Published in final edited form as:

Mol Genet Metab. 2014 April ; 111(4): 452–461. doi:10.1016/j.ymgme.2014.01.015.

Differential Effects of Low-Phenylalanine Protein Sources on Brain Neurotransmitters and Behavior in C57Bl/6-*Pah^{enu2}* Mice

Emily A. Sawin, Sangita G. Murali, and Denise M. Ney

Departments of Nutritional Sciences, University of Wisconsin-Madison, Wisconsin 53706

Emily A. Sawin: sawin@wisc.edu; Sangita G. Murali: murali@nutrisci.wisc.edu; Denise M. Ney: ney@nutrisci.wisc.edu

Abstract

Phenylketonuria (PKU) is an inborn error of metabolism caused by a deficiency of the enzyme phenylalanine hydroxylase, which metabolizes phenylalanine (phe) to tyrosine. A low-phe diet plus amino acid (AA) formula is necessary to prevent cognitive impairment; glycomacropeptide (GMP) contains minimal phe and provides a palatable alternative to the AA formula. Our objective was to assess neurotransmitter concentrations in brain and the behavioral phenotype of PKU mice (*Pah^{enu2}* on the C57Bl/6 background) and how this is affected by low-phe protein sources. Wild type (WT) and PKU mice, both male and female, were fed high-phe casein, low-phe AA, or low-phe GMP diets between 3–18 weeks of age. Behavioral phenotype was assessed using the open field and marble burying tests, and brain neurotransmitter concentration measured using HPLC with electrochemical detection system. Data were analyzed by 3-way ANOVA with genotype, sex, and diet as the main treatment effects. Brain mass and the concentrations of catecholamines and serotonin were reduced in PKU mice compared to WT mice; the low-phe AA and GMP diets improved these parameters in PKU mice. Relative brain mass was increased in female PKU mice fed the GMP diet compared to the AA diet. PKU mice exhibited hyperactivity and impaired vertical exploration compared to their WT littermates during the open field test. Regardless of genotype or diet, female mice demonstrated increased vertical activity time and increased total ambulatory and horizontal activity counts compared with male mice. PKU mice fed the high-phe casein diet buried significantly fewer marbles than WT control mice fed casein; this was normalized in PKU mice fed the low-phe AA and GMP diets. In summary, C57Bl/6-*Pah^{enu2}* mice showed an impaired behavioral phenotype and reduced brain neurotransmitter concentrations that were improved by the low-phe AA or GMP diets. These data support lifelong adherence to a low-phe diet for PKU.

Keywords

Phenylketonuria; glycomacropeptide; marble burying; open field; catecholamines; serotonin

© 2014 Elsevier Inc. All rights reserved.

Corresponding Author: Denise M. Ney, University of Wisconsin-Madison, Department of Nutritional Sciences, 1415 Linden Drive, Madison, WI 53706, ney@nutrisci.wisc.edu, Phone: (608) 262-4386, Fax: (608) 262-5860.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

1. INTRODUCTION

Phenylketonuria (PKU) is an autosomal recessive inborn error of metabolism caused by loss of function mutations of the gene encoding phenylalanine hydroxylase (EC 1.14.16.1, *PAH* in humans and *Pah* in mice), resulting in hyperphenylalaninemia due to an inability to convert phenylalanine (phe) to tyrosine. Untreated PKU is typically characterized by elevated blood phe concentrations and severe cognitive impairment. Introduction of a low-phe diet shortly after birth is necessary to prevent cognitive impairment, microcephaly, delayed speech, seizures, eczema, behavior abnormalities, and other symptoms associated with untreated PKU [1]. The low-phe diet is needed lifelong, and consists of limited intake of natural protein, which restricts intake of phe, combined with amino acid (AA) formula supplemented with trace elements to meet nutrient needs [2]. Even with continuous treatment initiated shortly after birth, neurophysiological and neuropsychological symptoms continue to persist [3]. Despite years of research the underlying mechanism(s) for the decrease in cognitive and executive functions associated with PKU is still unclear [4,5]. A correlation between high blood phe concentrations and poor cognitive outcomes has been established, and multiple theories exist to explain this correlation; including impaired brain neurotransmitter (NT) metabolism, myelination and protein synthesis. Multiple studies have demonstrated that high blood phe concentration is associated with decreased serotonin, dopamine, and norepinephrine in human and murine PKU [6–8]. This decrease in NT concentrations can potentially be explained by the effects of high phe on AA transport at the blood brain barrier (BBB) or the influence of phe on the enzymes involved in NT synthesis [7]. All large neutral amino acids (LNAA) utilize the same transporter, LAT1, which has a high affinity for phe, to cross the BBB [9]. In hyperphenylalaninemia this transporter may become saturated and reduce the concentration of tryptophan (trp) and tyrosine (tyr) available for NT synthesis. High phe has also been shown to be a competitive inhibitor of tyr and trp hydroxylase, and additionally, certain metabolites of phe inhibit 5-hydroxytryptophan decarboxylase and dopa decarboxylase, all of which are involved in NT synthesis [10–12].

The widely used animal model of PKU, the BTBR-*Pah*^{enu2} mouse, was developed to help determine neuropsychological deficits associated with PKU [13,14]. The *Pah*^{enu2} mouse has a missense point mutation in the *PAH* gene [14] that allows for it to closely mimic the phenotype of human PKU, including the deficits in the catecholamine NTs, dopamine and norepinephrine, and the indolamine NT serotonin [8,15–16]. A battery of behavior tests has been applied to the BTBR-*Pah*^{enu2} mouse including a T-maze alternation task [17], olfactory learning test [18], and special novelty and object discrimination tasks [19]. The conclusions reached from these studies are that there are mild behavioral impairments in BTBR-*Pah*^{enu2} mice, particularly in behavioral flexibility, the ability to alter previous behaviors in response to a changing environment [20]. The behavioral phenotype of *Pah*^{enu2} on the C57Bl/6 background, preferred for breeding [21], has not been reported. While much knowledge can be gained from murine behavior studies, an issue arises that cognitive impairment in untreated human PKU is severe whereas the impairment in untreated murine PKU tends to be more mild [20]. Moreover, the low-phe diet cannot be introduced until weaning after the majority of brain development has occurred in mice and it is more difficult to assess overall cognitive function in mice compared with humans [20]. Two potential

explanations for why the behavioral phenotype of murine PKU differs from human PKU include that rodents are not as sensitive to hyperphenylalaninemia and a single *Pah* mutation is insufficient to represent the entire spectrum of genetic mutations in human PKU [19]. Another gap in the literature exists in that most of the behavior studies focus solely on male mice, as they do not have an estrous hormonal cycle that may affect behavior.

Human dietary compliance with a low-phe diet is often poor due to the bitter taste and strong odor of the AA formulas as well as very limited food choices [22–25]. Also, quite a few suboptimal outcomes persist in patients with PKU treated with the AA-based diet [26]. Supplementation with LNAA has been used to increase tyr and trp concentrations by outcompeting phe at the BBB. Some success has been seen as dopamine and serotonin metabolite concentrations were increased in cerebrospinal fluid in individuals with PKU [27, 28]. LNAA supplementation also increases urine melatonin, a metabolite of serotonin, and urine dopamine albeit less than control values [29].

