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Of mice and men: Plasma phenylalanine reduction in PKU corrects neurotransmitter pathways in the brain

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ABSTRACT

In phenylketonuria (PKU), mutations of the phenylalanine hydroxylase (PAH) gene decrease the ability of PAH to convert phenylalanine (Phe) to tyrosine (Tyr), resulting in Phe accumulation in the blood and brain and disruption of neurotransmitter (NT) biosynthesis and metabolism. The following translational study explored the relationship between pegvaliase-mediated Phe correction in plasma and the NT biosynthesis and metabolism pathway in mice and humans with PKU. Lower plasma Phe levels were associated with normalization of the NT biosynthesis pathway which correlated with an improvement in inattention symptoms in subjects with PKU.

1. Introduction

Phenylketonuria (PKU) is an autosomal recessive disorder characterized by a deficiency of phenylalanine hydroxylase (PAH), an enzyme that converts phenylalanine (Phe) to tyrosine (Tyr), leading to an accumulation of plasma Phe [1]. Increased plasma Phe facilitates greater entry of Phe into the brain, outcompeting the transport of other large neutral amino acids (LNAA) causing a decrease in brain Tyr and tryptophan (Trp), competitive inhibition and disruption of the neurotransmitter (NT) biosynthesis pathway [2–4].

LNAAs rely on a complex network of luminal and abluminal LNAA transporters to help maintain homeostasis in the brain [5]. In the case of elevated plasma Phe, one of the most abundant LNAA transporters, LAT1, is responsible for increasing brain Phe, impacting several amino acids, NTs, and NT metabolites (NTMs) in the brain (Fig. 1). Tyr and Trp are transported by LAT1 into the brain and converted by their respective hydroxylases to L-dihydroxyphenylalanine (L-DOPA) and 5-hydroxytryptophan (5-HT). At this first step in the NT synthesis pathway, Phe has been shown to competitively inhibit these enzymes, especially Trp hydroxylase [6]. Phe, L-DOPA, Trp, and 5-HT are converted by L-amino acid decarboxylase (AADC) to the NTs phenethylamine (PEA), dopamine, tryptamine, and serotonin, respectively. Supraphysiological levels of brain Phe can competitively inhibit AADC, increasing the synthesis of PEA and reducing the synthesis of other NTs depending on the K_m of the other substrates. PEA may also competitively inhibit norepinephrine synthesis, as PEA and dopamine both rely on dopamine beta hydroxylase (DBH) for the synthesis of phenylethanolamine and norepinephrine, respectively. These NTs are ultimately metabolized into their NTMs: PEA and phenylethanolamine to phenylacetic acid (PAA) and mandelic acid (MA), respetively; dopamine and norepinephrine to homovanillic acid (HVA) and 3-methoxy-4-hydroxyphenyl glycol (MOPEG), respectively; and tryptamine and serotonin to indoleacetic acid (IAA) and 5-hydroxyindoleacetic acid (5HIAA), respectively. In contrast to NTs, NTMs are lipophilic and can permeate across the blood-brain barrier into the plasma, where they are eventually cleared by the kidneys. Interestingly, the majority of PAA is conjugated to glutamine in humans and glycine in rodents, forming phenylacetylglutamine (PAG) and phenylacetylglycine (PAGly) [7].

NTs have been associated with neurocognitive and behavioral deficits observed in subjects with PKU. Executive functioning, such as working memory, attention span, and inhibitory control, is often compromised with elevated brain Phe [8]. While the exact mechanisms contributing to these cognitive deficits are unclear, it is postulated that there is a cognitive impact from a buildup of toxic metabolites that can disrupt brain development and decrease NT biosynthesis [9–14]. However, no clinical studies to date have strongly correlated NT and NTM levels with the observed neurocognitive deficits.

A meta-analysis demonstrated neuropsychological symptoms in adults with PKU were associated with high Phe levels, and neurological performance improved with lowering of Phe [15]. Many of the symptoms evaluated by Bilder et al. have been associated with NT imbalances [15–17]. In the murine model of PKU, *Pah^{enu2}* mice on a Phe-restricted diet experienced correction of brain NTs and improved

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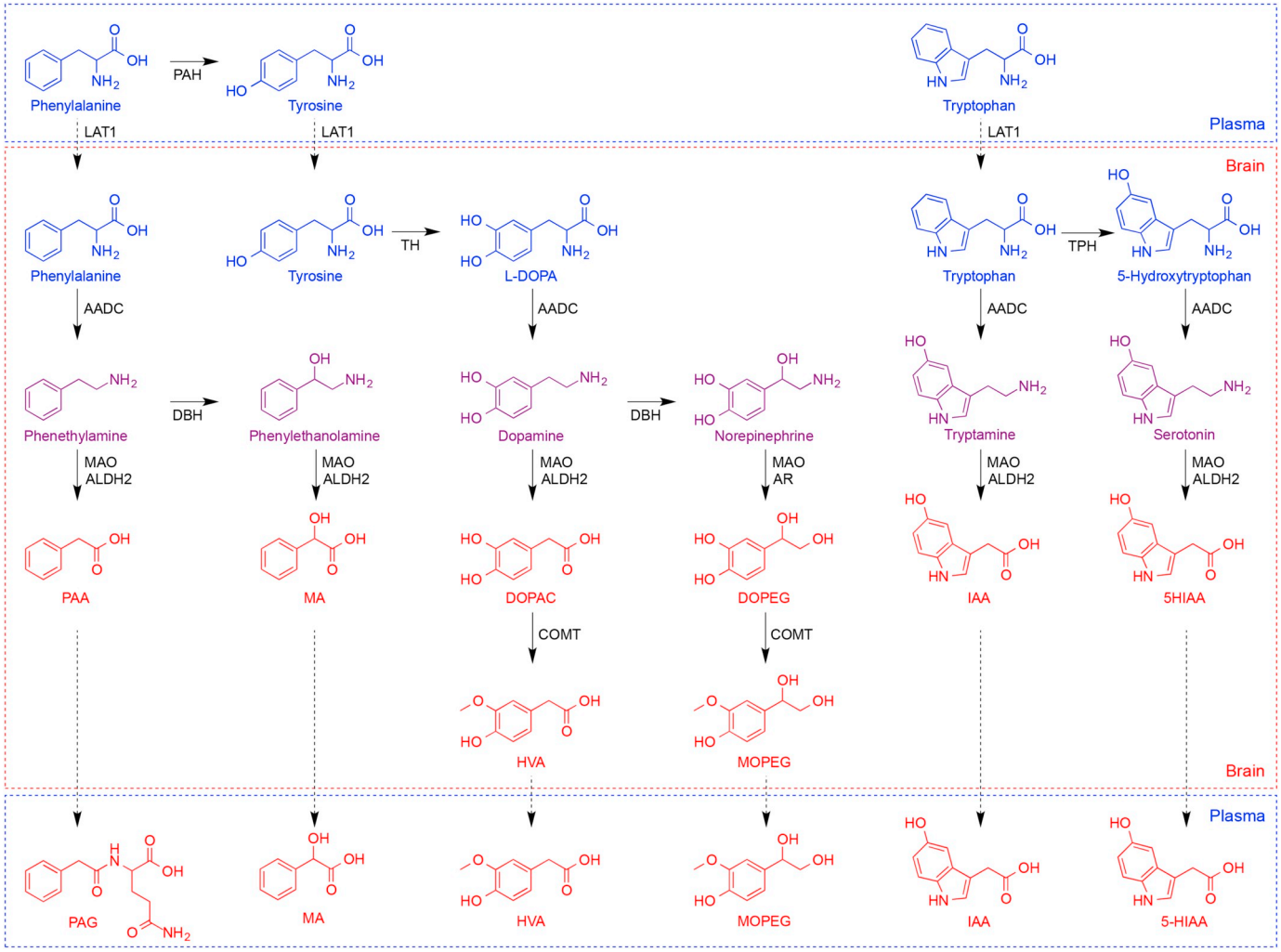

