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Phenylalanine hydroxylase contributes to serotonin synthesis in mice

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Abstract

Serotonin is an important signaling molecule in the periphery and in the brain. The hydroxylation of tryptophan is the first and rate-limiting step of its synthesis. In most vertebrates, two enzymes have been described to catalyze this step, tryptophan hydroxylase (TPH) 1 and 2, with expression localized to peripheral and neuronal cells, respectively. However, animals lacking both TPH isoforms still exhibit about 10% of normal serotonin levels in the blood demanding an additional source of the monoamine. In this study, we provide evidence by the gain and loss of function approaches in *in vitro* and *in vivo* systems, including stable-isotope tracing in mice, that phenylalanine hydroxylase (PAH) is a third TPH in mammals. PAH contributes to serotonin levels in the blood, and may be important as a local source of serotonin in organs in which no other TPHs are expressed, such as liver and kidney.

KEYWORDS
phenylalanine hydroxylase, serotonin, tryptophan hydroxylase

Abbreviations: 5-HT, serotonin; 5-HTP, 5-hydroxytryptophan; α-MPA, alpha-methylphenylalanine; AADC, aromatic amino acid decarboxylase; CYP, cytochrome-dependent monooxygenase; F, phenylalanine-residue; PAH, phenylalanine hydroxylase; PCA, perchloric acid; TPH, tryptophan hydroxylase; TH, tyrosine hydroxylase; W, tryptophan-residue.

Alexander Mordhorst and Priyavathi Dhandapani contributed equally to this work.

1 | INTRODUCTION

Serotonin (5-Hydroxytryptamine, 5-HT) is an ancient signaling molecule produced by some bacteria and most if not all Eukaryota including numerous plants.[1] Historically 5-HT has been first extracted from the gastrointestinal tract and blood of mammals, by the groups of Erspamer[2] and Rapport[3] in 1937 and 1948, respectively. Still, it would take them until 1952 to discover, that they had found the same molecule.[4] Only thereafter 5-HT was found in the central nervous system of vertebrates,[5] where it acts as the main neurotransmitter in a network influencing most aspects of behavior and cognition and in the enteric neurons in the gut,[6] being essential for normal gastrointestinal motility.[7] However, almost all 5-HT in mammals are located outside of the brain being generated by enterochromaffin cells in the gut and then taken up and distributed in the bloodstream by platelets.[8] Although less well-known, the peripheral 5-HT regulates a multitude of important bodily functions. Following its release from circulating platelets upon local activation or from tissue cells storing or producing 5-HT, it affects processes spanning from hemostasis[9] and vascular tone,[10] to metabolic regulation in gut, liver, fat tissue, and endocrine pancreas.[1] 5-HT is generated from the amino acid tryptophan (Trp) in a two-step enzymatic process. In the first and rate-limiting step, Trp is hydroxylated to 5-hydroxytryptophan (5-HTP), followed by its decarboxylation to 5-HT by aromatic amino acid decarboxylase (AADC). Tryptophan hydroxylase (TPH) was cloned in 1987 and found to be part of the aromatic amino-acid hydroxylase (AAAH) family of enzymes, also including phenylalanine hydroxylase (PAH) and tyrosine hydroxylase (TH).[11] Until 2003, when knockout (KO) mouse models for TPH were described by us[12] and others,[13] it was considered the only enzyme responsible for serotonin synthesis in the brain and periphery. While in these animals peripheral 5-HT levels were drastically reduced, they surprisingly showed normal 5-HT levels in the brain. This led to the discovery of the TPH2, the neuron-specific TPH isoenzyme, and the coining of the "duality" concept for the serotonin system: It consists of two completely independent networks in neuronal and non-neuronal tissues controlled by the gene products of two different paralogous genes, *Tph2* and *Tph1*, respectively and separated by the blood-brain barrier.[14] The exclusive activity of TPH2 for neuronal 5-HT synthesis was confirmed shortly thereafter by the identification of a mouse strain with a naturally occurring point-mutation,[15,16] and a few years later conclusively clarified by the generation of TPH2-KO models in mouse, rat, and pig, which were all lacking 5-HT in the brain[17-21] and the enteric nervous system.[22] However, further studies revealed that TPH1-deficient mice, although being totally devoid of TPH1 enzyme, still contain 5-HT in the bloodstream, amounting to about 10% of wild-type (WT) levels.[13] These amounts of peripheral 5-HT remain unchanged in TPH1/TPH2-double KO animals, arguing that they are not contributed by TPH2 in the brain or the enteric nervous system.[17] In this study, we aim to elucidate the source of the residual blood 5-HT, which together with the gut is the largest serotonin pool in the mammalian body, and identify the enzyme which functions as a third TPH *in vivo*.

2 | METHODS

2.1 | Animals

Mice were maintained at the MDC animal facility in individually ventilated cages (Tecniplast, Italy) under specific pathogen-free, standardized conditions in accordance with the German Animal Protection Law. Mice were group-housed at a constant temperature of 21 ± 2°C with a humidity of 65 ± 5%, an artificial 12 hours light/dark cycle, and with free access to water *ad libitum*. All experimental procedures were performed according to the national and institutional guidelines and have been approved by responsible governmental authorities (Landesamt für Gesundheit und Soziales (*LaGeSo)*, Berlin, Germany).

