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High dose sapropterin dihydrochloride therapy improves monoamine neurotransmitter turnover in murine phenylketonuria (PKU)

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Abstract

Central nervous system (CNS) deficiencies of the monoamine neurotransmitters, dopamine and serotonin, have been implicated in the pathophysiology of neuropsychiatric dysfunction in phenylketonuria (PKU). Increased brain phenylalanine concentration likely competitively inhibits the activities of tyrosine hydroxylase (TH) and tryptophan hydroxylase (TPH), the rate limiting steps in dopamine and serotonin synthesis respectively. Tetrahydrobiopterin (BH₄) is a required cofactor for TH and TPH activity. Our hypothesis was that treatment of hyperphenylalaninemic *Pah^{enu2/enu2}* mice, a model of human PKU, with sapropterin dihydrochloride, a synthetic form of BH₄, would stimulate TH and TPH activities leading to improved dopamine and serotonin synthesis despite persistently elevated brain phenylalanine. Sapropterin (20, 40, or 100 mg/kg body weight in 1% ascorbic acid) was administered daily for 4 days by oral gavage to *Pah^{enu2/enu2}* mice followed by measurement of brain biopterin, phenylalanine, tyrosine, tryptophan and monoamine neurotransmitter content. A significant increase in brain biopterin content was detected only in mice that had received the highest sapropterin dose, 100 mg/kg. Blood and brain phenylalanine concentrations were unchanged by sapropterin therapy. Sapropterin therapy also did not alter the absolute amounts of dopamine and serotonin in brain but was associated with increased homovanillic acid (HVA) and 5-hydroxyindoleacetic acid (5-HIAA), dopamine and serotonin metabolites respectively, in both wild type and *Pah^{enu2/enu2}* mice. Oral sapropterin therapy likely does not directly affect central nervous system monoamine synthesis in either wild type or hyperphenylalaninemic mice but may stimulate synaptic neurotransmitter release and subsequent metabolism.

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Keywords

Phenylketonuria; Tetrahydrobiopterin; Sapropterin dihydrochloride; Dopamine; Serotonin; Monoamine neurotransmitters

Introduction

Phenylketonuria (PKU) due to recessively-inherited deficiency of phenylalanine hydroxylase (PAH, EC 1.14.16.1) is one of the most common inborn errors of metabolism. Dietary therapy of PKU to reduce plasma phenylalanine (Phe) levels prevents the major manifestations of the disease (mental retardation, seizures, and growth failure) yet mild cognitive deficits persist in some treated children (Azen et al. 1991). Additionally, PKU therapy requires lifelong strict adherence to an unpalatable and complicated diet. In our clinical experience, nonadherence to this difficult diet is common in adolescents and adults. Up to 75% of adolescents and adults in some PKU treatment centers are unable to maintain blood Phe levels within the recommended target range (Walter and White 2004). Chronically elevated blood Phe is frequently associated with disturbed executive functioning (VanZutphen et al. 2007; Christ et al. 2010). The proximate central nervous system mechanisms that underlay the effects of hyperphenylalaninemia are incompletely understood, but abundant evidence implicates dysfunction of the dopaminergic and serotonergic neuronal systems, particularly of the prefrontal cortex, as having major roles in the symptoms of anxiety and impaired executive functioning associated with poorly treated PKU (Christ et al. 2010; de Groot et al. 2010; Feillet et al. 2010). Elevated brain Phe but decreased tyrosine and tryptophan, dopamine and serotonin have been measured in brains obtained at autopsy of individuals with untreated PKU (McKean 1972). Elevated blood phenylalanine in individuals with classical PKU has been associated with decreased urinary excretion of dopamine and serotonin or their metabolites (Curtius et al. 1972; Curtius et al. 1981), and in diet treated individuals, a phenylalanine challenge yielded a transient decrease in urine dopamine and serotonin levels along with further functional impairment on neuropsychological measures of higher executive functioning (Krause et al. 1985). In *Pah^{enu2/enu2}* mice, a model of human PKU, deficiencies of both dopamine and serotonin have been documented (Puglisi-Allegra et al. 2000; Pascucci et al. 2002; Harding et al. 2014). These deficiencies have been postulated to be involved in the memory deficit exhibited by these animals (Zagreda et al. 1999). Additionally, we have documented decreased exploratory behavior in an open field test in hyperphenylalaninemic *Pah^{enu2/enu2}* mice that could also be related to monoamine neurotransmitter deficiency (unpublished data). The likely mechanisms causing monoamine neurotransmitter deficiency in PKU are relative brain tyrosine and tryptophan deficiency due to competition with phenylalanine at the blood brain barrier (Binek-Singer and Johnson 1982; Knudsen et al. 1995; Hoeksma et al. 2009) and phenylalanine-mediated competitive inhibition of tyrosine hydroxylase (TH) and tryptophan hydroxylase (TPH), the rate limiting steps in dopamine and serotonin synthesis respectively (Ikeda et al. 1967; Ogawa and Ichinose 2006; Pascucci et al. 2009; Harding et al. 2014) (Figure 1).