Fig. 1. The neurotransmitter biosynthesis pathway. Metabolites of Phe and other neurotransmitters derived from Tyr and Trp can be detected in the plasma as surrogates for brain metabolism. Blue represents molecules originating within the plasma, whereas red represents metabolites that have their primary origin within the brain. 5HIAA, 5-hydroxyindoleacetic acid; AADC, amino acid decarboxylase; ALDH2, aldehyde dehydrogenase; AR, aldehyde reductase; COMT, catecho-*O*-methyltransferase; DBH, dopamine beta hydroxylase; DOPAC, 3,4-dihydroxypheyl-acetic acid; DOPEG, 3,4-dihydroxy-phenylethyleneglycol; HVA, homovanillic acid; IAA, indoleacetic acid; LAT1, large L-type neutral amino acid transporter 1; MA, mandelic acid; MAO, monoamine oxidase; MOPEG, 3-methoxy-4-hydroxyphenylglycol; PAA, phenylacetic acid; PAG, phenylacetylglutamine; PAH, phenylalanine hydroxylase; TH, tyrosine hydroxylase; TPH, tryptophan hydroxylase. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

nesting behaviors [18], suggesting that NT deficiencies may contribute to the cause of adverse behavioral effects associated with elevated Phe and that correction of NT levels can reverse this phenotype.

McKean et al. demonstrated that cerebrospinal fluid (CSF) levels of 5HIAA and HVA were inversely proportional to plasma Phe in subjects with PKU and returned to normal when subjects followed a Phe-restricted diet [19]. Decreasing plasma Phe by dietary restriction has been associated with an increase in dopamine and serotonin in urine, suggesting plasma Phe levels may be related [20–22]. Due to a lack of effective therapeutic options and sensitive assays, no comprehensive study comparing NT or NTM levels in treated versus untreated subjects with PKU has been conducted.

Pegvaliase (PALYNZIQ®, BioMarin Pharmaceutical Inc., Novato, CA), a subcutaneous injection of PEGylated recombinant phenylalanine ammonia lyase enzyme, is indicated for adults with PKU to lower plasma Phe. Plasma Phe levels ≤360 μM and within the range of unaffected adults (≤120 μM) have been reported in subjects administered pegvaliase in phase 3 clinical trials, as well as in *Pah*^{enu2} mice [23,24]. We hypothesized that reducing Phe with pegvaliase treatment would lead to an improvement in NT and NTM levels. Herein, we present preclinical data in the *Pah*^{enu2} mouse model treated with pegvaliase, correlating plasma Phe levels with amino acids, NT and NTM levels in brain and plasma. Next, we studied the relationship between changes in plasma NTMs for a cohort of subjects with PKU that had plasma Phe < 360 μM (the target blood Phe level per American College of Medical Genetics [ACMG] guidelines [25]) or > 900 μM (a group with poor blood Phe control) after 12 months of pegvaliase treatment in a phase 3 clinical trial. Finally, we present for the first time, a correlation between a clinically relevant neurocognitive measure and a plasma NTM in subjects with PKU.

2. Materials and methods

2.1. Mouse studies

All animal experiments followed the National Institutes of Health guidelines for the care and use of laboratory animals. Various dose levels of pegvaliase (10–80 mg/kg) or vehicle (Tris Buffered Saline/2 mM trans-cinnimate; 4 mL/kg) were administered to nine-week-old male C57BL6-*Pah*^{enu2} mice (n = 7), on unrestricted diet (Teklad Global 18% protein rodent diet #2918, containing 1% Phe, 0.6% Tyr, 0.2% Trp), by subcutaneous injection. Blood was sampled from each animal prior to