TPH1-deficient[12] and TPH2-deficient[18] mice were cross-bred to generate TPH1/TPH2-double KO mice. PAH^{enu2} mice (PAH-KO) were obtained from Jackson Laboratories, USA and then cross-bred with TPH1-KO mice to generate TPH1/PAH-double-KO animals. All animals except PAH-KO and TPH1/PAH-KO were fed a standard diet (0.25% sodium; ssniff, Soest, Germany). TPH1/PAH-double-KO and PAH-KO were routinely maintained under Phe-free diet (S0087-E745, Ssniff, Soest, Germany). To check the effect of standard Phe-rich diet on serotonin metabolism, animals were fed standard chow for 4 weeks prior to experiments.

The PAH inhibitor α-methyl-L-phenylalanine (α–MPA, Sigma-Aldrich, Germany) was administered intraperitoneally (ip) at a dose of 0.43 mg/g mouse weight every 12 hours for 2.5 days. The control animals were treated with saline.

^{13}C11 L-tryptophan (Cambridge Isotopes, USA) was administered ip to 18hrs starved mice at a dose of 30 mg/kg. Animals were sacrificed 4 hours after administration.

For organ and blood collection, animals were anesthetized by ip injection of ketamine (100 mg/kg)/xylazine (10 mg/kg)). Three hundred microliters of whole blood were collected into 1 ml syringes prefilled with 100 µL of heparin and quickly transferred to Eppendorf tubes containing 10 µL of perchloric acid (PCA) and 5 µL of 10 mg/mL of ascorbic acid, vortexed, centrifuged (20 000 ×*g*, 30 minutes, 4°C) and the supernatant was frozen at −80°C until HPLC and LC-MS/MS measurements. Immediately after the blood collection, mice were transcardially perfused with ice-cold

PBS to remove blood, containing platelet 5-HT. Tissues were snap-frozen in liquid nitrogen and kept at −80°C until further analysis by HPLC, LC-MS/MS or 5-HTP generation assay.

2.2 | HPLC

Frozen tissue samples were homogenized in 710 µM ascorbic acid and 2.4% perchloric acid (Sigma-Aldrich, Steinheim, Germany), and precipitated proteins were pelleted through centrifugation (20 minutes, 20000 ×*g*, 4°C) and the collected supernatant was analyzed for serotonergic metabolites (Trp, 5-HTP, and 5-HT) using high-sensitive reversed-phase high-performance liquid chromatography (HPLC) with fluorometric detection. Samples were separated over a C18 reversed phase column (LipoMare C18, AppliChrom, Oranienburg, and ProntoSIL 120 C18 SH, VDS Optilab, Berlin) at 20°C in a 10 mM potassium phosphate buffer (pH 5.0) (Sigma-Aldrich, Steinheim, Germany) with 5% methanol (Roth, Karlsruhe, Germany) and a flow rate of 0.8-1.0 mL/min. The excitation wavelength was 295 nm and the fluorescent signal was measured at 345 nm. CLASS-VP software (Shimadzu, Tokyo, Japan) was used to analyze the peak parameters of chromatographic spectra and quantify substance levels, based on comparative calculations with alternately measured external standards.

2.3 | 5-HTP generation assay in organ lysates and transfected cells

The 5-HTP generation assay was adapted from a previously published method from our lab.[23] Frozen organs were homogenized in 75 mM Tris-acetate (pH 7.5) (Roth, Karlsruhe, Germany), sonicated, and briefly centrifuged. Lysates were incubated with catalase (2 mg/mL), ferrous ammonium sulfate (100 µM), 1,4-dithiothreitol (25 mM, Sigma-Aldrich, Steinheim, Germany) for 10 minutes at 30°C. Tris-acetate of 15 mM (pH 6.4) (Roth, Karlsruhe, Germany), 50 µM L-tryptophan, and 2 mM NSD1015 (Sigma-Aldrich, Steinheim, Germany) were added in a second incubation step at 37°C. The reaction was stopped by adding 2.4% perchloric acid (70%) (Sigma-Aldrich, Steinheim, Germany). The tryptophan hydroxylating activity in organs was determined by the accumulation of 5-HTP after 30 minutes of incubation. For the pharmacological inhibition of PAH activity, 140 mM α-MPA was added to the reaction.

HEK293 cells were seeded in 6 well plates and transfected with a human PAH expression plasmid (Origene Technologies, USA) using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocols. Cell lysates were obtained 48 hours post-transfection and *in vitro* 5-HTP generation assay was performed as described for organ lysates.

2.4 | 5-HTP generation assay in primary hepatocytes

Murine primary hepatocytes were isolated and seeded with phenol red-free William E medium on collagen A coated dishes suitable for fluorescence microscopy. The cells were allowed to adhere overnight, serum-starved for 12 hours, and the ex vivo 5-HTP generation assay was performed with the addition of 250 µM Trp and 250 µM 6-methyl-5,6,7,8-tetrahydropterine for 2 hours. The assay was stopped by washing the cells with PBS and fixation with paraformaldehyde. NSD-1015 was not added to avoid cell death. For immunofluorescence analysis, the fixed cells were blocked for 30 minutes with 3% normal donkey serum in PBS, followed by incubation with the primary antibody against 5-HTP (1:250) (ImmunoStar, USA) in blocking solution overnight at 4°C. The next day, sections were washed with PBS three times for 15 minutes and incubated with the secondary antibody solution in PBS for 2 hours at room temperature. The cells were washed again three times with PBS and VECTASHIELD mounting medium containing DAPI was added. Images were taken using an inverted microscope (Keyence BZ-9000, Japan). Stainings were quantified using the BZ-II Analyzer software (Keyence, Japan).