The aromatic amino acid hydroxylases, PAH, TH, TPH1, and TPH2, all require (6R)-L-erythro-5,6,7,8-tetrahydrobiopterin (BH₄) for catalytic activity. Additionally, BH₄ exhibits chaperone activity stabilizing PAH protein (Blau and Erlandsen 2004; Thony et al. 2004), and BH₄ supplementation leads to decreased blood phenylalanine in a subset of individuals with PKU (Kure et al. 1999; Levy et al. 2007). BH₄ deficiency in mice is associated with decreased TH protein and activity in brain (Brand et al. 1996; Sumi-Ichinose et al. 2001), and BH₄ supplementation has been shown to increase striatal TH activity (Nagatsu et al. 1994; Thöny et al. 2008). BH₄ has also been shown to stabilize TH protein *in vivo*, and daily oral administration of BH₄ to wild type C57BL/6 mice resulted in increased brain TH protein and activity (Thöny et al. 2008). Sapropterin dihydrochloride (Kuvan™, BioMarin Pharmaceutical Corp., Novato, CA) is a synthetic form of BH₄ that is approved for the treatment of BH₄ responsive PKU. The purpose of this study was to evaluate the effect of sapropterin treatment upon monoamine neurotransmitter status in *Pah^{enu2/enu2}* mice, a model that is known to *not* be BH₄ responsive in terms of blood phenylalanine levels. Our hypothesis was that enteral treatment with sapropterin would lead to improved central nervous system monoamine neurotransmitter status in *Pah^{enu2/enu2}* mice even though blood and brain phenylalanine content is unaffected. A study of BH₄ penetration into brain following oral sapropterin supplementation was a secondary aim of this project.

Methods

In this experiment, sapropterin dihydrochloride (Kuvan™, BioMarin Pharmaceutical Inc., Novato, CA) was administered by gavage to hyperphenylalaninemic C57BL6-*Pah^{enu2/enu2}* mice (*Pah*^{-/-}) or to wild type C57BL6 mice (*Pah*^{+/+}) to evaluate the uptake of BH₄ into brain and the effect of enteral BH₄ administration upon brain monoamine neurotransmitter content. Sapropterin, 100 mg tablets, were dissolved in 1% ascorbic acid immediately prior to administration. The final sapropterin concentration was varied to yield different doses in identical volumes of solution to be administered to the mice. Mice received sapropterin (20, 40, or 100 mg/kg body weight) once daily for four days by gavage. A control group of mice received only 1% ascorbic acid vehicle without added sapropterin. During this period, the animals received standard mouse chow (21% protein by weight) and water *ad libitum*. On the fifth day, mice were euthanized at various time points following sapropterin administration for tissue harvest. During the time period between sapropterin gavage and euthanasia, mouse chow was withheld in order to minimize variability in blood amino acid concentrations caused by feeding. Animals were sedated using inhaled isoflurane anesthesia. Whole blood was collected by cardiac puncture, allowed to clot in an Eppendorf tube, and serum was separated by centrifugation. The mice were then euthanized by exsanguination and perfused with 20 ml normal saline via the left cardiac ventricle to clear blood from the cerebral circulation. Following decapitation, whole brain was rapidly excised from the cranium, split sagittally, and immediately submerged in liquid nitrogen. Half brains and serum samples were stored at -80°C until processing for amino acid or neurotransmitter analysis.

Amino acid analysis

Amino acid concentrations in sera or brain tissue were measured by precolumn derivitization with 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC, Waters AccQ Tag™ derivitization system) with separation by ultra high performance liquid chromatography and UV absorbance detection (Waters Acquity™ UPLC, Milford, MA) using the Waters Masstrak Amino Acid Analysis method. Serum samples were deproteinized by adding an equal volume of 10% sulfosalicylic acid containing the nonphysiologic amino acid norvaline (250 µM) as an internal recovery standard. For analysis of amino acid concentrations in brain, half-brains were mechanically homogenized in 5 volumes/weight 10% trichloroacetic acid containing norvaline (150 µM). The homogenates were clarified by centrifugation at 500 × g and supernatants stored at –80°C until analysis. Deproteinized serum or brain homogenate was neutralized by adding 20 µl sample to 60 µl borate buffer (Waters Masstrak kit) plus sodium hydroxide to a final pH = 8. Derivitization was accomplished by adding 20 µl AQC reagent and incubating for 10 minutes at 55°C according to the instructions of the Waters MassTrak amino acid analysis kit (final volume of the derivitization reaction = 100 µl). 1 µl of this final derivitized solution was injected onto the UPLC for analysis. The serum amino acid concentrations were reported as µM and corrected for dilution to reflect the actual concentrations in serum. Brain amino acid concentrations are also reported as µM, but these are the measured amino acid concentrations in the brain homogenate supernatants that have not been corrected for brain weight and are not the actual amino acid concentrations expected in undiluted brain tissue. Because all brain samples were homogenized in a measured amount of homogenizing solution per brain weight wet, these uncorrected amino acid concentrations are suitable for examining differences among experimental groups

Brain biopterin content

Mouse half-brains were mechanically homogenized in ice cold homogenizing buffer (50 mM Tris-HCl, pH 7.5, 0.1 M KCl, 1 mM EDTA, 1 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride, 1 µM leupeptin, and 1 µM pepstatin), 4 µl/mg tissue and further processed according to a previously published method (Elzaouk et al. 2003). Total biopterin concentration in brain was measured by HPLC and fluorescence detection (Blau and Thöny 2008). Total brain homogenate biopterin content was corrected for the protein content of the homogenate and expressed as pmol/mg protein.