Glycomacropeptide (GMP), a 64-AA protein produced during cheese making, contains minimal phe as well as high levels of LNAA [30]. GMP can be made into a variety of acceptable foods and beverages and has been shown to be a safe and effective alternative to AA formula for individuals with PKU in the short-term [31, 32]. Furthermore, the GMP diet versus the synthetic AA diet has been found to lower blood and brain phe concentrations in PKU mice fed for 6 weeks [33], which may also suggest that GMP would improve the brain NT profile. Our objective was to determine brain NT concentrations and the behavioral phenotype of male and female *Pah^{enu2}* mice, on the C57Bl/6 background, and how this is affected by low-phe protein sources. Our data indicate that *Pah^{enu2}* C57Bl/6 mice show alterations in both the behavioral phenotype and brain NT profile, which are improved with low-phe AA and low-phe GMP diets.

2. METHODS

2.1. Animals and Experimental Design

The University of Wisconsin-Madison Institutional Animal Care and Committee approved the facilities and protocols used in this study. Experimental animals were produced from a breeding colony of PKU mice by breeding C57BL/6J mice heterozygous for the *Pah^{enu2}* mutation to yield homozygous PKU mice and WT control mice [34,35]. Experimental mice were genotyped for the presence of the *Pah^{enu2}* mutation [36]. The experimental design was set to control for three main effects and their interactions; these being genotype (WT or PKU), sex (male or female), and diet (low-phe GMP, low-phe AA, or high-phe casein), in a 2×2×3 factorial design, Figure 1. WT mice fed the casein diet served as the control. At weaning (21 days) mice were randomized to one of the three diets and separated by sex and conventionally housed in open top cages with littermates. The facility was maintained at 22°C on a 12-12-h light-dark cycle with mice being fed ad-libitum and having free access to water. Mice were fed the experimental diet from weaning (3 weeks of age) through young adulthood, which resulted for an average feeding time of 18 ± 3 weeks (n = 284 mice). All the diets were isoenergetic with the protein source being the only source of variation (Harlan Teklad, Madison, WI; TD.09667 – TD.09669, TD.120645) [36]. The protein in the casein diet was provided by 20% (wt/wt) casein plus 0.3% L-cystine, the AA diet included 17.5%

free AAs [37], and the GMP diet had 20% GMP (BioPURE GMP, Davisco Foods International, LeSueur, MN or LACPORDEN CGMP-10, Arla Foods Ingredients a.m.b.a., Viby J, Denmark) plus 1.5 times the National Research Council (NRC) requirement for 5 limiting AA in order to provide a complete protein source. The AA profile of the diets was previously reported [36]. At the end of the study mice were placed under anesthesia using an isoflurane anesthesia machine and euthanized via exsanguination. Blood was collected by cardiac puncture into syringes containing a final concentration of 2.7 mmol/l EDTA, and plasma was isolated by centrifugation at 4°C. The brain was dissected, weighed and snap frozen in liquid nitrogen. The brain was ground in a frozen mortar/pestle to prevent thawing and divided into 2 frozen pre-weighed microphage tubes. Tubes with powdered brain were weighed again and frozen at -70°C.

2.2. Analytical Methods

The plasma AA profile was determined using a Hitachi L-8900 AA analyzer equipped with an ion chromatography system using postcolumn ninhydrin derivatization [38]. For NT extraction, homogenizing buffer (0.15M perchloric acid, 0.025% cysteine and 0.025% NaEDTA) was added in a ratio of 1:2.5, 1g powdered brain/2.5 ml buffer, and tissue was homogenized for 10 seconds on ice, and the supernatant was filtered through 0.2 µm syringe filter. Twenty µl samples of brain extract were injected using an auto sampler and analyzed with a Waters HPLC system, using the Waters 2465 electrochemical detector and an Atlantis Symmetry C18 3.5 µm column (Waters, Milford, MA). The mobile phase consisted of 4% acetonitrile, containing 2.3 mM sodium 1-octanesulphonate, 13.7 mM monosodium dihydrogenorthophosphate, 30 mM sodium citrate, 0.025 mM EDTA and diethylamine. Samples were fractionated at a flow rate of 0.7 ml/hr with a total run time of ~ 26 min. A NT cocktail containing norepinephrine (NE), epinephrine (EPI), dopamine (DA), 3,4-dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA) and 5-hydroxytryptamine (5HT or serotonin) at 200 ng/ml was serially diluted to make standards ranging from 200 ng/ml to 1.5 ng/ml; standard curves were run with each set of samples. Data was analyzed by using BreezeTM2 software (Waters).

2.3. Histology and Immunohistochemistry

Brain tissue from PKU and WT mice (n = 3–4) were dissected after transcardiac perfusion with 4% paraformaldehyde in PBS and embedded in paraffin. Sections were cut in the transverse plane and prepared for either staining with hematoxylin and eosin (H&E) or for immunohistochemical detection of glial fibrillary acidic protein [39]. Sections were also cut sagittally down the midline and prepared for staining with Cresyl Violet-Luxol Fast Blue.

2.4. Marble Burying

Marble burying has been proposed to be a genetically regulated behavior that reflects repetitive digging, a normal mouse behavior [40]. Mice will dig to find and hoard food, make a safe nesting area for their young, and hide from predators. Laboratory mice exhibit this same digging behavior with sensitivity to strain differences and drugs [41]. Marble burying behavior has not been reported in *Pah^{enu2}* mice. Both PKU and WT mice were acclimatized to a marble burying room for at least 15 min prior to testing. A clean cage was

prepared with 20 black marbles arranged into a 4×5 grid that covered 2/3 of the cage. One mouse was placed into the cage at the end that did not contain the marbles and allowed to explore the new cage for 30 min. After 30 min the mouse was returned to its home cage and the number of visible marbles, defined as greater than 50% of the marble being seen, were counted.

2.5. Open Field Test

Spontaneous exploratory activity can be quantitatively measured using the open field test [42]. Measurement of anxiety-like behavior can also be assessed using this behavioral test, quantified by the ratio of center distance, which is the distance travelled by the animal in the center of the chamber, to the total distance travelled [43]. Each mouse was placed in the center of an open-field apparatus (40 × 40 × 30cm; Accuscan Instruments, Columbus, OH) and left to freely explore. To assess overall activity, total distance traveled in the open field arena (in cm), vertical activity, horizontal activity, time spent in the center, and ambulatory and stereotypic behaviors were recorded according to infrared beam breaks. Data was collected in 10 min time intervals over a 30 min period.

2.6. Statistical Analysis

Data were analyzed by three-way ANOVA using PROC MIXED to identify the main treatment effects of genotype, sex, diet, as well as their two and three way interactions. Differences between the treatment groups were detected using a protected Fisher's Least Significant Difference (LSD) test (SAS Institute, 2007, Cary, NC). Data transformations were performed where appropriate to fit assumptions of normality and equal variance prior to statistical analysis. Data in tables are presented untransformed and analyzed per animal, with sample size indicated. Data are presented as mean ± SE. P-values < 0.05 are considered significant. If there was a significant sex effect with no significant sex interaction, males and females were analyzed separately, and where there was no significant interaction, data were pooled into treatment groups by their respective main effects.

3. RESULTS

3.1. Plasma AA Profile, Brain Mass and Structure

The low-phe diets, AA and GMP, produced similar growth and significantly reduced plasma phe concentration in both PKU and WT mice [36]. Plasma phe concentration in PKU mice fed the casein diet was 3-fold greater compared to PKU mice fed the low-phe AA and GMP diets ($2,103 \pm 92$ casein vs 722 ± 26 AA or 766 ± 18 GMP; $\mu\text{mol phe/L}$), but remained abnormally elevated compared to WT mice ($43 \pm 2 \mu\text{mol/L}$, average of all 3 diets) (Table 1). Plasma tyr concentration was reduced by over 50% in PKU mice compared with WT mice. The AA and GMP diets significantly reduced plasma tyr in both WT and PKU mice compared to the high-phe casein diet, which provided higher tyr content (8.4 vs. 4.4–4.8, g tyr/kg diet). There was a significant main effect of genotype as plasma trp was significantly greater in WT mice compared to PKU mice. Both the casein and GMP diets had higher plasma valine and the sum of the branched-chain AA (isoleucine, leucine, and valine) compared with the AA diet; this is consistent with a lower branched-chain AA content of the AA diet.