2.5 | LC-MS/MS

Tryptophan, its metabolites, and their ^{13}C marked analogs were analyzed using HPLC and the triple quadrupole mass spectrometer Agilent 6470/1290 equipped with an AJS electrospray ion source (Agilent Technologies Santa Clara, CA). An HPLC column (Phenomenex Synergi Hydro-RP 150 × 2 mm, 4 µm, Aschaffenburg, Germany) was used as a stationary phase and a gradient of 0.1 % formic acid in water and methanol as a mobile phase.

The gradient started at 0.5 % methanol, held for 0.5 minutes, and was increased to 40 % methanol after 6.5 minutes and 95 % after 7 minutes. The run stopped after 15 minutes, the flow rate was 0.4 mL/min, the injection volume 2.5 µL.

All solvents in LC/MS/MS purity were purchased from Merck (Darmstadt, Germany).

The MS/MS transitions and ion source parameters were optimized using authentic standards purchased from Sigma-Aldrich (Munich, Germany). The detailed list of all transitions is shown in Supplementay Table S1 and the ion source parameter in Supplementary Table S2.

The system was calibrated in the range from 0.2 to 200 ng/mL with eight levels for each compound. The calibration curves of ^{12}C analogs were used also for ^{13}C-metabolites. The linear regression was calculated using Agilent Mass Hunter software. The lower limit of quantification and the coefficient of determination are shown in Supplementary Table S3.

2.6 | Generation of multiple sequence alignment and phylogenetic tree

Protein sequences were retrieved from the NCBI protein database in "*FASTA*"-format. Multiple sequence alignment (MSA) was performed in RStudio using the "*msa*" package and "*ClustalW*" alignment algorithm. The phylogenetic tree was estimated via the maximum likelihood method using the "*phangorn*" package. The final figure was generated via the "*ggtree*" package.

2.7 | Statistics

All data were subject to statistical analysis using R (version 4.0.2) and RStudio (version 1.3.959). The results shown in this study represent mean values ± standard error of mean (SEM), unless stated otherwise. Tests defining the level of significance included Student's t test to compare independent pairs of means, while more than two groups were analyzed by one-way ANOVA with Bonferroni post hoc test. Results with $P < .05$ were considered to be significantly different.

2.8 | Schematic drawings

The schematic drawing shown in Figure 4A was created with BioRender (BioRender.com).

3 | RESULTS

3.1 | PAH is the most promising candidate for Trp hydroxylation

In order to confirm that TPH2 does not contribute to the production of residual serotonin in the blood of TPH1-KO mice, we measured the blood and organ levels of 5-HT in TPH1-KO and TPH1/TPH2-double KO animals. Indeed, both strains equally exhibited around 10% of WT 5-HT levels in the blood as previously reported (Figure 1A).[17] The 5-HT concentration in spleen, the main place of platelet breakdown and release of the serotonin contained within them, was coherent with 5-HT levels in the circulation (Figure 1A). In the duodenum, serotonin was nearly undetectable in both lines demonstrating that TPH1 is the main serotonin-synthesizing enzyme in