Brain monoamine neurotransmitter analysis

Mouse half-brains were mechanically homogenized in ice cold homogenizing buffer and processed as described above. Monoamine neurotransmitter concentrations (L-DOPA, dopamine, HVA, serotonin, and 5-HIAA) were measured in brain tissue by HPLC and electrochemical detection (Blau et al. 1999). Measured brain homogenate monoamine neurotransmitter concentrations were corrected for the protein content of the homogenate and expressed as pmol/mg protein.

Statistical analysis

All data are reported as means \pm standard error of the mean (SEM). Two-way ANOVA with *Pah* genotype and sapropterin treatment as independent variables was utilized in the statistical analysis of all data. Individual group differences were analyzed using Bonferroni post tests. Means were considered significantly different when $p < 0.05$. All statistical analyses were carried out with Prism 6.0 software (GraphPad Software, Inc.).

Results

Brain biopterin content

We administered sapropterin dihydrochloride in 1% ascorbic acid at three different doses (20, 40 or 100 mg/kg body weight) to both wild type (*Pah*^{+/+}) and *Pah*^{enu2/enu2} (*Pah*^{-/-}) mice by daily oral gavage. Separate control animals of both genotypes received only 1% ascorbic acid. On the fourth day of sapropterin treatment, animals were euthanized at various time points following the final gavage and tissues were collected for analysis. Half-brain total biopterin content of sapropterin-treated mice (both genotypes combined) are plotted versus time after sapropterin gavage in Figure 2. Only administration of the highest sapropterin dose (100 mg/kg) yielded a clearly demonstrable increase in brain biopterin content. At 2 hours post sapropterin dose (100 mg/kg), the average brain biopterin content of sapropterin treated mice (22.8 ± 10.9 pmol/mg protein) had increased by more than two fold in comparison to the brain biopterin content of mice that had received only 1% ascorbic acid vehicle (9.9 ± 2.8 pmol/mg protein). Brain biopterin content decreased at later time points but remained above the baseline level for the six hour duration of the experiment. Comparing all treatment groups (Figure 3a), statistical analysis using two-way ANOVA with genotype and sapropterin treatment as independent variables revealed a significant effect of sapropterin treatment upon brain biopterin content ($F(3,79) = 14.4$, $p < 0.0001$) but no significant effect of genotype nor any significant interaction between genotype and sapropterin treatment.

Serum and brain phenylalanine

Sapropterin treatment had no significant effect upon either serum (Figure 3b) or brain (Figure 3c) phenylalanine content in *Pah*^{+/+} or *Pah*^{-/-} mice. By two way ANOVA, only genotype had a significant effect upon serum ($F(1,77) = 1073$, $p < 0.0001$) or brain ($F(1,74) = 445.2$, $p < 0.0001$) phenylalanine. There was no significant interaction between genotype and sapropterin treatment with regards to either serum or brain phenylalanine. In the cohorts of *Pah*^{-/-} animals that had received sapropterin (any dose), the mean serum phenylalanine at euthanasia (2591 ± 367 μ M) was not different than the serum phenylalanine concentrations in *Pah*^{-/-} mice that had received only 1% ascorbic acid (2476 ± 634 μ M) confirming that this animal model is not responsive to BH₄ administration in terms of blood phenylalanine concentrations (Figure 3b). Likewise, neither blood nor brain phenylalanine concentrations in wild type mice were affected by sapropterin treatment.

Serum and brain tyrosine and tryptophan content

PAH deficiency causes decreased tyrosine synthesis, and in our experiment both serum and brain tyrosine concentrations were significantly decreased in *Pah*^{-/-} mice relative to *Pah*^{+/+} mice (Figures 4a and 4b). By two way ANOVA, the variance in serum tyrosine concentrations among the experimental groups was entirely attributed to the *Pah*^{enu2/enu2} genotype ($F(1;78) = 15.1, p = 0.0002$); there was no effect of sapropterin treatment ($F(3,78) = 1.795$) and no significant interaction between genotype and treatment. The mean serum tyrosine of *Pah*^{-/-} mice in all treatment groups ($47.8 \pm 4.7 \mu\text{M}$) was 65% of that in *Pah*^{+/+} mice ($74.0 \pm 3.5 \mu\text{M}$). Likewise, genotype had a significant effect upon brain tyrosine content ($F(1,75) = 5.108, p = 0.0267$), but surprisingly sapropterin treatment also had a statistically significant but inconsistent effect upon brain tyrosine ($F(3,75) = 12.49, p < 0.0001$). There was no significant interaction between genotype and sapropterin treatment. This apparent effect of sapropterin treatment upon brain tyrosine (predominantly at the highest sapropterin dose) remains unexplained and was not consistently seen in measurement of other large neutral amino acids (data not shown).