The plasma ratios of specific AAs to LNAAs are thought to reflect competition at the BBB and what AAs are available for protein and NT synthesis. For example, a higher ratio of tyr to LNAA means that more tyr is available for dopamine synthesis [3,29]. There was a significant genotype by diet interaction for the ratio of phe to LNAA (phe, tyr, trp, leucine, valine, histidine, and threonine) as well as for the tyr to LNAA and trp to LNAA ratios. As expected WT and PKU mice responded differently with respect to the ratio of phe to LNAA, $gt \times diet$, $p < 0.0001$. PKU mice fed the high-phe casein diet had the greatest phe to LNAA ratio, which was significantly reduced by the low-phe AA diet and even further reduced by the GMP diet. The tyr and trp to LNAA ratios were significantly reduced in PKU mice compared to WT mice, but the low-phe AA and GMP diets significantly improved the trp to LNAA ratio to a similar extent in PKU mice compared with the high-phe casein diet. The branched-chain AA to aromatic AA ratio, a marker for reduced systemic inflammation at high levels [44], was 7- to 14-fold higher in WT mice compared to PKU mice. Consistent with our observation of lower inflammatory cytokines in mice fed GMP diet [36], the GMP diet significantly increased the BCAA to aromatic AA ratio compared to both the AA and casein diets in both WT and PKU mice, $p < 0.0001$.

The absolute mass of brain showed significant treatment effects for genotype, sex, and diet. PKU mice had 10% smaller brains than WT mice ($p < 0.01$), as shown in Figure 2A. Regardless of genotype or sex, mice fed the high-phe casein diet had smaller brain mass, than mice fed the AA or GMP diets. Relative brain mass showed treatment effects similar to absolute brain mass, except that in female PKU mice the GMP diet increased relative brain mass compared to the AA diet (Fig. 2B).

The general central nervous system cytoarchitecture showed no difference between the groups, based on H&E stained brain sections from 3 male PKU mice and 2 male and 2 female WT mice fed the casein diet. There was also no difference in astrocyte reactivity amongst 3 PKU mice and 2 WT mice, both groups being fed the high-phe casein diet. Myelin content, based on brain sections stained with Cresyl Violet-LFB, did not differ between 2 mice from each of the four groups, WT casein-fed, WT AA-fed, PKU casein-fed, and PKU AA-fed (data not shown).

3.2. Neurotransmitters

Brain NT concentrations showed different responses in male and female mice, and are presented separately. PKU male mice had significantly lower catecholamine concentrations, including NE (18% lower), EPI (66% lower), DOPAC (27% lower), DA (14% lower), and HVA (37% lower), than WT male mice (Table 2). Female mice followed the same trend as the male mice, as female PKU mice had significantly decreased catecholamine concentrations, including NE (31% decrease), EPI (55% decrease), DOPAC (47% decrease), DA (23% decrease), and HVA (38% decrease), when compared to WT female mice (Table 3). The low-phe AA and GMP diets normalized DA concentrations in male PKU mice when compared to the control casein-fed WT male mice, and improved NE concentrations in male PKU mice (16% increase) compared to male PKU mice fed casein. In female PKU mice the low-phe AA and GMP diets increased NE concentration by 15% compared to female PKU mice fed the casein diet. Regardless of genotype or diet, female mice had 4-fold greater EPI

concentrations compared to male mice and male mice had greater NE and DOPAC concentrations compared to female mice.

Brain serotonin concentration was significantly reduced in PKU mice, both males (23% lower) and females (45% lower), compared to WT mice, $p = 0.0023$ (Figure 3). In both male and female PKU mice, the low-phe AA and GMP diets significantly increased (21% and 35% higher, respectively) brain serotonin concentration compared with the casein diet. In both male and female WT mice, the low-phe AA diet significantly increased brain serotonin compared to WT control mice fed the casein diet. Regardless of genotype or diet, female mice had 2-fold greater brain serotonin concentration than male mice.

3.3. Marble Burying

The number of marbles buried was assessed in WT and PKU mice fed the high-phe casein and the low-phe AA and GMP diets as a measure of repetitive digging, a normal behavior in mice [40]. Sex did not have a significant effect and the number of marbles buried is shown combined for male and female mice (Figure 4). PKU mice fed the casein diet buried significantly fewer marbles than the WT control mice ($p=0.0084$), which was normalized in the PKU mice fed the low-phe AA or GMP diets. WT mice fed the AA diet buried significantly fewer marbles compared to WT mice fed the GMP diet. Therefore marble burying, a normal repetitive digging behavior in mice, can be rescued in male and female PKU mice by feeding them low-phe diets in association with increased brain serotonin concentration.

3.4. Open Field Test

Spontaneous exploratory behavior, assessed by number of rearing events/vertical activity, was measured in both PKU and WT mice in an open field test for 30 min. Male and female mice responded differently to the vertical activity measures in the open field test, therefore data are presented separately. Male WT mice had significantly greater total vertical activity time when compared to male PKU mice, $p < 0.0001$ (Figure 5A). Male mice fed the GMP diet also had greater total vertical activity time than male mice fed the AA diet, $p = 0.0197$. A similar trend was seen in total vertical episode count as male WT mice had higher total vertical episode count than male PKU mice, $p = 0.0061$ (Figure 5B). Female mice differed from male mice in that there was a significant genotype by diet interaction for both total vertical activity time and total vertical episode count, shown in Figures 5C and 5D. Female PKU mice fed the high-phe casein diet have significantly lower total vertical activity time and total vertical episode counts than all other groups, but this was normalized in female PKU mice fed the low-phe AA and GMP diets to that of female WT control casein-fed mice. Vertical activity measurements were also taken at 10 minute intervals over the 30 min time period to look at initial exploratory behavior (Supp. Table 1).

Locomotor activity, measured in the horizontal direction, was assessed in the open field arena where it was seen that regardless of sex or diet, PKU mice had significantly greater total movement time than WT mice, $p = 0.0228$, and significantly less rest time compared to WT mice, $p = 0.0050$, as shown in Figure 6. Movement and rest time for each of the 12 groups is shown in supp. Table 2. A significant treatment effect of sex was seen in both total

ambulatory activity count and total horizontal activity count. Regardless of genotype and diet, female mice had significantly higher total ambulatory activity count and total horizontal activity count compared to male mice (Figure 7). Both PKU and WT mice fed the AA diet also had significantly higher total ambulatory activity count compared to casein-fed mice. Female mice had significantly greater distance traveled than male mice, shown in supp table 3.

Anxiety behavior as assessed by the open field test using ratio of center distance did not differ in WT and PKU mice. This suggests an absence of anxiety, consistent with results reported by Cabib et al. using the elevated plus maze, another test commonly used to assess anxiety [19].

4. DISCUSSION

PKU is a genetic disorder in which lifelong adherence to a low-phe diet is necessary to prevent cognitive impairment; however, neurophysiological and neuropsychological impairment continue to persist. A large number of studies have examined the cognitive impairment of PKU yet there remains a limited understanding of the underlying brain pathology [20]. This study furthers understanding of the C57Bl/6-*Pah^{enu2}* model by examining the relationships among plasma AA concentrations, brain NT concentrations, and behavior tests in PKU and WT mice fed either the high-phe casein diet or the low-phe AA or GMP diets. PKU mice fed the high-phe casein diet had lower brain NT concentrations and an impaired behavioral phenotype that was improved in PKU mice fed the low-phe AA or GMP diets.