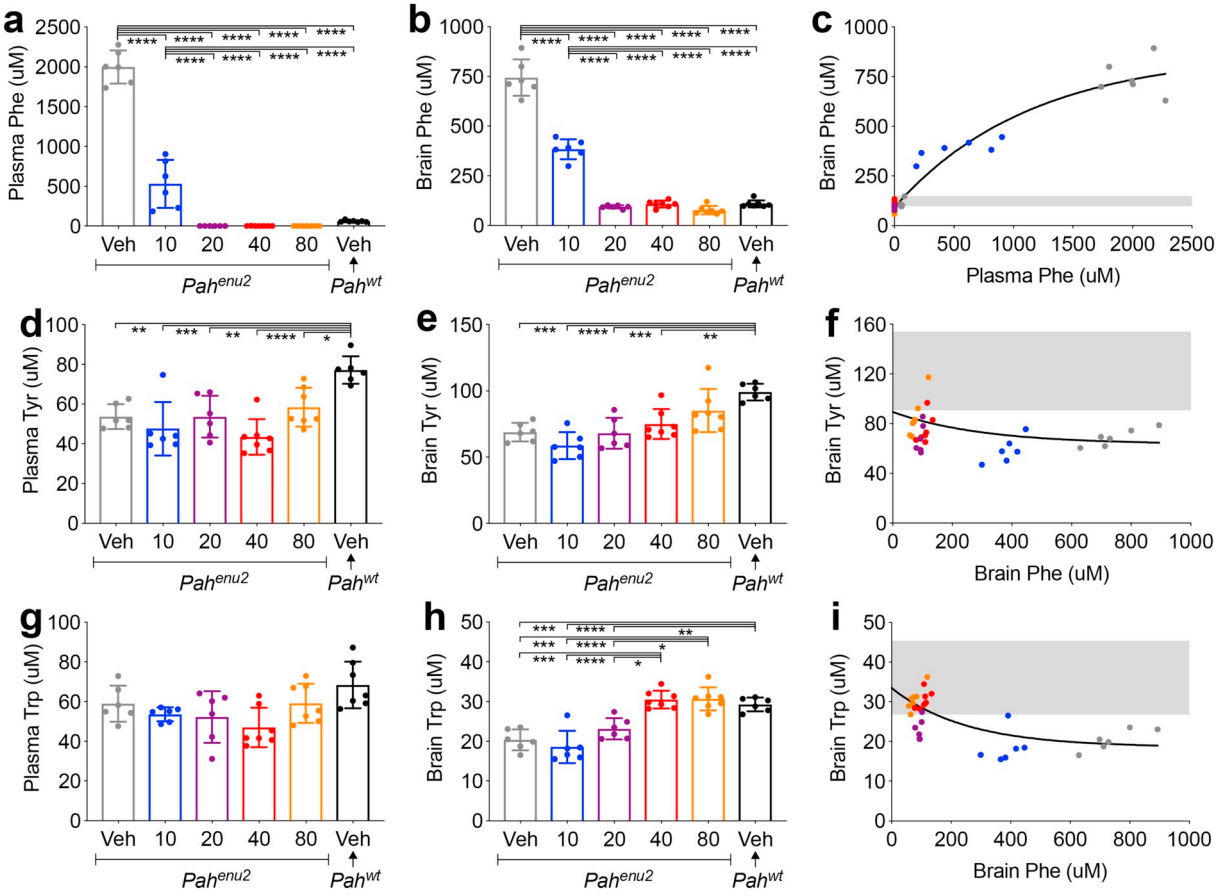

Fig. 2. Amino acid levels in plasma and brain of *Pah*^{enu2} mice were improved following pegvaliase treatment (x-axis: vehicle or pegvaliase dosage in mg/kg). *Pah*^{enu2} mice were treated with vehicle or increasing doses of pegvaliase while *Pah*^{wt} mice were treated with vehicle. Phe (A–C), Tyr (D–F), and Trp (G–I) were assessed in plasma and brain. Tukey's multiple comparison test with adjusted *p*-values was used to determine significance (****$p < .0001$; ***$p < .001$; **$p < .01$; *$p < .05$) in A, B, D, E, G, and H. For C, F, and I, *r*, *p*-values and 95% confidence intervals (CI) were calculated by linear fit to log-transformed data. Shaded regions represent upper and lower limit of *Pah*^{wt} mice. Brain Phe strongly correlated with plasma Phe with an *r* value of 0.89 (95% CI 0.81–0.94, $p < .0001$). Brain Tyr and Trp correlated with brain Phe with *r* values of -0.309 (95% CI -0.57–0.006, $p = .06$) and 0.58 (95% CI -0.76 to -0.33, $p = .0001$), respectively. PAH, phenylalanine hydroxylase; Phe, phenylalanine; Trp, tryptophan; Tyr, tyrosine.

and 24 and 72 h post-administration. Following whole-body perfusion with cold phosphate buffered saline (PBS; ~10–15 mL) via the left ventricle, brains were collected 72 h post-pegvaliase administration. In a separate study, nine-week-old male C57BL6-*Pah*^{enu2} mice were supplemented daily for 5 days with Tyr suspended in PBS (60 mg/kg) or PBS alone, three times per day via oral gavage, beginning one day before subcutaneous administration of pegvaliase or vehicle. Blood was sampled prior to Tyr dose initiation, and 1 and 4 days post-pegvaliase administration. Brains were collected 4 days post-pegvaliase dose and 1 h after the final Tyr dose, following whole-body perfusion with cold PBS.

Sectioned brains were weighed and homogenized in PBS (4:1 [*v/w*] containing 1% NP-40, 0.5% sodium deoxycholate, and 0.1% sodium dodecyl sulfate) using standard protocols on a FastPrep-24 5G instrument (MP Biomedicals 116,005,500). Supernatants were prepared by centrifuging brain homogenates at 21,000*g* for 15 min at 4 °C, then stored in aliquots at −80 °C.

2.2. Derivatization of plasma or brain homogenates

The same derivatization and LC/MS methods were applied to mouse brain homogenates and mouse and human plasma samples. In a 96-well plate, 20 µL of brain homogenate or plasma was precipitated with 80 µL of ice-cold acetonitrile containing internal standards. After centrifugation, 20 µL of supernatant was derivatized in a new plate with 10 µL of 100 mM sodium carbonate and 10 µL BzCl (6% [*v/v*] in acetonitrile). Reactions were quenched with 60 µL formic acid (0.16% [*v/v*] in water).

2.3. Neurotransmitter analysis by LC/MS

Derivatized samples were analyzed by LC/MS on an Acquity UPLC (Waters H-Class) coupled to a Sciex 6500 Q-Trap mass spectrometer using positive, scheduled MRM mode. 10 µL were injected on an Acquity HSS C18 column (2.1 mm × 150 mm, 1.8 µM, 100 A pore size, Waters 186,003,534) and eluted using a gradient of 10 mM ammonium formate with 0.15% formic acid (MPA) and acetonitrile (MPB) at a flow rate of 0.5 mL/min over 8 min. Settings for each analyte are found in Table S1. Peak integration was performed using MultiQuant software.

2.4. Human plasma samples

Plasma samples from 23 subjects that had participated in the phase 3 pegvaliase clinical trials (PRISM-1 [NCT01819727] or PRISM-2 [NCT01889862]) were evaluated for this preliminary work. All subjects provided informed written consent. Two cohorts were selected based on plasma Phe ≤360 µM, which is the upper limit of the target Phe range recommended in the ACMG PKU treatment guidelines [25], or plasma Phe > 900 µM, a Phe level substantially above both the ACMG and EU PKU treatment guidelines [25,26], at month 12 of pegvaliase treatment.