PAH deficiency was also associated with effects upon blood tryptophan content with mean *Pah*^{-/-} serum tryptophan ($62.7 \pm 1.6 \mu\text{M}$) measuring 74% of mean serum tryptophan in *Pah*^{+/+} mice ($84.2 \pm 3.4 \mu\text{M}$). Two way ANOVA revealed a significant effect of genotype upon serum tryptophan ($F(1,78) = 34.99, p < 0.0001$) with a modest effect from sapropterin treatment ($F(3,78) = 3.257, p = 0.026$); there was no significant interaction between genotype and sapropterin treatment. The mechanism behind serum tryptophan deficiency in *Pah*^{enu2/enu2} mice is unknown; dietary phenylalanine likely competes against dietary tryptophan for uptake into circulation across the intestinal enterocyte, but it is difficult to explain how hyperphenylalaninemia would interfere with intestinal tryptophan absorption. Phenylalanine is also known to compete against uptake of other large neutral amino acids across the blood brain barrier. Accordingly, the brain tryptophan concentration in *Pah*^{enu2/enu2} mice ($1.0 \pm 0.2 \mu\text{M}$) was approximately 1/5 of the concentration in wild type mice ($5.4 \pm 0.5 \mu\text{M}$). Two way ANOVA demonstrated a significant effect of genotype upon brain tryptophan ($F(1,75) = 52.03, p < 0.0001$) but no significant effect from sapropterin treatment ($F(3,75) = 1.676, p = 0.1794$) nor any significant interaction between genotype and treatment.

Brain monoamine neurotransmitter content

Both dopamine (Figure 4a) and serotonin (Figure 4b) content are depressed in brain of *Pah*^{-/-} mice in comparison to wild type mice as we and others have been reported previously (Harding et al. 2014). Additionally, the concentrations of homovanillic acid (HVA) (Figure 4c) and 5-hydroxyindoleacetic acid (5-HIAA) (Figure 4d), markers of dopamine and serotonin turnover respectively, are significantly depressed in *Pah*^{-/-} mice. Brain dopamine content in untreated *Pah*^{-/-} mice ($145.4 \pm 16.1 \text{ pmol/mg protein, } n = 6$) was reduced to approximately 54% of that in *Pah*^{+/+} mice ($271.0 \pm 15.2 \text{ pmol/mg, } n = 4$), while *Pah*^{-/-} HVA ($19.0 \pm 1.8 \text{ pmol/mg}$) was only 62% of HVA in wild type mice ($30.4 \pm 5.4 \text{ pmol/mg}$). Brain serotonin was more severely reduced with *Pah*^{-/-} serotonin content ($36.2 \pm 4.5 \text{ pmol/mg}$) measuring only 36% of wild type brain serotonin ($101.5 \pm 5.4 \text{ pmol/mg}$).

Similarly, *Pah*^{-/-} brain 5-HIAA (19.0 ± 1.8 pmol/mg) was reduced to 62% of *Pah*^{+/+} levels (30.4 ± 5.4 pmol/mg).

Sapropterin treatment had no effect upon brain dopamine or serotonin content in either wild type or *Pah*^{enu2/enu2} mice (Figure 4). Two-way ANOVA of brain dopamine in all experimental groups revealed a significant effect of genotype only ($F(1,79) = 15.54$, $p < 0.0001$) with no significant effect from sapropterin treatment ($F(3,79) = 2.182$, $p = 0.0967$) and no significant interaction between genotype and sapropterin treatment. Similarly, genotype significantly affected brain serotonin ($F(1,79) = 144.9$, $p < 0.0001$), but sapropterin treatment did not ($F(3,79) = 2.602$, $p = 0.0578$). Again, no significant interaction between genotype and sapropterin treatment was detected. Interestingly, brain HVA and 5-HIAA contents were significantly affected by both genotype and sapropterin treatment. For HVA, two way ANOVA revealed that both genotype ($F(1,79) = 10.8$, $p = 0.0015$) and sapropterin treatment ($F(3,79) = 4.077$, $p = 0.0096$) contributed equally to the variance among the experimental groups. Similar results were obtained for brain 5-HIAA content with genotype ($F(1,79) = 2.37$, $p < 0.0001$) and sapropterin treatment ($F(3,79) = 15.97$, $p < 0.0001$) having significant effects. Overall these results suggest that sapropterin treatment did not alter *de novo* dopamine or serotonin synthesis but did increase the metabolism of both dopamine and serotonin to HVA and 5-HIAA respectively.

Conclusions

Short term enteral administration by oral gavage of sapropterin dihydrochloride at 20, 40, or 100 mg/kg once per day for four days was well tolerated in both wild type and *Pah*^{enu2/enu2} mice. As expected, sapropterin treatment had no effect upon serum or brain phenylalanine concentration as the *Pah*^{enu2/enu2} model of human PKU is known to not be responsive to biopterin treatment in terms of lowering blood phenylalanine. Sapropterin treatment was associated with a statistically significant increase in brain total biopterin content but the greatest and most consistent effect was seen only in the animals that had received 100 mg sapropterin/kg. In these mice, brain biopterin content 2 hours after sapropterin gavage was more than double the brain biopterin content of untreated mice. This dose however is five fold higher than the oral dose typically prescribed to humans with BH₄-responsive PKU. These data are consistent with previous work demonstrating rather poor penetration of peripherally administered BH₄ across the blood-brain barrier of rodents except at high dosages (Kapatos and Kaufman 1981; Levine et al. 1987; Brand et al. 1996; Fiege and Blau 2006; Thöny et al. 2008) (summarized in Table 1) with better penetration into brain following parenteral BH₄ injection. Our previous experience demonstrated that BH₄ administered by intravenous injection to mice was primarily and rapidly taken up by liver and kidney with very little increase in BH₄ content of other tissues (Harding et al. 2004). Data concerning biopterin uptake into human brain following BH₄ administration is extremely limited. Oral BH₄ administration of 7.5–20 mg/kg/day to humans is associated with a significant increase in biopterin content of cerebrospinal fluid (CSF) (Kaufman et al. 1982; Ponzzone et al. 2006) but with no or only partial correction of CSF HVA and 5-HIAA concentrations in patients with inherited defects in BH₄ synthesis suggesting minimal functional correction of brain monoamine neurotransmitter metabolism following oral BH₄ administration at these lower doses (Ponzzone et al. 2006).