PKU mice with the *Pah^{enu2}* mutation on a BTBR background demonstrate cognitive deficits [19]. However, to our knowledge the behavioral phenotype of the *Pah^{enu2}* mutation has yet to be characterized in the C57Bl/6 background, a strain recognized to be a good performer in behavioral phenotyping tests [45] and possess heartier breeding capabilities compared to the BTBR strain [21]. Our data reveal that deficits in brain NT, in both catecholamines and serotonin, are observed in C57Bl/6-*Pah^{enu2}* mice consistent with what was reported in BTBR-*Pah^{enu2}* mice [8,15]. A behavioral phenotype in our mice was characterized by using the open field to assess activity and, for the first time in PKU mice, the marble burying test. The marble burying test reflects a normal repetitive behavior for mice [40,41]; however, it has also been used as a measure of anxiety. Interestingly PKU mice fed the high-phe casein left the majority of the 20 marbles untouched similar to a reduced environmental awareness noted in children with PKU who are off diet [46,47]. Moreover, the low-phe AA and GMP diets normalized marble burying activity in PKU mice to the level noted in WT mice fed the casein diet, which suggests that decreased phe helps to restore normal repetitive behavior, environmental awareness, and possibly reduce anxiety. PKU mice in the current study (age 4 mos.) exhibited hyperactivity as noted by increased movement time in open field, in contrast to the hypoactivity reported by Mochizuki et al. in 10 month old BTBR-*Pah^{enu2}* mice [48]. Hyperactivity agrees with the greater energy expenditure we observed in PKU vs. WT mice, regardless of diet they were fed [36]. Parallel to our findings in mice, studies in humans with PKU have noted hyperactivity as one of the behavioral impairments associated with high phe levels [49,50].

All measured catecholamine concentrations, include DA, NE, EPI, HVA, and DOPAC, were reduced in our PKU mice, similar to what was reported by Puglisi-Allegra et al. in their BTBR *Pah^{enu2}* mice [15]. Puglisi-Allegra et al. found that DA, NE, HVA, and DOPAC were all reduced in the prefrontal cortex, a brain region associated with attention-deficit hyperactivity disorder (ADHD) [51]. Dopamine and NE play a key role in attention and psychomotor behaviors, and their dysregulation may contribute to hyperactivity in rats [52]. This may help to explain the hyperactivity seen in the PKU mice, based on increased open field movement time in association with reduced brain DA and NE content [52]. Human PKU is associated with ADHD and the potential dysregulation of the monoamine NTs with decreased availability of tyr and trp to the brain [53]. Ney et al. found an inverse correlation between phe concentration in the cerebellum and plasma concentrations of threonine + valine + isoleucine in PKU mice fed the AA and GMP diets [33]. This is likely due to competition at the BBB for the LAT1 transporter as supported by an improved plasma phe/LNAA ratio in PKU mice fed the GMP diet compared to PKU mice fed the AA diet. The plasma phe/LNAA ratio also helps to explain the decrease in tyr and trp available for NT synthesis in PKU mice as phe outcompetes the other LNAAs for transport into the brain.

To our knowledge this is the first report of brain NTs and behavioral tests in female *Pah^{enu2}* mice. This is likely due to the difficulty in accounting for changes based on the estrous cycle of the female mice. However, research has assessed how estrogen and ovariectomy in female mice affects NT concentrations and behavioral tasks and demonstrated that estrogen increases or decreases catecholamines and serotonin in specific regions of the brain [54–56], similar to what we observed when comparing whole brain NT concentrations in male and female mice. Our finding, that WT female control mice fed the casein diet have greater brain serotonin concentration than WT male control mice fed the casein diet, is novel, although sex-based differences in brain serotonin receptor binding have been reported [57]. The differences in NT concentrations and effects of estrogen may help to explain why male and female mice responded differently to open field testing. Similar to the findings of Luine et al., who found that short-term estrogen treatment increased the number of rearings and crossings in the open field test, our female mice had increased ambulatory activity, horizontal activity and vertical activity time compared to male mice [55]. We also found that the GMP diet increased relative brain mass in female PKU mice compared to the AA diet, but this was not the case for male PKU mice. Female PKU mice showed more severe responses to the high-phe casein diet reflected in reduced vertical activity in the open field test compared to male PKU mice, as well as negative effects on body growth, and renal and liver mass [36]. These sex differences persist into human PKU as female behaviors were rated differently by their teachers compared to males with PKU [58]. Taken together, the evidence suggests that males and females respond differently to elevated phe levels or the stress that accompanies having PKU [58].

Mice were put on diet at 21 days of age, time of weaning, and thus most of brain development had occurred before the low-phe diet was introduced. However, introduction of the low-phe diets was still able to improve brain NT concentrations and performance in the marble burying test and multiple measures of the open field test. These findings suggest that phe levels correlate with behavior and NT concentrations throughout the lifespan of a

mouse. Our results are consistent with the clinical recommendations that the low-phe diet be continued for life to improve cognitive and emotional functioning and optimize school and work performance.

An interesting finding from our study is that the AA diet had sub-optimal effects on the WT mice compared to both the casein and GMP diets. WT male mice fed the AA diet had greater NE and serotonin concentrations than WT male mice fed casein or GMP diets and female WT mice fed the AA diet had greater serotonin than the female WT mice fed casein or GMP diets. This may reflect that the AA diet is composed of free AAs that are more rapidly absorbed compared to intact protein and that allow the NT precursors to reach the BBB more quickly. The increase in NT concentrations may also help to explain why the WT mice fed the AA diet buried fewer marbles than their WT counterparts and also had greater ambulatory and horizontal activity than WT mice fed either the casein or GMP diets. Cases et al. found that knockout mice with increased serotonin concentrations had increased aggressive behavior and other behavioral alterations [59]. Increased serotonin has generally been associated with decreased locomotion, yet the findings are more controversial when the mice are placed in novel environments where abnormal increased movement has been seen, similar to what was seen in our WT mice fed the AA diet [60]. Another difference is that the AA diet contains 22% glycine compared with 2–3% in the casein and GMP diets [36]. Interestingly, AA formulas for human PKU contain 3–12% glycine compared with negligible glycine in human GMP formulas. Glycine is a neurotransmitter in the central nervous system and an excess of glycine has been shown to be neurotoxic and increase excitability by activating N-methyl-D-aspartate receptors [61]. This may help to explain the significantly greater ambulatory activity seen in mice fed the AA diet, as modulation of glycine transport has been associated with hyperactivity in mice [62]. These data suggest that intact sources of dietary protein rather than mixtures of synthetic AAs support behavioral health.

We are the first to examine a low-phe alternative to the AA diet and its effects on behavior and brain NT concentrations in PKU mice. GMP enhanced vertical exploration, especially in PKU male mice, which agrees with the finding that GMP is able to improve learning and memory in mammals [63]. GMP contains a high concentration of sialic acid, a compound shown to accumulate rapidly in the frontal cortex during infancy [64]. Sialic acid, along with cholesterol, have higher concentrations in breast milk suggesting their importance for early brain development [64]. Supplementation with sialic acid was also able to improve learning and memory in developing piglets [65], which suggests that the high concentration of sialic acid in GMP may contribute to the improved behavioral phenotype in our PKU mice.