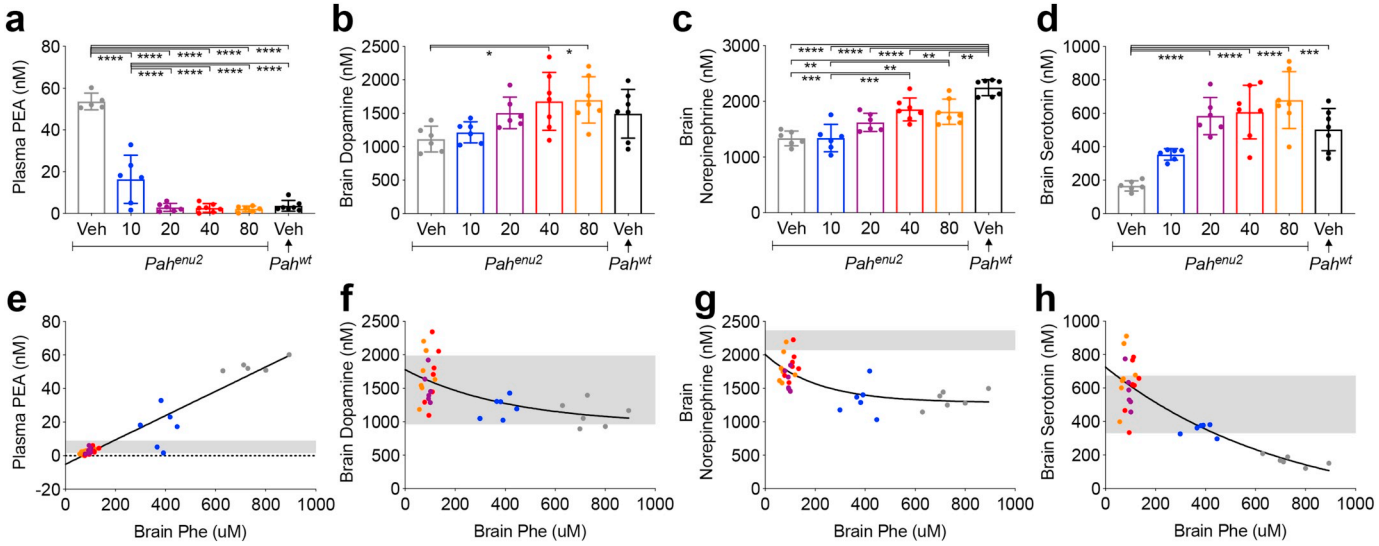

Fig. 3. Brain neurotransmitters were improved in *Pah*^{enu2} mice 72 h after pegvaliase treatment (x-axis: vehicle or pegvaliase dosage in mg/kg). (A) Plasma PEA was determined in plasma of *Pah*^{enu2} mice at baseline and following increasing doses of pegvaliase while *Pah*^{wt} mice were treated with vehicle. (B–D) Brain levels of dopamine, norepinephrine, and serotonin were measured in brain homogenates of *Pah*^{enu2} and *Pah*^{wt} mice. (E) Plasma PEA, (F) brain dopamine, (G) brain norepinephrine, and (H) brain serotonin levels were analyzed in samples from *Pah*^{enu2} and *Pah*^{wt} mice. Tukey's multiple comparison test with adjusted *p*-values was used to determine significance (****$p < .0001$; ***$p < .001$; **$p < .01$; *$p < .05$) in A–D. For E–H, *r*, *p*-values and 95% confidence intervals were calculated by linear fit to log-transformed data. Shaded regions represent upper and lower limit of *Pah*^{wt} mice. PAH, phenylalanine hydroxylase; PEA, phenethylamine; Phe, phenylalanine.

Baseline characteristics were matched for the 2 cohorts based on age, sex, body mass index (BMI), Attention Deficit Hyperactivity Disorder Rating Scale IV (ADHD-RS IV), and plasma Phe. NTM levels were evaluated in all plasma samples at 0, 6, and 12 months of treatment using the same derivatization and LC/MS methods described for animal studies. Twelve subjects were in Group 1 (Phe ≤360 μM) and 11 subjects were in Group 2 (Phe > 900 μM). Both groups were statistically similar for baseline Phe, age, BMI, ADHD-RS IV, and sex (Table S2). Adult control plasma samples were obtained from Discovery Life Sciences (Los Osos, CA) and were not statistically different from the phase 3 pegvaliase subjects for age and sex. BMI, ADHD-RS IV, and Phe were not available for controls. The control samples were de-identified remnant samples leftover from a diagnostic procedure not related to PKU. Methods for plasma Phe and ADHD-RS IV assessments in the pegvaliase clinical trials have been previously described [23].

2.5. Statistical analyses

Tukey's multiple comparison test with adjusted *p*-values was used to determine significance of amino acid, NT, and NTM levels in each treatment group. For correlation plots, *r*, *p*-values and 95% confidence intervals were calculated by linear fit to semi-log transformed data.

3. Results

3.1. Amino acid levels in brain correlate with plasma Phe reduction in mice

Amino acid levels were quantified in plasma and brain homogenates from *Pah*^{enu2} and *Pah*^{wt} mice 72 h after vehicle or pegvaliase treatment (Fig. 2). Pegvaliase above 10 mg/kg reduced plasma Phe levels in *Pah*^{enu2} mice from 1999.0 ± 85.0 μM (mean ± SEM) to < 1.0 μM (*Pah*^{wt} = 60.4 ± 4.4 μM) (Fig. 2A). In contrast to plasma, brain Phe levels were normalized relative to *Pah*^{wt} upon pegvaliase treatment and decreased from 743.7 ± 37.4 to 107.7 ± 6.6 μM (*Pah*^{wt} = 108.8 ± 6.7 μM) (Fig. 2B). Although brain Phe levels did not reduce below *Pah*^{wt} as observed in plasma Phe, a strong correlation was measured between the two compartments with an *r* value of 0.89 (95% confidence interval [CI] 0.81–0.94, $p < .0001$) (Fig. 2C).

While plasma Tyr remained low in all *Pah*^{enu2} groups relative to *Pah*^{wt} (Fig. 2D), brain Tyr in pegvaliase treated *Pah*^{enu2} mice increased from 68.8 ± 2.9 to 85.1 ± 6.1 μM (*Pah*^{wt} = 99.0 ± 2.6 μM) (Fig. 2E). Brain Tyr correlated with a reduction in brain Phe below 400 μM (Fig. 2F).

Plasma Trp levels remained normal in all treatment groups (Fig. 2G). Interestingly, brain Trp in *Pah*^{enu2} mice treated with pegvaliase normalized relative to *Pah*^{wt}, and increased from 20.4 ± 1.1 μM to 30.5 ± 0.8 μM (*Pah*^{wt} = 29.2 ± 0.7 μM) (Fig. 2H). Brain Trp levels were normalized when brain Phe dropped below 400 μM (Fig. 2I).

3.2. Neurotransmitter levels correlate with amino acid correction in mouse brains

All NT levels were normalized relative to *Pah*^{wt} upon pegvaliase treatment: plasma PEA decreased from 53.6 ± 1.8 to 2.7 ± 0.8 nM (*Pah*^{wt} = 3.7 ± 1.0 nM), brain dopamine increased from 1115 ± 78.6 to 1506 ± 96.5 nM (*Pah*^{wt} = 1492 ± 138.4 nM), brain norepinephrine increased from 1335 ± 54.0 to 1855 ± 78.0 nM (*Pah*^{wt} = 2247 ± 54.9 nM), and brain serotonin increased from 166 ± 12.5 to 583.8 ± 45.6 (*Pah*^{wt} = 502 ± 47.6 nM) (Fig. 3A–D).

A strong positive correlation between plasma PEA and brain Phe was observed with an *r* value of 0.95 (95% CI 0.89–0.97, $p < .0001$). Brain dopamine, norepinephrine, and serotonin correlated inversely with brain Phe, with *r* values of -0.61 (95% CI -0.79 to -0.34, $p = .0002$), -0.63 (95% CI -0.80 to -0.35, $p = .0001$), and 0.92 (95% CI -0.96 to -0.84, $p < .0001$), respectively (Fig. 3E–H).

3.3. Neurotransmitter metabolite levels correlate with neurotransmitter levels in mice

All NTM levels in brain increased relative to vehicle-treated *Pah*^{enu2} upon pegvaliase treatment: brain HVA increased from 869 ± 41.01 to 1073 ± 67.5 nM, brain MOPEG increased from 640.5 ± 55.22 to 746 ± 40.7 nM, and brain 5HIAA increased from 280.2 ± 20.2 to 1318.0 ± 37.4 nM (Fig. 4A–C).