CNS dopamine and serotonin deficiencies have been previously documented in *Pah^{enu2/enu2}* mice (Puglisi-Allegra et al. 2000; Pascucci et al. 2002; Harding et al. 2014) and were again confirmed in this study. Chronic deficiency of these critical monoamine neurotransmitters are thought to contribute to the neurobehavioral abnormalities associated with human hyperphenylalaninemia (Feillet et al. 2010). Given that oral BH₄ treatment has been shown to increase TH protein abundance and activity in rodent brain (Thöny et al. 2008), we hypothesized that sapropterin treatment of *Pah*^{-/-} mice would improve brain dopamine status despite persistent hyperphenylalaninemia. Our data suggest however that the absolute brain dopamine content was unaffected by sapropterin treatment in our experiment. Likewise, sapropterin administration had no significant positive effect upon brain serotonin content. It is likely that phenylalanine-mediated inhibition of tyrosine hydroxylase and tryptophan hydroxylase activities was sustained even in the presence of increased brain biopterin. This result is consistent with previously published *in vitro* data showing that the abundance and activity of TPH are unaffected by increased BH₄ concentration (Calvo et al. 2010). Alternately, brain tyrosine and tryptophan content in *Pah^{enu2/enu2}* brain may have been insufficient to fully support dopamine and serotonin synthesis even if TH and TPH activities were increased via sapropterin-mediated stimulation. We did not attempt to directly measure brain TH or TPH activities in this experiment as these assays, carried out *ex vivo*, are performed under optimal conditions with the addition of BH₄ to the assay; measurement of TH or TPH activity in brain homogenate would likely not accurately reflect the *in vivo* status of dopamine and serotonin synthesis.

Sapropterin treatment was associated however with statistically significant increases in brain HVA and 5-HIAA content. These results suggest that dopamine and serotonin turnover had increased in sapropterin treated mice, both wild type and *Pah^{enu2/enu2}* mice, despite unchanged brain dopamine and serotonin content. BH₄-stimulated synaptic release of dopamine or serotonin followed by metabolism to HVA and 5-HIAA respectively is a possible mechanistic explanation for our result. Previous investigators have documented, using brain microdialysis, that BH₄ added to the perfusate of microdialysis probes placed in wild type rat striatum was associated with significantly increased synaptic dopamine release and HVA production (Koshimura et al. 1990; Tsukada et al. 1994; Koshimura et al. 1995), although a prior similar experiment had failed to show this association (Nakahara et al. 1988). The existence of a presynaptic BH₄ receptor that stimulated monoamine release into the synaptic cleft upon BH₄ binding has been proposed (Koshimura et al. 1995). Our data suggest that sapropterin treatment can affect CNS monoamine neurotransmitter metabolism status in a manner that is independent of any effect of BH₄ treatment upon blood and brain phenylalanine concentrations and would be consistent with a BH₄-mediated effect upon synaptic monoamine neurotransmitter release.

This experiment did not examine possible sapropterin effects upon specific brain regions such as the striatum or prefrontal cortex, areas that are rich in dopaminergic and serotonergic neurons, but rather relied upon measurements of monoamine neurotransmitters in half-brain extracts. We have initiated experiments using brain microdialysis to directly measure the effect of sapropterin therapy upon neurotransmitters specifically in those brain regions. Also, microdialysis will allow us to evaluate whether sapropterin treatment has any effect

upon the synaptic release of dopamine or serotonin independent of any effects upon dopamine or serotonin synthesis.

Ultimately, regardless of measurable changes in brain monoamine status in sapropterin treated mice, any effects upon the neurobehavioral phenotype of *Pah^{enu2/enu2}* mice remain to be studied. For instance, hyperphenylalaninemic mice exhibit consistent and quantifiable deficits of memory and exploratory behavior; reduction of blood phenylalanine through restriction of dietary phenylalanine intake is associated with a measurable improvement in exploratory behavior (unpublished data). We plan to evaluate whether oral sapropterin treatment will alter behavior in the animals in the face of persistent hyperphenylalaninemia. In summary, oral sapropterin therapy in both wild type C57BL/6 and C57BL/6-*Pah^{enu2/enu2}* mice is associated with increased brain biopterin content, but sapropterin doses that are much larger than have typically been used in clinical practice are necessary to measure a significant increase in brain biopterin content. At the largest dose of 100 mg/kg/day, we have documented evidence of increased HVA and 5-HIAA content in brain but no effect upon the absolute amounts of dopamine or serotonin. Any behavioral effects associated with increased sapropterin-induced monoamine neurotransmitter turnover need yet to be evaluated.