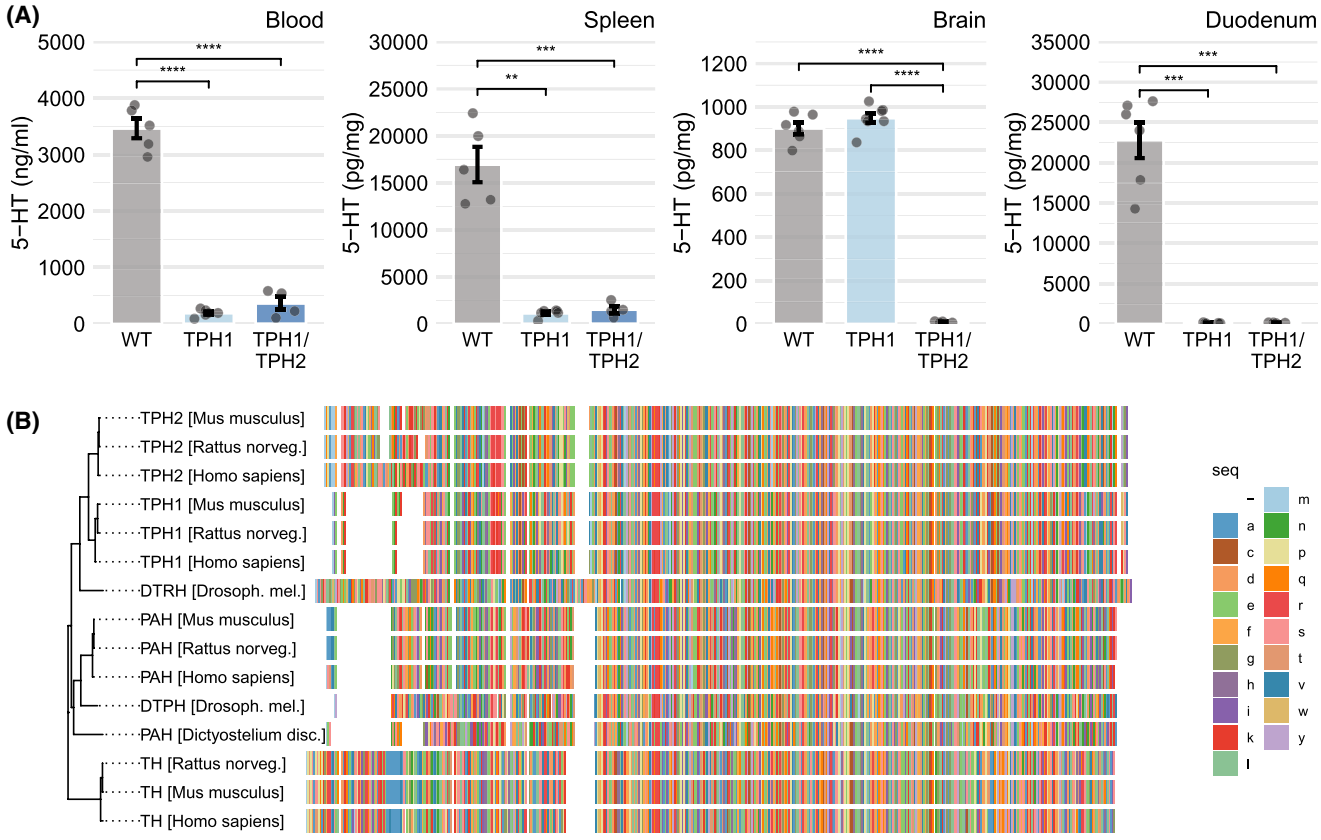

FIGURE 1 PAH is a candidate for serotonin synthesis in mammals. A, 5-HT levels in blood, spleen, brain, and duodenum of wild-type (WT), TPH1-KO, and TPH1/TPH2-double KO mice. $^{**}P < .01$, $^{***}P < .001$, $^{****}P < .0001$, one-way ANOVA with Bonferroni post hoc test. B, Multiple sequence alignment and derived phylogenetic tree showing stronger homology between TPH- and PAH families than to THs or either TPHs or PAHs

this organ and that the contribution of TPH2 in myenteric neurons is negligible (Figure 1A). Moreover, we verified the mere absence of serotonin in the brain of TPH2-KO animals[18] and the unchanged central levels in TPH1-KO mice[12] (Figure 1A). Next, we searched the literature for enzymes that could potentially complement TPHs by catalyzing the rate-limiting 5-hydroxylation of the indole-ring. This reaction is reported to be performed by only two families of enzymes: cytochrome-dependent monooxygenases (CYPs)[24] and biopterin-dependent monooxygenases of the AAAH family.[25] Since the conversion of tryptamine to serotonin by CYPs was only shown in plants,[24] we focused on the AAAH-family of monooxygenases that include the two known TPH enzymes, as well as PAH and TH. To narrow down a possible candidate, we checked for sequence homology between the two TPHs, PAH and TH from human, mouse, and rat and compared them to *Drosophil*a tryptophan-phenylalanine (DTPH) and tryptophan (DTRH) hydroxylases and the ancient PAH from the early metazoan/communal protozoan *Dictyostelium discoideum* (DictyoPAH), thought to be one of the most primal AAAHs (Figure 1B). Homology between the TPH- and PAH-families was overall higher than between THs and either of both families. DTRH showed high homology to the TPH-family, DTPH, and DictyoPAH to the PAH-family. This fits data from the published literature showing that DTRH, DTPH, and dicytoPAH are all able to hydroxylate both, Phenylalanine (Phe) and Trp, but not tyrosine.[26,27] Moreover, partially purified preparations of mammalian PAH displayed Trp hydroxylating activity *in vitro*.[25,28,29] Furthermore, TH is unlikely to contribute to the production of 5-HT since brain serotonin levels in both TPH2-KO[18] and TPH1/TPH2-double KO animals (Figure 1A) are close to zero and TH is primarily expressed in the brain. These considerations prompted us to prioritize PAH as a potential third key mammalian enzyme able to catalyze the first step of serotonin synthesis.

3.2 | PAH readily hydroxylates Trp to 5-HTP *in vitro*

First, we expressed mouse PAH in HEK cells. Cells expressing PAH were able to hydroxylate Trp to 5-HTP, while untransfected cells or cells transfected with an empty vector as expected were unable to do so due to the lack of endogenous expression of enzymes required for 5-HTP synthesis (Figure 2A).

Since PAH is primarily expressed in the liver, we used its homogenates for the *in vitro* 5-HTP generation assay. Trp hydroxylation to 5-HTP was readily observed in liver lysates from WT mice (Figure 2B). However, when we added the specific PAH-inhibitor alpha-methylphenylalanine (α-MPA),[30] a significant decrease of nearly 80% in Trp hydroxylation was observed (Figure 2B). We further confirmed that liver Trp hydroxylation was independent of TPH expression since 5-HTP synthesis rates in liver lysates from both TPH1 and TPH1/TPH2-KO animals were similar to WT (Figure 2C). In contrast, 5-HTP synthesis was nearly abolished in liver lysates from PAH-deficient mice (Figure 2C). PAH-deficient animals develop phenylketonuria characterized by high plasma and tissue concentrations of Phe, which are known to inhibit AAAHs.[31] To exclude any inhibitory effect of Phe on TPH activity, we confirmed the lack of Trp hydroxylating activity in liver lysates from PAH-KO mice under a Phe-free diet (Figure 2C).