Plasma PAGly levels were normalized relative to *Pah*^{wt} upon pegvaliase treatment and decreased from 205.7 ± 24.8 nM to

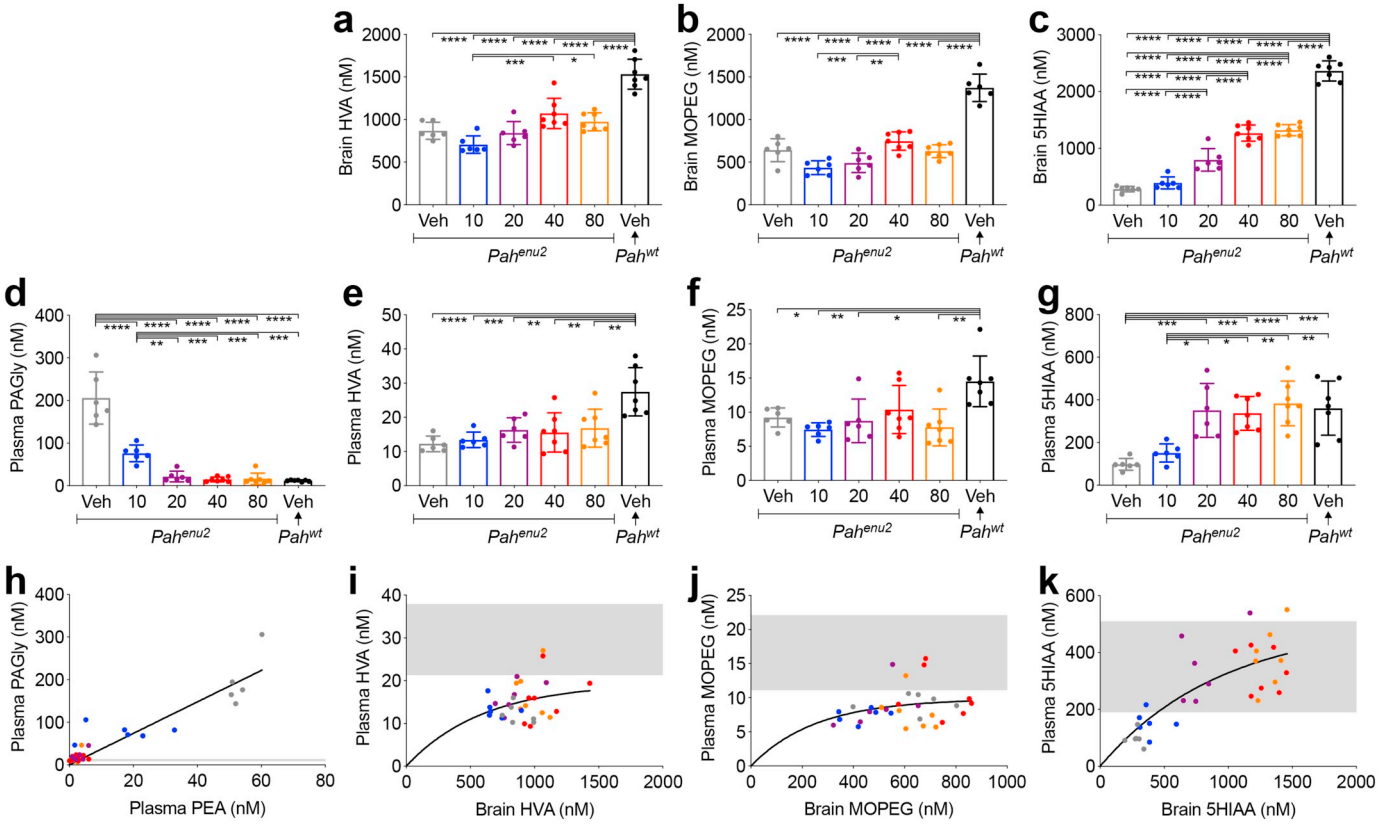

Fig. 4. Changes in neurotransmitter metabolites in the brain (A–C) and plasma (D–G), and the correlation of brain and plasma levels in Pah^{enu2} and Pah^{wt} mice after pegvaliase treatment (A–G x-axis: vehicle or pegvaliase dosage in mg/kg). Tukey's multiple comparison test with adjusted *p*-values was used to determine significance (****$p < .0001$; ***$p < .001$; **$p < .01$; *$p < .05$) in A–G. For E–H, *r*, *p*-values and 95% confidence intervals were calculated by linear fit to log-transformed data. Shaded regions represent upper and lower limit of Pah^{wt} mice. 5HIAA, 5-hydroxyindoleacetic acid; HVA, homovanillic acid; MOPEG, 3-methoxy-4-hydroxyphenylglycol; PAGly, phenylacetylglycine; PAH, phenylalanine hydroxylase; PEA, phenethylamine.

14.69 ± 2.2 nM (Pah^{wt} = 11.9 ± 0.7 nM) (Fig. 4D). Plasma HVA and MOPEG were similar to their corresponding brain levels; few Pah^{enu2} mice reached plasma Pah^{wt} levels (Fig. 4E, F). Plasma 5HIAA levels were similar to brain 5HIAA levels, until reaching a plateau at 400 nM in both high dose and Pah^{wt} groups (Fig. 4G).

Plasma PAGly directly correlated with plasma PEA with an *r* value of 0.93 (95% CI 0.86–0.96, $p < .0001$), confirming a direct relationship with the metabolite. Plasma HVA, MOPEG, and 5HIAA correlated with the corresponding brain NTM levels with *r* values of 0.62 (95% CI 0.37–0.78, $p < .0001$), 0.56 (95% CI 0.29–0.74, $p = .0003$), and 0.66 (95% CI 0.40–0.79, $p < .0001$), respectively.

3.4. Tyrosine supplementation with pegvaliase increased norepinephrine levels in mouse brains

A second study was conducted in Pah^{enu2} mice to mimic the phase 3 trial protocol in subjects, combining pegvaliase treatment with Tyr supplementation (180 mg/kg/day). Supplementation increased plasma Tyr levels in vehicle and pegvaliase-treated Pah^{enu2} mice (Fig. S1C). Tyr supplementation slightly increased brain Tyr levels in vehicle-treated Pah^{enu2} mice but had no effect in pegvaliase-treated Pah^{enu2} mice (Fig. S1D). Brain dopamine reached Pah^{wt} levels in pegvaliase-treated Pah^{enu2} mice regardless of Tyr supplementation (Fig. S1E). Most strikingly, Tyr supplementation in pegvaliase-treated Pah^{enu2} mice increased brain norepinephrine over PBS supplementation ($p = .0002$) from 1481 ± 34.8 to 1818 ± 62.7 nM (Fig. S1F).