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Abbreviations

BH₄	Tetrahydrobiopterin
TH	Tyrosine hydroxylase
TPH	Tryptophan hydroxylase
HVA	Homovanillic acid
5-HIAA	5-hydroxyindoleacetic acid

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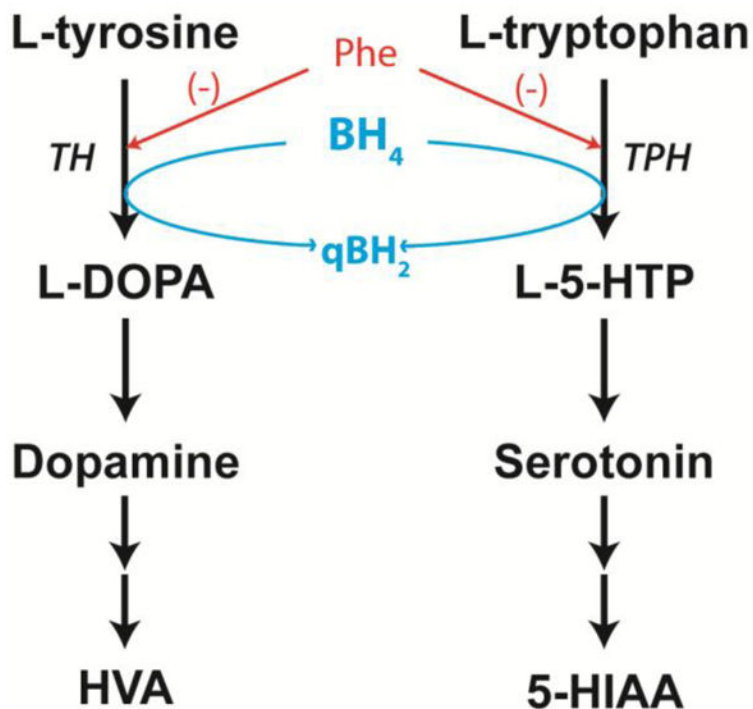


Figure 1. Pathways of dopamine and serotonin synthesis

Dopamine and serotonin are synthesized from the amino acids L-tyrosine and L-tryptophan respectively, and after release into the neuronal synapse, metabolized to homovanillic acid (HVA) or 5-hydroxyindoleacetic acid (5-HIAA). The rate limiting steps in these pathways are catalyzed by tyrosine hydroxylase (*TH*) and tryptophan hydroxylase (*TPH*) (*TPH2* in brain); both reactions require molecular oxygen (not shown) and BH_4 cofactor to complete substrate hydroxylation. Elevated brain phenylalanine (Phe) competitively inhibits both enzymes and is associated with deficient brain dopamine and serotonin synthesis. Our hypothesis was that increased brain biopterin content following enteral sapropterin treatment would lead to functionally increased *TH* and *TPH* activity and correction of brain dopamine and serotonin content in hyperphenylalaninemic *Pah^{enu2/enu2}* mice.

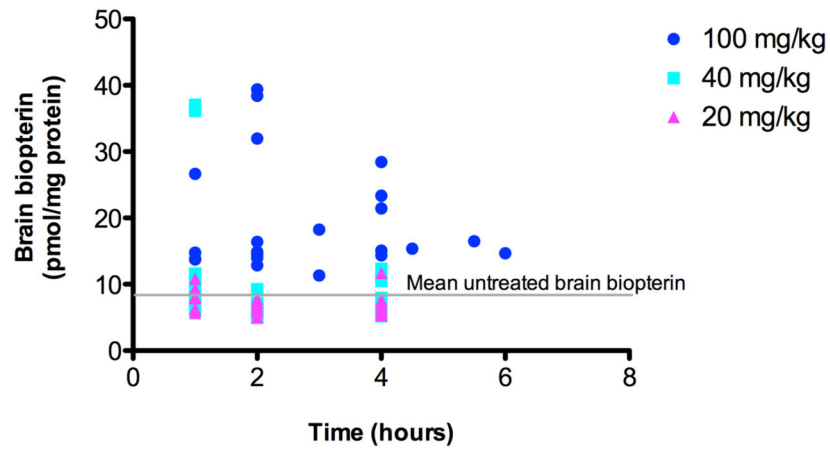


Figure 2. Brain bipterin content vs. time following enteral sapropterin administration
 Total bipterin was measured in homogenates of wild type and *Pah^{enu2/enu2}* mouse half-brains excised at various times after enteral sapropterin administration at three different doses: 20 mg/kg body weight (n = 28), 40 mg/kg (n = 27), or 100 mg/kg (n = 23). Brain bipterin concentration was corrected for the concentration of protein in the homogenate and expressed as pmol/mg protein. The brain bipterin content in six *Pah^{enu2/enu2}* mice that had received only 1% ascorbic acid by gavage feeding (mean untreated brain bipterin, gray line) was 9.2 ± 1.4 pmol/mg protein (mean \pm SEM).