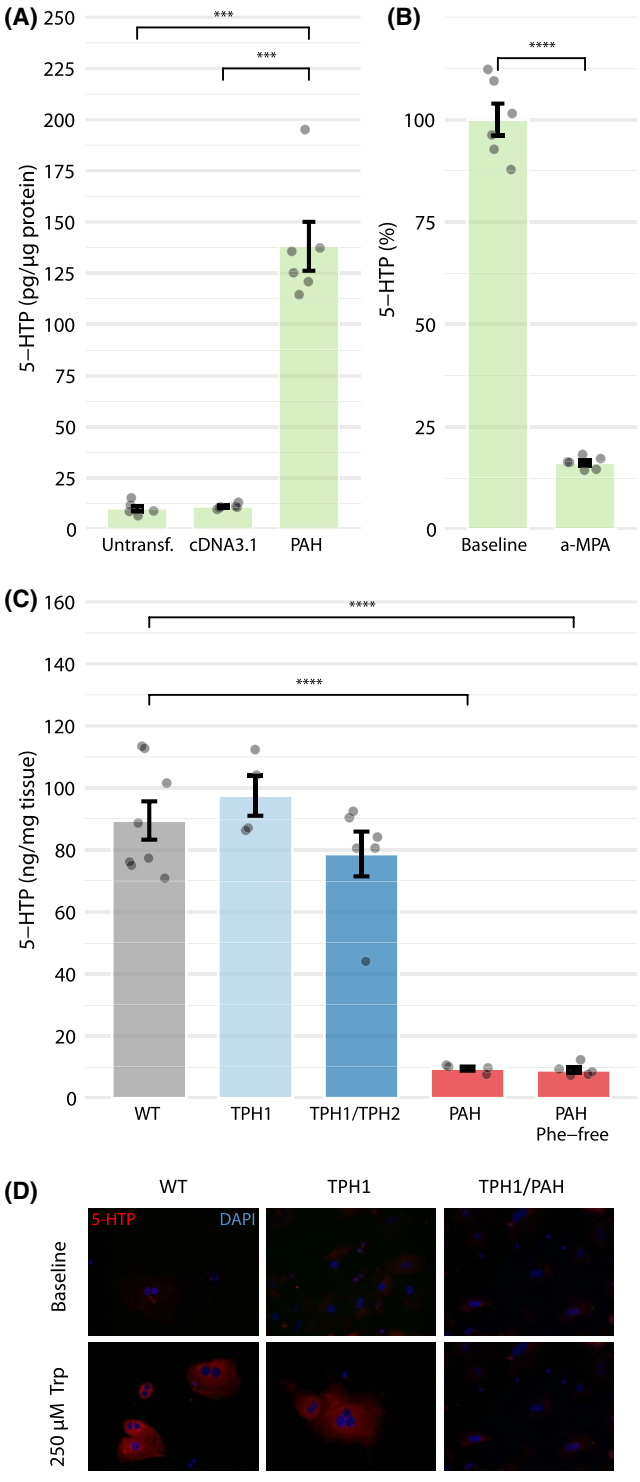

FIGURE 2 PAH is a tryptophan hydroxylating enzyme *in vitro*. A, *In vitro* 5-HTP generation in lysates from HEK293 cells either untransfected or transfected with empty vector (pcDNA3.1) or vector expressing PAH. B, *In vitro* 5-HTP production at baseline and following PAH-inhibition by α-MPA in liver lysates harvested from wild-type (WT) animals. C, *In vitro* 5-HTP generation in liver lysates harvested from WT, TPH1, TPH1/TPH2, and PAH-deficient animals under standard chow or phenylalanine-free (Phe-free) diet. D, 5-HTP accumulation in primary hepatocytes from WT, TPH1, and TPH1/PAH animals incubated with Trp for 2 hours. Micrographs represent cells immunofluorescently labeled with DAPI (blue) and anti-5-HTP antibodies (red). 5-HTP can be detected in hepatocytes harvested from WT and TPH1 animals but not in hepatocytes harvested from TPH1/PAH animals. ***$P < .001$, ****$P < .0001$, one-way ANOVA with Bonferroni post hoc test

Next, we cultured primary cells from the mouse liver to investigate the ability of hepatocytes to hydroxylate Trp to 5-HTP. While the supplementation of culture medium with Trp led to increased 5-HTP generation in WT and TPH1-KO hepatocytes as shown by immunohistochemistry, PAH-deficient cells isolated from TPH1/PAH-double KO mice were unable to generate 5-HTP (Figure 2D).

3.3 | PAH contributes to 5-HT-generation *in vivo*

Next, we treated TPH1-KO mice with the PAH inhibitor α-MPA. Here we saw a reduction of whole-blood 5-HT levels by about 75%, indicating that the remaining blood 5-HT in TPH1-KO-mice is mainly dependent on PAH-mediated Trp hydroxylation (Figure 3A).

In order to further evaluate the importance of PAH-dependent Trp hydroxylation for blood 5-HT levels, we used TPH1/PAH double-KO mice. Here we also fed animals with Phe-free diet to prevent TPH inhibition by excessive amounts of Phe. Indeed, PAH-KO animals under normal diet exhibited markedly reduced blood 5-HT levels, while under a Phe-free diet they showed peripheral 5-HT levels comparable to WT mice (Figure 3B). These data are in line with previously reported effects of Phe-free diet on brain 5-HT levels in PAH-KO mice and in patients suffering from phenylketonuria.[31] Strikingly, in the novel TPH1/PAH, double-KO mouse model 5-HT in the blood was close to the lowest detection limit and amounted to about 1% of WT levels independently of the diet (Figure 3B).