3.5. Neurotransmitter metabolite levels increased with reduced plasma Phe in human subjects with PKU

The data in Pah^{enu2} mice with pegvaliase treatment suggest lowering plasma Phe restores NTs to Pah^{wt} levels in brain, with plasma NTMs showing similar trends. To determine whether these findings could translate to human subjects with PKU, NTM levels were evaluated in plasma samples from a subset of subjects in phase 3 clinical trials of pegvaliase with baseline blood Phe levels > 900 µM. Subjects were divided into two cohorts, those that maintained plasma Phe levels > 900 µM or those that decreased plasma Phe to < 360 µM after 12 months of treatment, and compared to samples from a control group without PKU.

After 12 months of pegvaliase treatment, plasma PAG in the > 900 µM Phe group remained unchanged from 4177 ± 446.3 to 3565 ± 481.9 nM; PAG levels in the < 360 µM Phe group normalized relative to controls and decreased from 3133 ± 452.2 to 1133 ± 317.3 nM (control = 1167 ± 150.0 nM) (Fig. 5A).

Plasma HVA remained unchanged (27.6 ± 3.2 to 24.35 ± 1.72 nM) and below controls (42.1 ± 2.45 nM) in the > 900 µM group; plasma HVA normalized relative to controls in the < 360 µM group, increasing from 30.6 ± 3.9 to 44.8 ± 5.6 nM (Fig. 5B).

Plasma MOPEG increased from 38.2 ± 2.9 to 50.9 ± 5.3 nM in the > 900 µM group; plasma MOPEG normalized relative to controls in the < 360 µM group, increasing from 49 ± 6.4 to 70 ± 5.4 nM (control = 61.0 ± 4.1 nM) (Fig. 5C).

Finally, plasma 5HIAA remained unchanged (13.4 ± 0.8 to

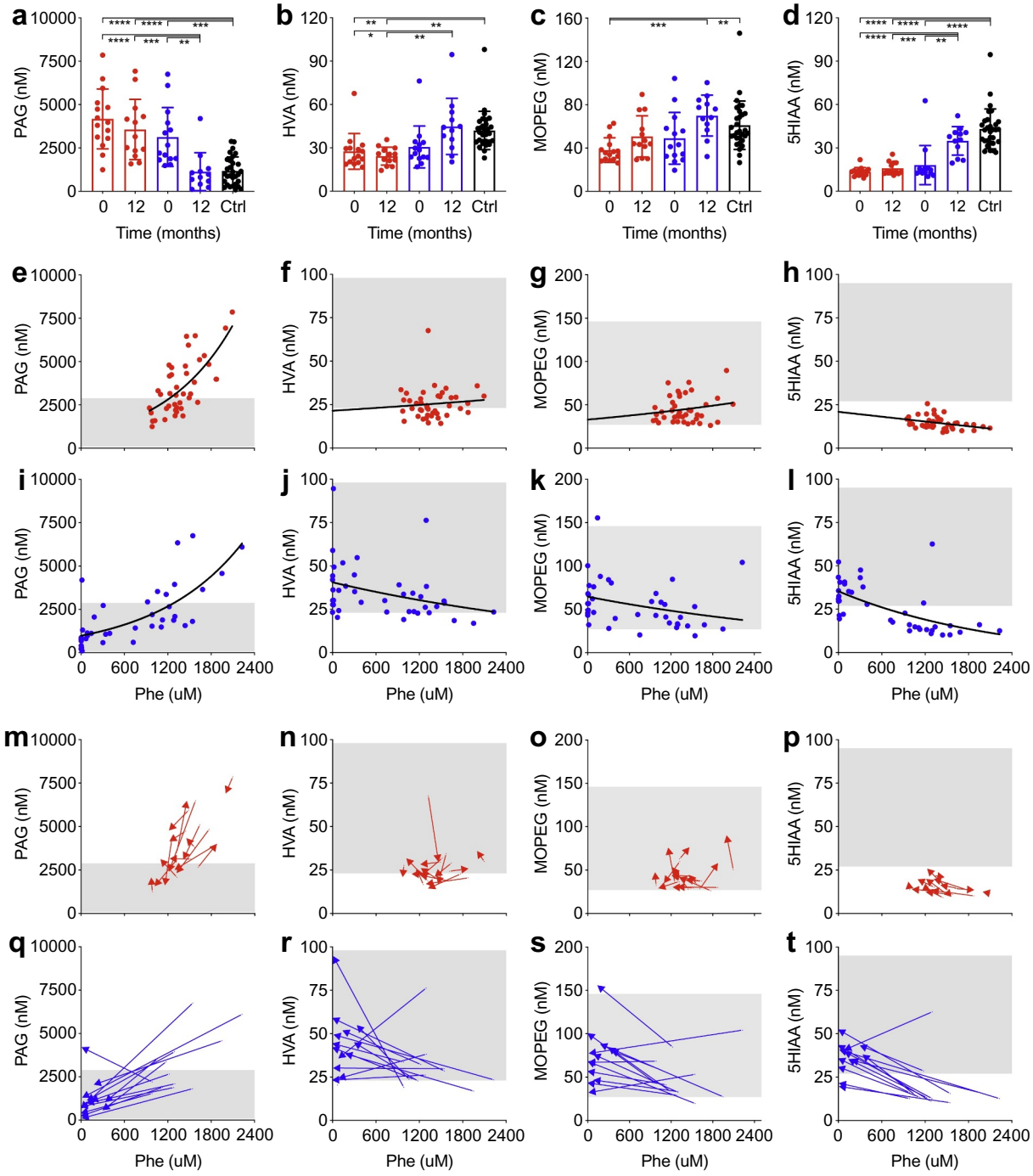

Fig. 5. Plasma levels of key neurotransmitter metabolites were influenced by a reduction of Phe after 12 months of pegvaliase. All data refer to samples from a subset of subjects that achieved either Phe levels < 360 μM (Group 1; blue) or > 900 μM (Group 2; red). (A–D) All neurotransmitter metabolites trended toward control values by 12 months. Tukey's multiple comparison test with adjusted *p*-values was used to determine significance (****$p < .0001$; ***$p < .001$; **$p < .01$; *$p < .05$). (E–L) Correlation of plasma neurotransmitter metabolites with plasma Phe were plotted for the > 900 μM group (E–H) and < 360 μM group (I–L). Data available at baseline, month 6 and month 12 are included. Shaded regions represent upper and lower limit of control subjects. For E–L, r, *p*-values and 95% confidence intervals calculated by linear fit to log transformed data. (M-T) Subject level trajectories of each neurotransmitter metabolite were plotted for the > 900 μM group (M–P) and < 360 μM group (Q–T) after 12 months of pegvaliase treatment. 5HIAA, 5-hydroxyindoleacetic acid; Ctrl, control; HVA, homovanillic acid; MOPEG, 3-methoxy-4-hydroxyphenylglycol; PAG, phenylacetylglutamine; Phe, phenylalanine. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

15.9 ± 1.22 nM) and below controls (43.1 ± 2.5 nM) in the > 900 μM group; while plasma 5HIAA approached control levels in the < 360 μM group, increasing from 18.1 ± 3.6 to 34.9 ± 2.8 nM (Fig. 5D).