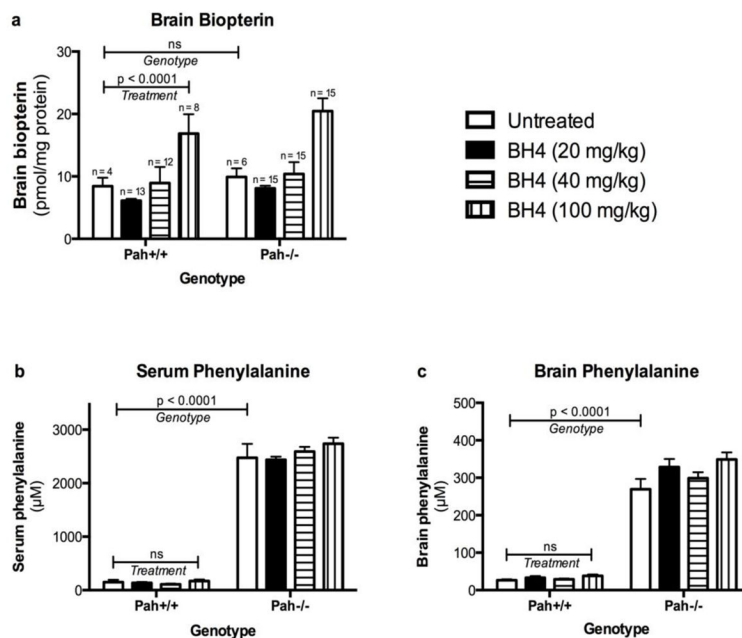


Figure 3. Effect of *Pah* genotype and sapropterin treatment upon brain biopterin, serum and brain phenylalanine

Brain biopterin content (pmol/mg protein, mean \pm SEM) (a) following either sapropterin treatment (BH4) or 1% ascorbic acid by gavage feeding in wild type (Pah^{+/+}) and *Pah^{enu2/enu2}* (Pah^{-/-}) mice. The data are the means of brain biopterin content from all animals at all time points up to six hours following gavage. Sapropterin treatment yielded a significant increase in brain biopterin content (two-way ANOVA, $p < 0.0001$) across all groups but only the 100 mg/kg sapropterin dose yielded a significant increase ($p < 0.01$) in brain biopterin in comparison to 1% ascorbic acid when analyzed by Bonferroni posttest intergroup analysis. There was no significant effect of genotype (Pah^{+/+} vs. Pah^{-/-}) upon brain biopterin content. Serum (b) and brain (c) phenylalanine concentration (μ M, mean \pm SEM) in Pah^{+/+} or Pah^{-/-} mice treated with sapropterin (BH4) or 1% ascorbic acid. The horizontal bars represent the statistical significance of the effects of either *Genotype* or sapropterin *Treatment* upon the brain biopterin, serum phenylalanine and brain phenylalanine concentrations as calculated by two way ANOVA. The *Pah* genotype had no effect upon brain biopterin content following sapropterin treatment. As expected, both serum and brain phenylalanine were extremely elevated in Pah^{-/-} mice in comparison to Pah^{+/+} animals. Sapropterin treatment had no significant (ns) effect upon either serum or brain phenylalanine.

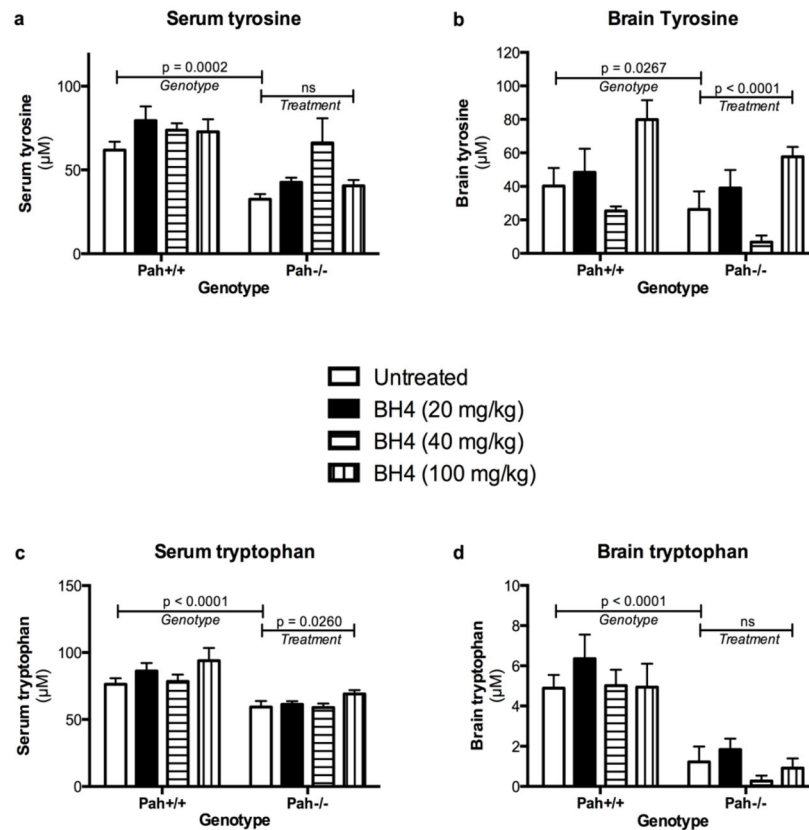


Figure 4. Effect of *Pah* genotype and sapropterin treatment upon serum and brain tyrosine and tryptophan

The concentrations (μM , mean \pm SEM) of tyrosine in serum (a) and brain (b) and of tryptophan in serum (c) and brain (d) from Pah+/+ and Pah-/- mice following gavage with sapropterin or 1% ascorbic acid are displayed. The data are the means from all animals obtained at all time points up to six hours after gavage. The horizontal bars represent the statistical significance of the effects of either *Genotype* or sapropterin *Treatment* upon the analytes as calculated by two way ANOVA. Pah-/- mice consistently exhibit tyrosine and tryptophan deficiency in both serum and brain relative to Pah+/+ animals. Sapropterin treatment had no effect upon serum tyrosine or brain tryptophan concentrations but did have apparent effects upon brain tyrosine and serum tryptophan, primarily in the highest sapropterin dose groups.

