animal models [62,63]; however, the effectiveness of TPH1 inhibitors as treatment for osteoporosis has been debated [114]. The TPH inhibitor telotristat–etiprate (LX-1032) has been shown to provide symptomatic relief in patients with carcinoid syndrome [65]. Inhibition of TPH1 of the enterochromaffin cells of the gut has been shown to decrease levels of serotonin in the periphery and increase bone formation [62]. Peripherally acting inhibitors of TPH1 have also shown promising effects in animal models of ulcerative colitis [71], irritable bowel disease [68], obesity [70], pulmonary arterial hypertension [74], asthma [115], and lung fibrosis [116].

4. Novel opportunities for targeting TH and TPH in monoamine therapy

4.1. PCs

PCs are a relatively new concept in the development of therapeutics. PCs are small molecular weight compounds that bind selectively to unstable protein conformations and stabilize their native state and/or aid the refolding of misfolded conformations, recovering (totally or partially) the native structure and proper function and trafficking [14,117]. Proof of principle has been obtained for PCs that restore protein function in unstable and partly misfolded mutants associated with a number of loss-of-function misfolding diseases, due to their rescue of the mutant proteins from degradation by the cellular quality control system [118]. Several PCs against lysosomal disorders are already in clinical trials demonstrating a promising approach toward small molecule-based therapies [119,120]. These therapeutics may be classified as active site-specific PCs, and non-active site PCs [121]. The initially described PCs rather belonged to the first type, which were developed through derivatization of natural ligands and agonists [117,121], largely based on the realization that supplementation with cofactor and coenzymes increases the activity and stability of numerous variant enzymes [122]. Indeed, a chaperone response on mutant PAH seems to be an important molecular mechanism behind BH4-responsive PKU [123]. Similarly, BH4 treatment increases TH activity and protein levels in treated mice [124]. On the other hand, it is increasingly recognized that development of non-active site PCs, especially those that show allosteric activating function, is to be preferred [121,125], since they may induce a more active, stable conformation on the target enzyme even during catalysis, without inhibiting the activity. For the AAAHs, it has been shown that several compounds effectively preserve TH activity by their weak binding to the catalytic iron through a pyrimidine nitrogen atom [126]. Thus, for TH, and likely also for the TPHs, therapeutic options including combinations of different types of PCs might be preferential [126].

4.2. Targeting PPIs

PPIs represent a large group of potential therapeutic targets both inside and outside the cell. PPIs are central to most biological processes and their dysregulation is therefore involved in many disorders [127]. The human interactome is estimated to be >650,000 interactions [128] and based on this, it can be argued that PPI modulation is one of the areas in drug discovery that holds the most promise for novel therapeutic interventions. PPIs can be targeted

using both inhibitors and stimulators. However, in general, PPI stabilization has been less successful than PPI inhibition [129]. Indeed, PPIs are more susceptible to disruption, and potent inhibitors of PPI interactions have been developed, with a few of recent rationally discovered PPI disruptors reaching clinical trials [127]. Nevertheless, small peptide analogs and small molecule stabilizers of relevant PPIs are also being discovered, such as stabilizers of the retromer which limits APP processing in hippocampal neurons [130], or of specific oligomeric forms, such as the non-aggregating tetrameric conformation of α-synuclein [131].

In the case of 14-3-3 proteins, both inhibitors and stabilizers have been discovered and both categories of compounds interact within the main phosphate groove of 14-3-3 [132]. Proteins that bind to 14-3-3, including TH and TPH, interact with 14-3-3 at the phosphopeptide binding site involving the specific arginines and tyrosine residues (Figure 5). Direct targeting of the phospho-Ser/Thr recognition site would be expected to inhibit broadly among 14-3-3 target proteins, but with higher potency toward low affinity binders. Although 14-3-3 proteins interact with many different targets, there are still several possibilities to improve selectivity, such as to target surface regions outside the phospho-peptide recognition site. Also, in a kinetics modeling study of 14-3-3 interactions, we found that sensitivity toward changes in affinity, as well as changes in signaling, input differed among target types [133].