3.6. Neurotransmitter metabolite levels correlate with plasma Phe levels in human subjects with PKU

Plasma PAG correlated with plasma Phe in the > 900 μM ($r = 0.68$; 95% CI 0.48–0.72; $p < .0001$) and < 360 μM groups ($r = 0.72$; 95%

CI 0.52–0.84; $p < .0001$; Fig. 5E), suggesting that higher plasma Phe leads to a correspondingly higher brain Phe level, and subsequently PAG. Similar to *Pah*^{enu2} mice, subjects with plasma Phe levels < 30 μM maintained PAG levels in the same range as controls.

Plasma HVA and MOPEG only correlated with plasma Phe in the < 360 μM group (HVA: $r = -0.42$, 95% CI -0.64 to -0.12, $p = .0075$; MOPEG: $r = -0.35$, 95% CI -0.60 to -0.049, $p = .0246$), suggesting lower plasma Phe levels are needed for an appreciable impact on dopamine and norepinephrine metabolite levels. Plasma 5HIAA correlated with plasma Phe levels in both the > 900 μM ($r = -0.38$; 95% CI -0.61 to -0.088; $p = .0126$) and < 360 μM ($r = -0.72$; 95% CI -0.84 to -0.53; $p < .0001$) groups (Fig. 5E–L). Plasma 5HIAA more strongly correlated with plasma Phe in the < 360 μM group, where 5HIAA levels reached the range of control samples.

Subject-level trajectories of each NTM and Phe were analyzed for the baseline to 12 month samples assayed. In the > 900 μM group, the subject-level trajectories for PAG and 5HIAA with Phe were modest in magnitude and were observed in a minority of subjects, and no consistent trends in trajectory were observed for HVA or MOPEG in this group (Figs. 5M–P). The strongest and most consistent changes in NTM levels relative to Phe changes were observed in the < 360 μM group. More strikingly, in the < 360 μM group, individual subject's trajectory trends were greater in magnitude and all but one subject experienced a reduction in PAG with a reduction in plasma Phe (Fig. 5Q). For HVA, a positive trend in trajectories was observed; all but three subjects experienced an increase in HVA with a reduction of Phe (Fig. 5R). Similarly, for MOPEG, all but three subjects experienced an increase in MOPEG with a reduction in plasma Phe (Fig. 5S). For 5HIAA, all but one subject experienced an increase in 5HIAA with a reduction in plasma Phe after 12 months (Fig. 5T).

3.7. Inattention score improvements correlate with plasma MOPEG levels in subjects with PKU

The inattention subscale domain of the ADHD RS-IV (ADHD RS-IV IA) was used to evaluate neuropsychological symptoms of inattention in clinical studies of subjects with PKU treated with pegvaliase. An improvement in mean inattention subscale scores was associated with reductions in mean plasma Phe in subjects continuing long-term pegvaliase treatment. The association was particularly evident in subjects reporting inattention symptoms at baseline, indicated by an ADHD RS-IV IA score ≥ 9 [23].

Reductions in norepinephrine and dopamine levels have been associated with ADHD symptoms. Therefore, associations between changes in ADHD RS-IV IA scores and the dopamine and norepinephrine NTMs, HVA and MOPEG respectively, were explored (Fig. 6). No correlation was found between a change in HVA and ADHD RS-IV IA in subjects with baseline ADHD RS-IV IA scores ≥ 9 or < 9. A correlation was found between a change in MOPEG and change in ADHD RS-IV IA in subjects with a baseline ADHD RS-IV IA score ≥ 9 ($r = -0.5434$, $p = .0242$). As MOPEG levels increased (for both the > 900 μM and < 360 μM groups), the change in ADHD RS-IV IA scores was negative, indicating improvement in scores. No correlation between HVA and change in ADHD RS-IV IA score was found in subjects with baseline ADHD RS-IV IA score < 9 (Fig. 6C, D).

4. Discussion

The current studies demonstrate for the first time a connection between plasma Phe reduction by pegvaliase and correction of the NT biosynthesis pathway in both *Pah*^{enu2} mice and subjects with PKU. Amino acid transport into the brain inversely correlated with plasma Phe levels and these amino acids, except for Tyr, are corrected in the brains of *Pah*^{enu2} mice when plasma Phe dropped below 120 μM. In addition, competitive inhibition by brain Phe on enzymes in the monoamine NT synthesis pathway was restored with pegvaliase treatment. While these studies do not prove that the plasma NTMs originate from the brain, the peripheral effects of Phe on competitive inhibition of the NT pathway is likely similar.

Plasma NTM levels in pegvaliase-treated subjects were presented for a cohort of subjects who had either < 360 μM plasma Phe (demonstrating good Phe control) or > 900 μM plasma Phe (demonstrating poor Phe control) after 12 months of treatment. While the exact Phe level threshold requires a larger sample size, these data support our hypothesis that lowering plasma Phe to < 360 μM can increase NT and NTM levels closer to normal ranges in subjects with PKU while also improving inattention symptoms in subjects with PKU. Interestingly, when plasma Phe levels were < 30 μM in pegvaliase-treated *Pah*^{enu2} mice, brain Phe, plasma PEA and PAGly levels all remained within the normal range. These data support the hypothesis that plasma PEA may originate from the brain. Consistent with these findings, pegvaliase-treated subjects with plasma Phe levels < 30 μM had PAG levels in the same range as controls, providing indirect evidence that brain Phe, PEA and PAA were at normal levels. Given the elevated PEA and PAGly in PKU mice and PAG in subjects with PKU, it is reasonable to assume that subjects also experience elevated brain PEA. Acting as an endogenous amphetamine, excess PEA in urine and CSF has been linked to 'deviant behaviors,' hostility and suspiciousness in paranoid schizophrenia and manic disorders [27]. Restoration of PAG levels, and likely PEA levels by inference, to the control range in subjects reaching Phe < 360 μM was intriguing and warrants further investigation given the link between psychologic symptoms and elevated PEA levels.