Next, we directly measured the *in vivo* 5-HT neo-synthesis by a ^{13}C stable-isotope resolved mass spectrometry (SIRM)-based approach (Figure 4A). Trp has been shown to be prone to label-loss when a ^{3}H-label is used[32] and therefore previous studies have used ^{15}N-labeled[33] or ^{13}C-labeled Trp[34,35] to assess the peripheral 5-HT synthesis in dogs, rats, and humans. When we injected animals with ^{13}C-labeled Trp, it was readily converted to 5-HT in WT and PAH-KO mice as detected in blood by LC-MS/MS (Figure 4B,C). As expected, conversion was markedly reduced, but still measurable in TPH1 and TPH1/TPH2-double KO mice. However, no ^{13}C-labeled 5-HT could be detected in the blood of TPH1/PAH-double KO mice (Figure 4C) finally confirming the contribution of PAH to Trp hydroxylation *in vivo*.

4 | DISCUSSION

In this study, we provide several lines of evidence revealing PAH as the third tryptophan hydroxylating enzyme in mice and likely in other mammals: (1) mice lacking both known TPH isoforms, TPH1 and TPH2, still have about 10% of normal serotonin levels in the blood; (2) additional genetic or pharmacological blockade of PAH drastically reduces this leftover; (3) cells overexpressing PAH gain Trp hydroxylating activity; (4) *in vitro* Trp hydroxylation takes place in liver extracts and cultured hepatocytes from WT but not from PAH-KO mice; 5) isotope-labeled Trp is metabolized to serotonin in the periphery of WT and TPH1-KO mice but not of TPH1/PAH-double KO animals.

PAH has already been described as a Trp hydroxylase in invertebrates. In fact, PAH, encoded by the *Henna* gene, was for a long time the only known TPH in *Drosophila* and is still called DTPH. This error was based on the strong reduction of serotonin in the head of Henna mutant flies.[36] Later it turned out that the absence of PAH caused a dramatic increase in Phe and thereby an inhibition of the real TPH, which was then discovered as the product of the CG9122 gene, now called DTRH.[26] Nevertheless, PAH also contributes significantly to serotonin production in flies and is proposed to be responsible for peripheral, but not central 5-HT synthesis in these species.

Historically PAH was first suggested to be the main TPH also in mammals,[25,37] until organs that lack PAH expression, such as the intestines, were found to contain high amounts of 5-HT.[38] Nevertheless, the first TPH gene was cloned by screening an expression library from the rabbit pineal body with an—obviously cross-reacting—antibody against rat PAH.[11]

The efficiency of PAH to hydroxylate Trp is lower than that of TPH.[29] This is due to a crucial tryptophan-residue (W) in the active site of PAH that reduces the affinity to the substrate Trp.[39] In TPH, a conserved phenylalanine residue (F) is found at the homologous position (Figure 5). If this active site amino acid residue is mutated to W, the preference of TPH for the substrate Trp is reduced. Vice versa, a mutation of W to F causes PAH to be converted into a very efficient TPH.[39] Figure 5 presents the superimposition of the crystal structures of the catalytically active domains of PAH and TPH and illustrates the general ability of PAH to bind Trp as substrate. However, the side chain of the W residue is positioned only 3 Ångstrom away from the substrate-binding

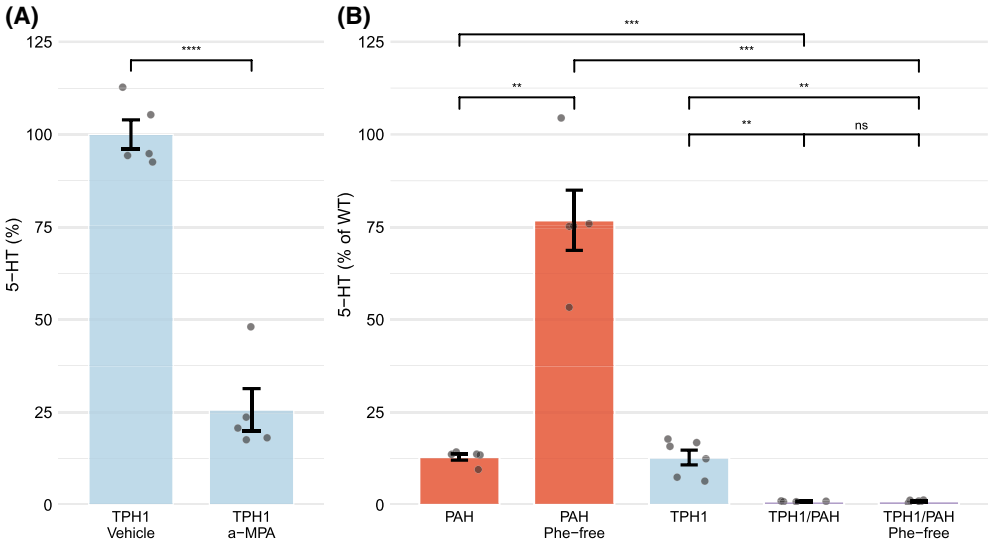

FIGURE 3 Pharmacological inhibition or genetic ablation of PAH activity reduces blood 5-HT levels *in vivo*. A, Blood 5-HT levels in TPH1-KO mice treated either with saline (vehicle) or PAH-inhibitor α-MPA. B, Blood 5-HT levels in TPH1-KO, PAH-KO, and TPH1/PAH-double KO animals either on a standard chow or a phenylalanine-free (Phe-free) diet. Data are presented as % of WT blood 5-HT levels. $^{**}P < .01$, $^{***}P < .001$, $^{****}P < .0001$, ns, not significant, one-way ANOVA with Bonferroni post hoc test