In conclusion, we report for the first time the behavioral phenotype of C57Bl/6-*Pah^{enu2}* mice, which is characterized by decreased brain catecholamine and serotonin concentrations as well as hyperactivity and reduced digging behavior. The low-phe AA and GMP diets are able to improve some of the behavioral impairments and, in some behavioral tests, normalize it to that of the WT casein control mice. Our findings support the view that the low-phe diet be maintained for life to improve cognitive functioning in those with PKU. Given our findings of sex-based differences in brain NT concentrations and behavior in the *Pah^{enu2}* mouse model, further research in murine and human PKU is needed to understand

how males and females respond differently to hyperphenylalaninemia. Overall our results suggest that the *C57Bl/6-Pah^{enu2}* mouse model is suitable for mechanistic studies to further understanding of the human PKU neurophysiological phenotype.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Funding: This work was supported by United States Department of Agriculture HATCH Grant WIS01517 and Department of Health and Human Services grant P30-HD-03352. Funding sources had minimal input regarding study design, collection, analysis, and interpretation of the data, and no input regarding the writing of the report and the decision to submit the paper for publication.

We thank Dr. Albee Messing for performing and interpreting brain histology and immunohistochemistry work, Mike Grahn for his assistance with NT analyses, undergraduate students Jennifer Mallon and Therese Breunig for their assistance with the PKU mouse colony, and Dr. Adam Brinkman and Dr. Patrick Solverson for their help with tissue processing.

Abbreviations

PKU	phenylketonuria
pah	phenylalanine hydroxylase
phe	phenylalanine
AA	amino acid
NT	neurotransmitter
BBB	blood brain barrier
LNAA	large neutral amino acid
trp	tryptophan
tyr	tyrosine
WT	wild type
GMP	glycomacropeptide
NE	norepinephrine
EPI	epinephrine
DA	dopamine
DOPAC	3,4-dihydroxyphenylacetic acid
HVA	homovanillic acid
5HT	5-hydroxytryptamine
ADHD	attention-deficit hyperactivity disorder

References

1. National Institutes of Health. Phenylketonuria (PKU): Screening and management. NIH consensus statement. 2000; 17(3):1–33.
2. MacLeod, Erin L.; Ney, Denise M. Nutritional management of phenylketonuria. *Ann Nestlé* (English ed). 2010; 68:58–69.
3. De Groot MJ, Hoeksma M, Blau N, Reijngoud DJ, Van Spronsen FJ. Pathogenesis of cognitive dysfunction in phenylketonuria: review of hypotheses. *Mol Genet Metab*. 2010; 99:S86–S89. [PubMed: 20123477]
4. Azadi B, Seddigh A, Tehrani-Doost M, Alaghband-Rad J, Ashrafi MR. Executive dysfunction in treated phenylketonuric patients. *Eur Child & Adolescent Psychiatry*. 2009; 18:360–368.
5. Christ SE, Huijbregts SC, de Sonnevile LM, White DA. Executive function in early-treated phenylketonuria: profile and underlying mechanisms. *Mol Genet Metab*. 2010; 99:S22–S32. [PubMed: 20123466]
6. Landvogt C, Mengel E, Bartenstein P, Buchholz HG, Schreckenberger M, Siessmeier T, Scheurich A, Feldmann R, Weglage J, Cumming P, Zepp F, Ullrich K. Reduced cerebral fluoro-L-dopamine uptake in adult patients suffering from phenylketonuria. *J of Cereb Blood Flow & Metab*. 2007; 28:824–831. [PubMed: 17971791]
7. Güttler F, Lou H. Dietary problems of phenylketonuria: effect on CNS transmitters and their possible role in behaviour and neuropsychological function. *J of Inherit Metab Dis*. 1986; 9:169–177. [PubMed: 2877115]
8. Pascucci T, Ventura R, Puglisi-Allegra S, Cabib S. Deficits in brain serotonin synthesis in a genetic mouse model of phenylketonuria. *Neuroreport*. 2002; 13:2561–2564. [PubMed: 12499868]
9. Pardridge WM. Blood-brain barrier carrier-mediated transport and brain metabolism of amino acids. *Neurochemical Res*. 1998; 23:635–644.
10. McKean CM. The effects of high phenylalanine concentrations on serotonin and catecholamine metabolism in the human brain. *Brain Res*. 1972; 47:469–476. [PubMed: 4642573]
11. Pare CMB, Sandler M, Stacey RS. Decreased 5-hydroxytryptophan decarboxylase activity in phenylketonuria. *The Lancet*. 1958; 272:1099–1101.
12. Sandler M. Inborn errors and disturbances of central neurotransmission (with special reference to phenylketonuria). *J Inherit Metab Dis*. 1982; 5:65–70. [PubMed: 6820424]
13. Shedlovsky A, McDonald JD, Symula D, Dove WF. Mouse models of human phenylketonuria. *Genetics*. 1993; 134:1205–1210. [PubMed: 8375656]
14. McDonald JD, Charlton CK. Characterization of mutations at the mouse phenylalanine hydroxylase locus. *Genomics*. 1997; 39:402–405. [PubMed: 9119379]
15. Puglisi-Allegra S, Cabib S, Pascucci T, Ventura R, Cali F, Romano V. Dramatic brain aminergic deficit in a genetic mouse model of phenylketonuria. *Neuroreport*. 2000; 11:1361–1364. [PubMed: 10817622]
16. Pascucci T, Andolina D, Ventura R, Puglisi-Allegra S, Cabib S. Reduced availability of brain amines during critical phases of postnatal development in a genetic mouse model of cognitive delay. *Brain Res*. 2008; 1217:232–238. [PubMed: 18502400]
17. Sarkissian CN, Boulais DM, McDonald JD, Scriver CR. A heteroallelic mutant mouse model: a new orthologue for human hyperphenylalaninemia. *Mol Genet Metab*. 2000; 69:188–194. [PubMed: 10767173]
18. Mihalick SM, Langlois JC, Krienke JD, Dube WV. An olfactory discrimination procedure for mice. *J Exp Analysis Behav*. 2000; 73:305–318.
19. Cabib S, Pascucci T, Ventura R, Romano V, Puglisi-Allegra S. The behavioral profile of severe mental retardation in a genetic mouse model of phenylketonuria. *Behav Genet*. 2003; 33:301–310. [PubMed: 12837019]
20. Martynyuk AE, Van Spronsen FJ, Van der Zee EA. Animal models of brain dysfunction in phenylketonuria. *Mol Genet Metab*. 2010; 99:S100–S105. [PubMed: 20123463]
21. Ding Z, Georgiev P, Thöny B. Administration-route and gender-independent long-term therapeutic correction of phenylketonuria (PKU) in a mouse model by recombinant adeno-associated virus 8