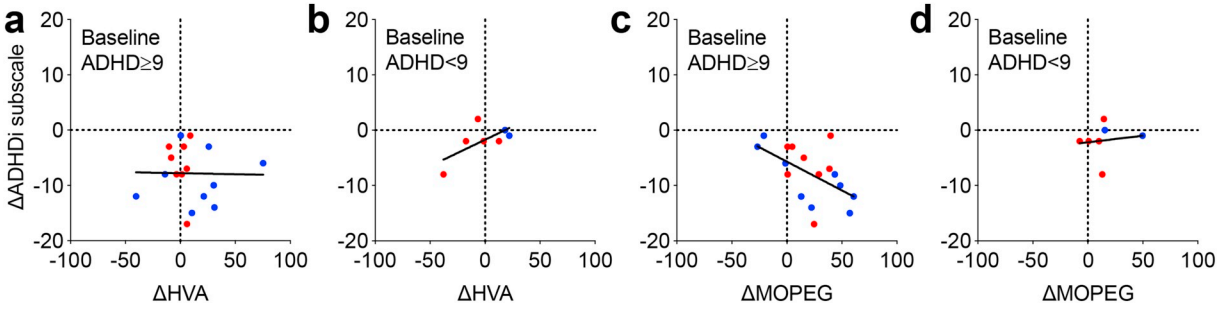

Fig. 6. Inattentive ADHD (ADHDi) subscale for a subset of subjects with ADHDi scores ≥9 at baseline were inversely correlated with an increase in MOPEG. Correlation of change in ADHDi subscale and change in plasma HVA with ADHDi scores greater than or equal to 9 ($n = 17$) (A) or < 9 ($n = 7$) (B) at baseline or MOPEG with ADHDi scores greater than or equal to 9 (C) or < 9 (D) at baseline; score greater than or equal to 9 indicates symptoms of inattention. Blue and red dots indicate subjects from < 360 and > 900 μM groups, respectively. The r and p-values for each data set were determined after fitting a linear regression to each data set. 5HIAA, 5-hydroxyindoleacetic acid; ADHDi, inattention subscale domain of Attention Deficit Hyperactivity Disorder Rating Scale IV; HVA, homovanillic acid; MOPEG, 3-methoxy-4-hydroxyphenylglycol. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

High plasma Phe has been shown to competitively inhibit the LAT1 transporter located on the apical and basolateral membrane of endothelial cells, reducing the bioavailability of Tyr and Trp in the brain [28]. A single dose of pegvaliase in *Pah*^{enu2} mice over a 72 h time period led to complete reduction of plasma Phe, normalization of brain Phe, and an increase in brain Tyr despite low plasma Tyr. Remarkably, these amino acid levels in brain were sufficient to normalize dopamine and increase norepinephrine. Tyr supplementation in pegvaliase-treated *Pah*^{enu2} mice further increased norepinephrine levels, suggesting sensitivity to Tyr bioavailability. The impact of plasma Phe reduction in *Pah*^{enu2} mice was even more apparent with Trp, where normal bioavailability of plasma Trp led to complete normalization of Trp and serotonin in the brain. While brain levels of dopamine and norepinephrine could not be measured directly in subjects with PKU, their metabolites, HVA and MOPEG, moderately correlated with plasma Phe in the < 360 μM Phe group. While these subjects were given Tyr supplementation, this moderate albeit not strong correlation could be related to variability in total intake of Tyr. Conversely, the strong correlation between plasma Phe and 5HIAA may be related to the bioavailability of plasma Trp as was observed in the *Pah*^{enu2} mouse studies. Another possible explanation is that elevated Phe competitively inhibits Trp hydroxylase, the first enzyme in the serotonin biosynthesis pathway, while Tyr hydroxylase, the first enzyme in the dopamine biosynthesis pathway, is unaffected.

At 72 h after a single dose of pegvaliase, *Pah*^{enu2} mice had an increase in NT levels and a moderate increase in NTM levels; one possible explanation is that brain NTM metabolites require more time to equilibrate than NTs. Nevertheless, NT and NTM data in mouse studies indicate plasma NTMs as reasonable surrogate biomarkers of NT levels in subjects where access to brain tissue or CSF is not readily available. While there are peripheral sources of some NTs, the results in *Pah*^{enu2} mice indicate trends in NT and NTMs in the brain are reflected in blood NTMs. Prior studies evaluating post mortem brain tissue, CSF, and a peripheral matrix (i.e., plasma or urine) also support the theory that NTs are disrupted in subjects with PKU and this disruption is reflected in peripheral NTM levels [21,22,29]. NTMs in plasma were likely to be in equilibrium with NTs after 12 months of pegvaliase treatment and likely representative of brain NT levels based on data in the literature and our findings in the *Pah*^{enu2} mouse. The serotonin metabolite, 5HIAA, was significantly decreased at baseline in both PKU groups and correction of this metabolite to mean control values was observed only in those subjects with Phe < 360 μM, suggesting serotonin levels had been improved, if not restored to normal levels. Reduction of serotonin levels has been associated with a number of symptoms that have been observed in PKU such as disturbance in sleep and mood [30]. Mean HVA and MOPEG levels were reduced at baseline in both PKU groups; however, the differences between these dopamine and norepinephrine metabolite levels and control levels were less drastic than observed for PAG and 5HIAA, perhaps due to variability in Tyr intake. An increase in these metabolites was primarily observed in subjects with Phe < 360 μM, where the LAT1 transporter to the brain was less inhibited by elevated plasma Phe. Both dopamine and norepinephrine have been associated with ADHD, and symptoms of ADHD have been described in patients with PKU [16,23,31]. Atomoxetine, a selective norepinephrine reuptake inhibitor, has been found to increase norepinephrine throughout the brain of rat models and be an effective treatment in both inattentive and combined forms of ADHD [32,33]. Indeed, this study found that a change in inattention symptoms as measured by ADHD RS-IV IA in subjects symptomatic at baseline was correlated with a change in MOPEG levels. This early result was intriguing and additional studies will be necessary to further explore this potential connection between plasma MOPEG levels and symptoms of inattention in PKU.

The present study correlating plasma Phe reduction to brain Phe, NTs and NTMs in *Pah*^{enu2} mice and NTMs in subjects with PKU suggests that elevated plasma Phe reduces transport of LNAAs into the brain while a reduction of plasma Phe < 360 μM increases NT precursor availability in the brain and may have clinical benefits not yet observed to date. NTMs may be useful as biochemical markers to evaluate and understand the heterogeneous neurological and psychological sequelae described in PKU. Additional studies on the entire cohort of subjects from this clinical study are warranted to investigate the role of pegvaliase in the restoration of the NT biosynthesis pathway in the brain, how they correlate with plasma NTMs, and how this may contribute to improved cognitive function at varying plasma Phe levels. Of particular interest is the effect of plasma Phe of < 360 μM vs 360–600 μM, given the differences in the recommended target Phe levels for adults in treatment guidelines from the US and Europe. Furthermore, evaluating NTMs in subjects with other neurological diseases may yield clues on disrupted NT pathways to guide more effective treatments.

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Author contributions

GYB conceived of the assays and wrote the manuscript. AYC and GYB optimized and performed the assays. SB, LZ, LX, and GYB participated in the design of the animal studies. GP, LZ, RM, and LX performed all animal studies. GYB, NTM, JO, and HHW contributed to the design of the human sample study. GYB and NTM performed the analysis and interpretation of the human sample study. GYB, NTM, and SB participated in drafting and all authors contributed to the review the manuscript.

Conflict of interest

All authors are employees of BioMarin Pharmaceutical Inc.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ymgme.2019.08.004.

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