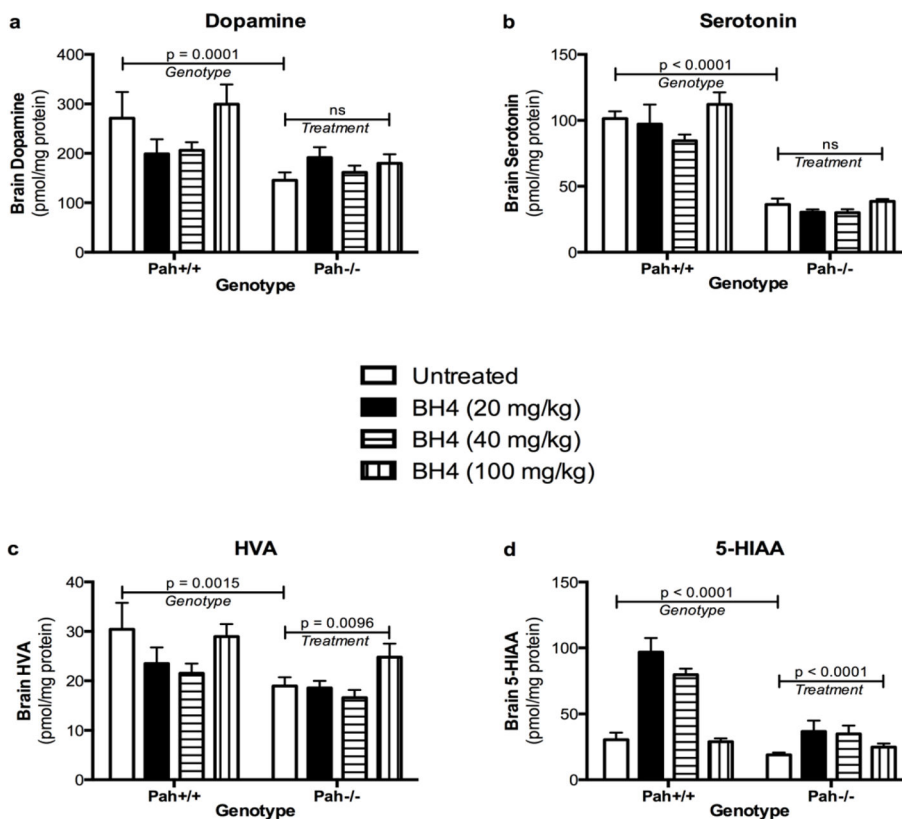


Figure 5. Effect of *Pah* genotype and sapropterin treatment upon brain monoamine neurotransmitters

The concentrations (pmol/mg protein, mean \pm SEM) of the monoamine neurotransmitters dopamine (a) and serotonin (b), their metabolites homovanillic acid (HVA) (c) and 5-hydroxyindoleacetic acid (5-HIAA) (d) in half-brain homogenates from wild type (Pah^{+/+}) and *Pah*^{enu2/enu2} (Pah^{-/-}) mice following gavage with sapropterin or 1% ascorbic acid are displayed. The data are the means of brain neurotransmitters from all animals obtained at all time points up to six hours after gavage. The horizontal bars represent the statistical significance of the effects of either *Genotype* or sapropterin *Treatment* upon the brain monoamine neurotransmitter concentrations as calculated by two way ANOVA.

Effect of peripherally administered BH₄ upon bipterin content in rodent brain as summarized from the literature

Table 1

Reference	Species	Route of BH ₄ administration	Dose (mg/kg)	Fold increase in brain bipterin
Kapatos and Kaufman 1981	Rat	IP	24	1.8
Levine et al 1987	Rat	IP	10	1
			20	3
Brand et al 1996	Mouse	SQ	30	2
Hayashi et al 1992	Rat	Oral	10	1
			100	1.4
Thöny et al 2008	Mouse	Oral	20	1.3
			100	1.6
This report	Mouse	Oral	20	1
			40	1
			100	2.1

This table lists the available literature reports of brain bipterin content following peripheral administration of BH₄ to rodents. 'Fold increase in brain bipterin' reports the ratio of brain bipterin following BH₄ administration to the brain bipterin content of untreated animals in each individual study. A ratio of 1 reflects no measurable increase in brain bipterin of BH₄ treated animals above untreated controls. The data from Hayashi T, Ogata A, Takehisa M, Komoridani K, Oonuma N, Studies on metabolism and disposition of sapropterine hydrochloride (SUN-0588) L-erythro-tetrahydrobipterin dichloride in rats, *Clin Report* 26:3471-95, 1992 were translated for and reported in Fiege and Blau, 2006.