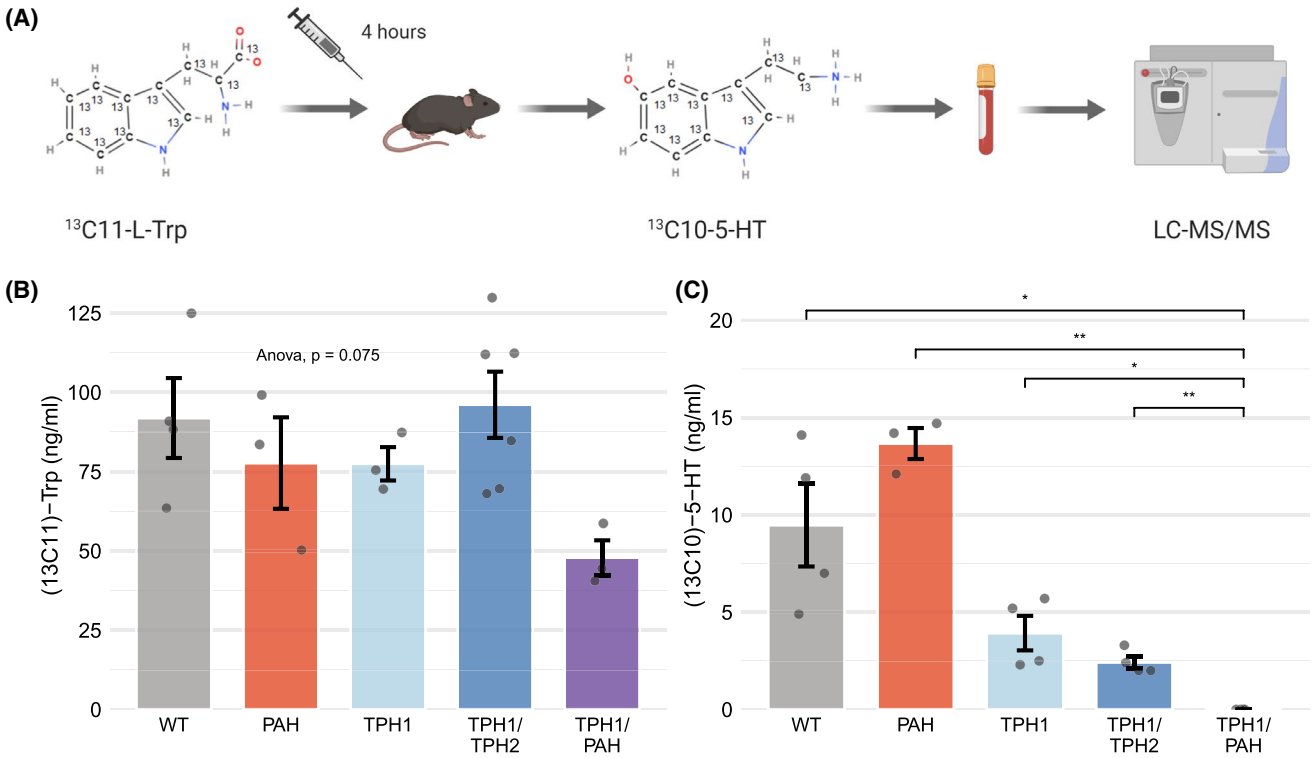

FIGURE 4 Animals lacking TPH1 and PAH show no 5-HT neo-synthesis as detectable via stable-isotope resolved mass spectrometry. A, Experimental setup. Mice were treated with ^{13}C-labeled Trp and their blood was evaluated for fully ^{13}C-labeled 5-HT via LC-MS/MS. B, ^{13}C-labeled Trp and c ^{13}C-labeled 5-HT levels in the blood of WT, TPH1-KO, TPH1/TPH2-double KO, PAH-KO, and TPH1/PAH-double KO mice 4 hrs after ^{13}C-Trp treatment. PAH-KO and TPH1/PAH-double KO animals were kept on a Phe-free diet. $^{*}P < .05$, $^{**}P < .01$, one-way ANOVA with Bonferroni post hoc test

site and thus influences substrate binding both sterically and by hydrophobic interactions.

The 5-HT levels in TPH1/PAH-double KO mice were drastically reduced in comparison to TPH1-KO animals, but still detectable at about 1% of WT mice. This finding raises the question: What are the possible sources for this leftover? One option is a spillover of TPH2-generated 5-HT from brain.[40] Enteric serotonergic neurons could also serve as a