- pseudotyped vector-mediated gene transfer. *Gene Therapy*. 2005; 13:587–593. [PubMed: 16319947]
22. Koch R, Burton B, Hoganson G, Peterson R, Rhead W, Rouse B, Scott R, et al. Phenylketonuria in adulthood: a collaborative study. *J Inherit Metab Dis*. 2002; 25:333–346. [PubMed: 12408183]
 23. MacDonald A. Diet and compliance in phenylketonuria. *Eur J Pediatr*. 2000; 159:S136–S141. [PubMed: 11043160]
 24. MacDonald A, Daly A, Davies P, Asplin D, Hall SK, Rylance G, Chakrapani A. Protein substitutes for PKU: What's new? *J Inherit Metab Dis*. 2004; 27:363–371. [PubMed: 15190194]
 25. Walter JH, White FJ. Blood phenylalanine control in adolescents with phenylketonuria. *Int J Adolesc Med Health*. 2004; 16:41–46. [PubMed: 15148857]
 26. Enns GM, Koch R, Brumm V, Blakely E, Suter R, Jurecki E. Suboptimal outcomes in patients with PKU treated early with diet alone: Revisiting the evidence. *Mol Genet Metab*. 2010; 101:99–109. [PubMed: 20678948]
 27. Nielsen, JB. In *Dietary Phenylalanine and Brain Function*. Birkhäuser; Boston: 1988. Effect of dietary tryptophan supplement on neurotransmitter metabolism in phenylketonuria; p. 261–264.
 28. Lykkelund C, Nielsen JB, Lou HC, Rasmussen V, Gerdes AM, Christensen E, Güttler F. Increased neurotransmitter biosynthesis in phenylketonuria induced by phenylalanine restriction or by supplementation of unrestricted diet with large amounts of tyrosine. *Eur J Pediatr*. 1988; 148:238–245. [PubMed: 2463918]
 29. Yano S, Moseley K, Azen C. Large Neutral Amino Acid Supplementation Increases Melatonin Synthesis in Phenylketonuria: A New Biomarker. *J Pediatr*. 2012
 30. Etzel MR. Manufacture and use of dairy protein fraction. *J Nutr*. 2004; 134:996S–1002S. [PubMed: 15051860]
 31. Ney DM, Gleason ST, Van Calcar SC, MacLeod EL, Nelson KL, Etzel MR, Rice GM, Wolff JA. Nutritional management of PKU with glycomacropeptide from cheese whey. *J Inherit Metab Dis*. 2009; 32:32–39. [PubMed: 18956251]
 32. van Calcar SC, MacLeod EL, Gleason ST, Etzel MR, Clayton MK, Wolff JA, Ney DM. Improved nutritional management of phenylketonuria by using a diet containing glycomacropeptide compared with amino acids. *Am J Clin Nutr*. 2009; 89:1068–1077. [PubMed: 19244369]
 33. Ney DM, Hull AK, van Calcar SC, Liu X, Etzel MR. Dietary glycomacropeptide supports growth and reduces the concentrations of phenylalanine in plasma and brain in a murine model of phenylketonuria. *J Nutr*. 2008; 138:316–322. [PubMed: 18203898]
 34. McDonald JD V, Bode C, Dove WF, Shedlovsky A. Pahhph-5: a mouse mutant deficient in phenylalanine hydroxylase. *Proc Natl Acad Sci*. 1990; 87:1965–1967. [PubMed: 2308957]
 35. Harding CO, Gillingham MB, Bird A, Koeberl DD. Long-term correction of hyperphenylalaninemia following liver-directed, AAV2/8-mediated gene therapy in murine phenylketonuria. *J Inherit Metab Dis*. 2006; 29:30–30. [PubMed: 16601865]
 36. Solversen P, Murali SG, Brinkman AS, Nelson DW, Clayton MK, Yen Chi-Liang E, Ney DM. Glycomacropeptide, a low-phenylalanine protein isolated from cheese whey, supports growth and attenuates metabolic stress in the murine model of phenylketonuria. *Am J Phys-Endo Metab*. 2012; 302:E885–E895.
 37. Rogers QR, Harper AE. Amino acid diets and maximal growth in the rat. *J Nutr*. 1965; 87:267–273. [PubMed: 5859253]
 38. Slocum, RH.; Cummings, JG. *Techniques in diagnostic human biochemical genetics: a laboratory manual*. New York: Wiley-Liss; 1991. Amino acid analysis of physiological samples; p. 87–126.
 39. McCall MA, Gregg RG, Behringer RR, Brenner M, Delaney CL, Galbreath EJ, Zhang CL, Pearce RA, Chiu SY, Messing A. Targeted deletion in astrocyte intermediate filament (Gfap) alters neuronal physiology. *Proc Natl Acad Sci*. 1996; 93:6361–6366. [PubMed: 8692820]
 40. Thomas A, Burant A, Bui N, Graham D, Yuva-Paylor LA, Paylor R. Marble burying reflects a repetitive and perseverative behavior more than novelty-induced anxiety. *Psychopharmacol*. 2009; 204:361–373.
 41. Deacon RMJ. Digging and marble burying in mice: simple methods for in vivo identification of biological impacts. *Nature Protocols*. 2006; 1:122–124.

42. Holmes A, Wrenn CC, Harris AP, Thayer KE, Crawley JN. Behavioral profiles of inbred strains on novel olfactory, spatial and emotional tests for reference memory in mice. *Genes, Brain and Behav.* 2002; 1:55–69.
43. Veeraragavan S, Graham D, Bui N, Yuva-Paylor LA, Wess J, Paylor R. Genetic reduction of muscarinic M₄ receptor modulates analgesic response and acoustic startle response in a mouse model of fragile X syndrome (FXS). *Behav Brain Res.* 2012; 228:1–8. [PubMed: 22123412]
44. Berg RM, Taudorf S, Bailey DM, Lundby C, Larsen FS, Pedersen BK, Møller K. Cerebral net exchange of large neutral amino acids after lipopolysaccharide infusion in healthy humans. *Crit Care.* 2010; 14:R16. [PubMed: 20149231]
45. Dobkin C, Rabe A, Dumas R, Idrissi AE, Haubstock H, Brown WT. *Fmr 1* knockout mouse has a distinctive strain-specific learning impairment. *Neurosci.* 2000; 100:423–429.
46. Bickel H, Gerrard J, Hickmans EM. Influence of phenylalanine intake on phenylketonuria. *Lancet.* 1953; 265:812. [PubMed: 13098090]
47. Bickel H, Gerrard J, Hickmans EM. The influence of phenylalanine intake on the chemistry and behaviour of a phenylketonuria child. *Acta Paediatrica.* 1954; 43:64–77. [PubMed: 13138177]
48. Mochizuki S, Mizukami H, Ogura T, Kure S, Ichinohe A, Kojima K, Matsubara Y, et al. Long-term correction of hyperphenylalaninemia by AAV-mediated gene transfer leads to behavioral recovery in phenylketonuria mice. *Gene therapy.* 2004; 11:1081–1086. [PubMed: 15057263]
49. Yannicelli S, Ryan A. Improvements in behaviour and physical manifestations in previously untreated adults with phenylketonuria using a phenylalanine-restricted diet: a national survey. *J Inherit Metab Dis.* 1995; 18:131–134. [PubMed: 7564227]
50. Arnold GL, Vladutiu CJ, Orlowski CC, Blakely EM, DeLuca J. Prevalence of stimulant use for attentional dysfunction in children with phenylketonuria. *J Inherit Metab Dis.* 2004; 27:137–143. [PubMed: 15159644]
51. Russell V, de Villiers A, Sagvolden T, Lamm M, Taljaard J. Altered dopaminergic function in the prefrontal cortex, nucleus accumbens and caudate-putamen of an animal model of attention-deficit hyperactivity disorder—the spontaneously hypertensive rat. *Brain Res.* 1995; 676:343–351. [PubMed: 7614004]
52. Bymaster FP, Katner JS, Nelson DL, Hemrick-Luecke SK, Threlkeld PG, Heiligenstein JH, Morin SM, Gehlert DR, Perry KW. Atomoxetine increases extracellular levels of norepinephrine and dopamine in prefrontal cortex of rat: a potential mechanism for efficacy in attention deficit/hyperactivity disorder. *Neuropsychopharmacol.* 2002; 27:699–711.
53. Stevenson M, McNaughton N. A comparison of phenylketonuria with attention deficit hyperactivity disorder: Do markedly different aetiologies deliver common phenotypes? *Brain Res Bulletin.* 2013; 99:63–83.
54. Heikkinen T, Puoliväli J, Liu L, Rissanen A, Tanila H. Effects of ovariectomy and estrogen treatment on learning and hippocampal neurotransmitters in mice. *Hormones Behav.* 2002; 41:22–32.
55. Luine VN, Richards ST, Wu VY, Beck KD. Estradiol enhances learning and memory in a spatial memory task and effects levels of monoaminergic neurotransmitters. *Hormones Behav.* 1998; 34:149–162.
56. Vries GJ. Sex differences in neurotransmitter systems. *J neuroendocrinology.* 1990; 2:1–13. [PubMed: 19210390]
57. Parsey RV, Oquendo MA, Simpson NR, Ogden RT, Van Heertum R, Arango V, Mann JJ. Effects of sex, age, and aggressive traits in man on brain serotonin 5-HT_{1A} receptor binding potential measured by PET using [C-11] WAY-100635. *Brain Res.* 2002; 954:173–182. [PubMed: 12414100]
58. Stermerdink BA, Kalverboer AF, Van der Meere JJ, Van der Molen MW, Huisman J, de Jong LWA, Slijper FME, Verkerk PH, van Spronsen FJ. Behaviour and school achievement in patients with early and continuously treated phenylketonuria. *J Inherit Metab Dis.* 2000; 23:548–562. [PubMed: 11032330]
59. Cases O, Seif I, Grimsby J, Gaspar P, Chen K, Pournin S, Müller U, et al. Aggressive behavior and altered amounts of brain serotonin and norepinephrine in mice lacking MAOA. *Science.* 1995; 268:1763. [PubMed: 7792602]