4.2.1. Targeting the complexes of TH-pSer19 and TPH2-pSer19 with 14-3-3 proteins

As presented above (Section 2.4.3), Ser19-dependent activation of both TH and TPH requires the binding to 14-3-3 proteins. Stabilization of this interaction might be a therapeutic option. In the case of the 14-3-3 proteins, both inhibitors (R18, PDB: 1A38 [50]; FOBISIN101, PDB: 3RDH [134]) and stabilizers (fusicoccin, PDB: 5EXA [135]; epibestatin, PDB: 3M50 [136]) have been found [129,132]. However, to achieve specificity for the multipartner 14-3-3 proteins, which have hundreds of partners, this becomes very challenging. Recently, a semi-synthetic natural product derivative has been discovered that specifically stabilizes the cancer-relevant interaction of Gab2 phosphorylated at Thr391 with 14-3-3 (PDB ID: 5EXA) [135]. Whereas several experimental and virtual screening strategies, as well as derivatization of natural ligands, are methods customary used for the identification of compound hits that stabilize unstable protein targets and may be potential PCs [14], the discovery of PCs to stabilize PPI requires the integration of a large number of biochemical and biophysical techniques with access to structural information of the PPI interface [130,131].

5. Expert opinion

Most drugs that are effective against human neuropsychiatric disorders and some drugs used in cardiovascular diseases target a few receptors, transporters, and enzymes, mainly within the classical monoaminergic neurotransmitter systems. However, these proteins constitute a small fraction of potential targets within the ubiquitous and complex monoamine signaling systems.

In the past, amino acid analogs have been developed as inhibitors of the key regulatory enzymes TH, TPH1, and TPH2. Such inhibitors have been essential in dissecting the physiological roles of serotonin and catecholamines. More recently, access to genetically modified animal models and genomic data from large patient cohorts have revealed that catecholamines and serotonin also are essential endocrine and paracrine signaling molecules in a diverse range of physiological conditions and disease states. In particular, TPH1 and TPH2 inhibitors have shown promising results in the treatment of carcinoid syndrome, gastrointestinal dysfunction, and possibly asthma, osteoporosis, and bone homeostasis. This has triggered a new interest in developing more potent and selective inhibitors.

Here, we have reviewed this historical development, with a focus on recent structural and mechanistic insights into the TH, TPH1, and TPH2 enzymes and how these data can be exploited in the search for new therapeutic compounds.

We point to the following areas of research that should be further developed:

(1) High-resolution 3D structures are available for all these three enzymes, notably for the catalytic domain including complexes with substrates and inhibitors. This has paved the way for rational drug design, where *in silico* docking and modeling can be combined with *in vitro* screening and combinatorial chemistry to develop more effective compounds.
(2) While amino acid analogs have been successfully used as inhibitors of all these enzymes, the BH4, and iron-binding sites, as well as allosteric ligand binding modes should be further explored.
(3) A first generation of PCs has been discovered that can partially restore the functions of misfolded TH, TPH1, TPH2, and PAH due to missense mutations. These data are encouraging and provide hope of developing new therapies for protein misfolding diseases, including THD syndromes.
(4) Genetically modified organisms with selective knockout or knock-in of these enzymes, or other signaling components, are valuable disease models and experimental tools. In particular, knock-in mice harboring unstable missense variants of these enzymes are useful to test the effects of PCs *in vivo*.
(5) TH, TPH1, and TPH2 are regulated by an intricate combination of site-specific phosphorylation and interactions in protein complexes. In theory, modulation of the phosphorylation of hydroxylases and their interaction with 14-3-3 proteins provide novel opportunities for monoamine targeting that should be investigated.
(6) Successful application of novel therapies will require more structural information, as well as understanding of the cellular kinetics of regulatory mechanisms. This information, together with more quantitative experimental data, could be implemented in mathematical models of monoamine synthesis. Such predictive models could become valuable tools to better understand

the regulation of monoamine synthesis, release, and recycling to allow effective targeting strategies to be tested and evaluated.

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Declaration of interest

A. Martinez has a patent on the on the use of the first generation pharmacological chaperones for AAAHs mentioned in the text (compositions for the treatment of hyperphenylalaninemia). The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

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