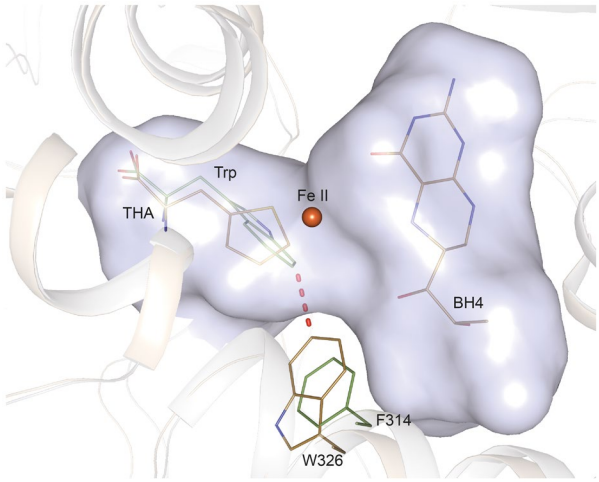

FIGURE 5 Human PAH crystal structure analysis. A superimposition of the crystal structures of the catalytic domains of human PAH (PDB ID 1mmk,[57]) and chicken TPH1 (PDB ID 3e2t,[58]) is shown. The cartoon model of the overall fold is displayed in light orange (PAH) and grey (TPH1), respectively. The PAH crystal structure is complexed with iron (Fe^{II}, orange sphere), the cofactor tetrahydrobiopterin (BH4), and the substrate analog 3-(2-thienyl)-L-alanine (THA) (orange stick models), TPH1 is bound to tryptophan (Trp, green stick model). The interior surface (light blue) of the active-site cavity of PAH was calculated with the program Hollow and displayed with PyMOL Molecular Graphics System (Version 1.3, Schrödinger, LLC). The superimposition illustrates that the PAH active site is generally capable of Trp binding. However, in PAH, the efficiency of Trp conversion is diminished by the presence of the bulky side chain of a Trp residue (W326 in human PAH, orange stick model) that is positioned only 3 Ångstrom away from the substrate-binding site and thus influences substrate binding both sterically and by hydrophobic interactions (red dashed line). In comparison, in TPH, a phenylalanine residue is conserved at this position (F314 in chicken TPH1, green stick model)

source of these minute amounts of 5-HT. Although 5-HT released at synapses are unlikely to escape the enteric nervous system without prior metabolism due to the high activity of the serotonin transporter and organic cation transporters,[41] this scenario can also not be excluded. Since we were as of yet unable to generate TPH1/TPH2/PAH-triple-KO mice, we could not test these hypotheses.

A second possibility is the uptake of 5-HT from the gut lumen, where it is either present in the food or produced by gut bacteria. Food serves as the primary supply of Trp for mammals and even contains detectable amounts of 5-HT.[42] Besides their stimulating effect on serotonin synthesis in the enterochromaffin cells of the gut,[43,44] enteric bacteria have also been shown to synthesize and react to 5-HT and may contribute to its luminal levels.[44,45] Its uptake from the gut lumen is possible in small amounts, as already shown in earlier studies by giving food rich in serotonin, eg, bananas[46] or by the oral administration of the monoamine in high concentration.[47] The standard mouse food used in our study contains about 0.5 μg/g 5-HT. Mice consume about 4 g of food daily. This, together with the bacterial 5-HT production and metabolism, results in about 2-4 μg 5-HT daily passing through the gut. This food- or bacteria-supplied 5-HT may contribute to the residual traces found in the blood of mice lacking serotonin-producing enzymes in the periphery (TPH1/PAH-double KO). That being said, the mechanisms of such uptake would still have to be clarified.

The physiological role of PAH-derived 5-HT remains to be determined, but it may be important under certain (patho) physiological conditions. Moreover, since local 5-HT generation and signaling, with no spillover into the reservoir of the blood, has been shown in several organs, such as pancreas, fat, and mammary glands,[1,48-50] it is reasonable to expect the physiological effects of PAH-derived 5-HT in the main PAH-expressing organs (liver and kidney) in which no other TPHs are expressed.

For the liver, numerous physiological and pathophysiological effects have been assigned to serotonin, such as the stimulation of regeneration, aggravation of fibrosis, and amelioration of virus infection.[51-53] However, most of these effects were abolished in TPH1-KO mice showing that gut- and platelet-derived serotonin is the main mediator in these situations.

Little is known about the possible 5-HT functions in the kidney, the second site of PAH expression in mammals.[54,55] Since kidneys do not express either TPH,[54] PAH is likely to be the main source of the locally generated 5-HT. There are several 5-HT receptors including 5-HT2A and 2B in kidney and their effects on blood flow and sodium absorption have been described.[55,56] However, the relevance of these findings for normal kidney physiology and renal diseases is yet to be established.

The role of PAH-derived 5-HT in physiology could be studied in PAH-KO mice, but these animals suffer from phenylketonuria and the high phenylalanine levels confound physiological analyses. Nevertheless, our study is the first step in understanding the importance of PAH as third 5-HT synthesizing enzyme and future studies are warranted to clarify its role in particular in liver and kidney possibly using animal models with organ or cell-type-specific reduction of PAH activity.

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CONFLICTS OF INTEREST

The authors have no conflicts of interest to declare.

AUTHOR CONTRIBUTIONS

M. Bader and N. Alenina supervised the study. A. Mordhorst, P. Dhandapani, S. Matthes, V. Mosienko, M. Todiras, J. Self, and N. Alenina performed biochemical, cell culture, and animal experiments. M. Rothe performed mass spectrometry, and A. Schütz did the structural modeling. A. Mordhorst, P. Dhandapani, S. Matthes, V. Mosienko, M. Rothe, W.H. Schunck, and N. Alenina carried out data analysis. A. Mordhorst, M. Bader and N. Alenina drafted the manuscript with input from all co-authors.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the Supporting Information section.

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