60. Gerson SC, Baldessarini RJ. Motor effects of serotonin in the central nervous system. *Life Sciences*. 1980; 27:1435–1451. [PubMed: 6160367]
61. Newell DW, Barth A, Ricciardi TN, Malouf AT. Glycine causes increased excitability and neurotoxicity by activation of NMDA receptors in the hippocampus. *Exp Neurol*. 1997; 145:235–244. [PubMed: 9184125]
62. Lipina T, Labrie V, Weiner I, Roder J. Modulators of the glycine site on NMDA receptors, D-serine and ALX 5407, display similar beneficial effects to clozapine in mouse models of schizophrenia. *Psychopharmacol*. 2005; 179:54–67.
63. McMahon, RJ.; Wang, B.; Brand-Miller, J.; Rumsey, SC. Method Of Improving Learning and Memory in Mammals. WIPO Patent. 2006118719. issued November 10, 2006
64. Scholtz SA, Gottipati BS, Gajewski BJ, Carlson SE. Dietary Sialic Acid and Cholesterol Influence Cortical Composition in Developing Rats. *J Nutr*. 2013; 143:132–135. [PubMed: 23256147]
65. Wang B, Yu B, Karim M, Hu H, Sun Y, McGreevy P, Petocz P, Held S, Brand-Miller J. Dietary sialic acid supplementation improves learning and memory in piglets. *Am J Clin Nutr*. 2007; 85:561–569. [PubMed: 17284758]

Figure 1.

Experimental design that controlled for the main effects of genotype (WT or PKU), sex (male or female) and diet (high-phe casein, low-phe AA, or low-phe GMP) in mice randomized to 12 treatment groups.

Figure 2.

Brain mass (A) and relative brain mass (B) in PKU and WT mice fed Casein, AA, and GMP diets. Values are mean + SE; nos. in parentheses indicate sample size. Brain mass showed a significant effect of genotype ($P < 0.0001$), sex ($P = 0.0011$), and diet ($P = 0.017$); greater brain mass was seen in WT vs. PKU and female vs. male mice and the GMP and AA diets vs. the casein diet.

Relative brain mass showed a significant 3-way interaction between genotype, sex, and diet. Means without a superscripted letter ^{a,b,c,d,e,f} in common are significantly different ($P < 0.05$). For example relative brain mass in female PKU mice fed GMP was significantly different from female PKU fed the AA diet, but are not significantly different from female WT mice fed the casein diet.

Figure 3.

Brain serotonin concentration in male (A) and female (B) WT and PKU mice fed Casein, AA, and GMP diets. Values are mean + SE; nos. in parentheses indicate sample size. Female mice had 2-fold greater brain serotonin concentration compared with male mice. Brain serotonin concentration in male mice showed a significant effect of genotype ($P = 0.0023$) and diet ($P = 0.0326$); greater brain serotonin was seen in WT vs. PKU and the AA diet vs. the casein and GMP diets. Brain serotonin concentration in female mice showed a significant effect of genotype ($P < 0.0001$) and diet ($P = 0.0277$); greater brain serotonin was seen in WT vs. PKU and the AA diet vs. the casein diet. $**p < 0.005$, $***p < 0.0005$ for genotype treatment effect. Means without a superscripted letter^{a,b} in common are significantly different ($P < 0.05$) for diet treatment effect.

Figure 4.

Number of marbles buried among WT and PKU mice fed Casein, AA, and GMP diets. Values are means $\pm$ SE; nos in parentheses indicate sample size. Means without a superscripted letter^{a,b,c} in common are significantly different ($p < 0.05$), reflecting the significant interaction of genotype and diet on marble burying. Sex did not have a significant effect and the number of marbles buried is shown combined for male and female mice.

Figure 5.

Total vertical activity time and total vertical episode count in male (A and B) and female (C and D) mice fed Casein, AA, or GMP diets. Total vertical activity time (A and C) and total vertical episode count (B and D) are assessed over a 30 min period. Male mice showed a significant main effect for genotype and dietary treatment without significant interaction for total vertical activity time (A). Male PKU mice had significantly lower total vertical episode count than male WT mice (B). Female mice showed significant genotype $\times$ diet interaction, as reflected in presentation of the six groups (C and D). Nos. in parentheses indicate sample size. $*p < 0.05$, $**p < 0.005$, $***p < 0.0005$ for genotype treatment effect. Means without a superscripted letter^{a,b,c} in common are significantly different ($P < 0.05$).

Figure 6.

Total movement time (A) and total rest time (B) assessed over 30 min in the open field arena for WT and PKU mice. Values are means $\pm$ SE; p-values represent main effect of genotype. Sample size is shown in parenthesis. Regardless of sex and diet, PKU mice had significantly greater movement time and significantly less total rest time compared to WT mice. $*p < 0.05$, $**p < 0.005$ for genotype treatment effect.

Figure 7.

Total ambulatory activity count (A) and total horizontal activity count (B) assessed for 30 min in the open field arena in WT and PKU mice fed Casein, AA, and GMP diets. Values are means + SE; nos in parenthesis indicate sample size. Total ambulatory activity count showed significant treatment effects for both sex ($P = 0.0358$) and diet ($P = 0.0405$); greater total ambulatory activity count was seen in AA-fed mice vs. casein-fed mice and female mice vs. male mice. A significant sex effect was seen for total horizontal activity count ($P = 0.0390$) where female mice had a higher total horizontal activity count vs. male mice. * $p < 0.05$ for sex treatment effect. Means without a superscripted letter^{a,b} in common are significantly different ($P < 0.05$) for diet treatment effect.

Table 1
Concentrations and ratios of amino acids in plasma of WT and PKU mice fed diets containing casein, GMP or amino acids

Amino Acid	WT Mice			PKU Mice			ANOVA P Value	
	Casein	Amino Acid	GMP	Casein	Amino Acid	GMP	Genotype	Diet
N	15	13	13	14	15	14		
Phenylalanine	51 ± 2 ^c	36 ± 4 ^d	41 ± 4 ^d	2,103 ± 92 ^a	722 ± 26 ^b	766 ± 18 ^b	<0.0001	<0.0001
Tyrosine	102 ± 11	57 ± 12	71 ± 17	50 ± 7	22 ± 3	19 ± 3	<0.0001	<0.0001
Tryptophan	61 ± 4	75 ± 10	72 ± 7	64 ± 25	52 ± 4	65 ± 4	0.0033	0.0040
Leucine	162 ± 12	113 ± 11	148 ± 11	186 ± 20	134 ± 10	127 ± 5		0.0003
Isoleucine	110 ± 8 ^{cd}	83 ± 9 ^d	243 ± 30 ^a	134 ± 12 ^c	95 ± 7 ^{cd}	185 ± 24 ^b	<0.0001	0.0355
Valine	315 ± 23	219 ± 17	401 ± 42	352 ± 28	227 ± 14	333 ± 31		<0.0001
Histidine	73 ± 3	67 ± 4	81 ± 14	82 ± 4	69 ± 5	76 ± 2		
Threonine	317 ± 23	409 ± 53	971 ± 125	305 ± 24	384 ± 37	807 ± 98		<0.0001
BCAA	587 ± 42	415 ± 32	792 ± 80	672 ± 60	456 ± 28	644 ± 55		<0.0001
Phe/LNAA	0.044 ± 0.002 ^d	0.036 ± 0.004 ^d	0.024 ± 0.004 ^e	0.645 ± 0.015 ^a	0.428 ± 0.013 ^b	0.339 ± 0.023 ^c	<0.0001	<0.0001
Tyr/LNAA	0.083 ± 0.006 ^a	0.054 ± 0.010 ^b	0.038 ± 0.007 ^c	0.015 ± 0.002 ^d	0.013 ± 0.002 ^d	0.008 ± 0.001 ^d	<0.0001	0.0232
Trp/LNAA	0.054 ± 0.004 ^{ab}	0.074 ± 0.010 ^a	0.041 ± 0.006 ^{bc}	0.022 ± 0.010 ^d	0.031 ± 0.002 ^c	0.029 ± 0.003 ^c	<0.0001	0.0009
BCAA/AAA	4.00 ± 0.24	5.58 ± 0.76	8.79 ± 1.27	0.31 ± 0.02	0.61 ± 0.03	0.84 ± 0.08	<0.0001	<0.0001

Values are means ± SE in μmol/l; WT, wild type; PKU, phenylketonuria; BCAA, branched chain amino acid; LNAA, large neutral amino acid; AAA, aromatic amino acid. There was a significant genotype effect where WT mice had higher plasma concentrations of tyrosine and tryptophan compared with PKU mice. WT mice had a higher BCAA to AAA ratio than PKU mice. GMP resulted in higher plasma tryptophan than casein. Casein had higher plasma concentrations of leucine and tyrosine than both the GMP and amino acid diets. Both casein and GMP resulted in higher plasma valine and BCAA compared to the amino acid diet. The GMP diet had higher plasma threonine than both the casein and amino acid diets. The GMP diet had the significantly greatest BCAA to AAA ratio, and the amino acid diet had a significantly higher BCAA to AAA ratio than the casein diet. There was a significant genotype by diet interaction for phenylalanine and isoleucine. There was also a significant genotype by diet interaction for the ratio of phenylalanine to LNAA, the ratio of tyrosine to LNAA, and the ratio of tryptophan to LNAA.

^{a,b,c,d,e} Means in a row with superscripted letters without a common letter differ. $P < 0.05$. BCAA, sum of isoleucine, leucine, and valine. LNAA, sum of phenylalanine, tyrosine, tryptophan, leucine, isoleucine, valine, histidine, and threonine. AAA, sum of phenylalanine and tyrosine. P values not listed are nonsignificant.

Table 2
Brain catecholamine concentrations in male WT and PKU mice fed casein, amino acid, and GMP diets

Catecholamine	Male WT Mice			Male PKU Mice			ANOVA <i>P</i> Value	
	Casein	Amino Acid	GMP	Casein	Amino Acid	GMP	Genotype	Diet
<i>N</i>	11	11	10	11	8	8		
NE	177±10 ^{bc}	218±18 ^a	189±10 ^{ab}	142±10 ^c	180±12 ^b	158±13 ^{bc}	0.0022	0.0123
EPI	15.3±1.7 ^a	15.7±2.4 ^a	15.2±3.2 ^a	6.00±0.56 ^b	5.10±0.58 ^b	4.45±0.52 ^b	<0.0001	
DOPAC	36.6±3.3 ^a	33.4±5.6 ^{ab}	35.3±4.7 ^a	26.1±3.0 ^{ab}	21.8±3.82 ^a	28.9±3.2 ^{ab}	0.0080	
DA	297±23 ^{bc}	360±24 ^a	347±23 ^{ab}	270±15 ^c	300±24 ^{abc}	294±13 ^{bc}	0.0109	
HVA	63.3±4.3 ^b	76±3.9 ^a	68±6.4 ^{ab}	43.1±2.4 ^c	44.3±3.9 ^c	42.5±2.7 ^c	<0.0001	

Values are means ± SE in ng/g brain weight; n, no. of mice; WT, wild type; PKU, phenylketonuria; NE, norepinephrine; EPI, epinephrine; DOPAC, 3,4-Dihydroxyphenylacetic acid; DA, dopamine; HVA, Homovanillic acid. There was a significant genotype effect where WT mice had higher brain concentrations of the five catecholamine neurotransmitters measured compared with PKU mice. The amino acid diet resulted in higher concentrations of norepinephrine compared with casein.

a,b,c, Means in a row with different superscripted letters are significantly different, *P* <0.05. *P* values not listed are nonsignificant.

Table 3
Brain catecholamine concentrations in female WT and PKU mice fed casein, amino acid, and GMP diets.

Catecholamine	Female WT Mice			Female PKU Mice			ANOVA P Value	
	Casein	Amino Acid	GMP	Casein	Amino Acid	GMP	Genotype	Diet
N	9	7	5	11	7	5		
NE	153±16 ^{ab}	183±11 ^a	162±19 ^a	102±3.9 ^c	120±12 ^{bc}	120±14 ^{bc}	<0.0001	
EPI	77±14 ^a	67±17 ^a	53±16.5 ^{ab}	31.6±3.1 ^{bc}	26.6±2.6 ^c	30.6±3.3 ^{bc}	<0.0001	
DOPAC	27.5±2.8 ^{ab}	31.8±6.7 ^a	22.2±5.8 ^{abc}	14.2±1.7 ^c	12.1±1.1 ^c	16.6±4.7 ^{bc}	0.0002	
DA	308±31 ^{ab}	362±26 ^a	299±10 ^{abc}	251±14 ^{bc}	237±18.2 ^c	259±26 ^{bc}	0.0005	
HVA	60±7.3 ^{ab}	68±5.9 ^a	48.4±6.9 ^{bc}	39.5±4.3 ^c	32.9±3.2 ^c	37.6±2.9 ^c	<0.0001	

Values are means ± SE in ng/g brain weight; n, no. of mice; WT, wild type; PKU, phenylketonuria; NE, norepinephrine; EPI, epinephrine; DOPAC, 3,4-Dihydroxyphenylacetic acid; DA, dopamine; HVA, Homovanillic acid. There was a significant genotype effect where WT mice had higher brain concentrations of the five catecholamine neurotransmitters measured compared with PKU mice.

^{a,b,c} Means in a row with different superscripted letters are significantly different, $P < 0.05$. P values not listed are nonsignificant.