Chapter 5

C57Bl/6 Pah-enu2 PKU mice show motor deficits, altered exploration behavior, and increased depression-like behavior associated with reduced brain concentrations of monoaminergic neurotransmitters

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In preparation
Abstract

In phenylketonuria (PKU) research, the development of the genetic Pah-enu2 PKU mouse model has greatly increased pathophysiological knowledge. Recently, we found that C57Bl/6 PKU mice do not display behavioral phenotypes reflecting learning and memory deficits (manuscript under revision). In the current study, behavioral phenotypes reflecting mood and motor function of C57Bl/6 PKU mice were investigated in relation to brain concentrations of monoaminergic neurotransmitters. We hypothesized that C57Bl/6 PKU mice would show several behavioral phenotypes reflecting mood and motor function impairments compared to C57Bl/6 wild type (WT) mice, paralleled by reduced brain concentrations of monoaminergic neurotransmitters known to induce such phenotypes. In line with this hypothesis, PKU mice displayed an increase in time spent in the corners of the open field test (WT 239 ± 32 s vs. PKU 301 ± 31 s, p<0.001), increases of the number of steps and number of slips in the balance beam (WT 39 ± 3 vs. PKU 54 ± 4, p<0.001 and WT 5 ± 4 vs. PKU 27 ± 10, p<0.001, respectively), increased open arm times and open arm entries in the elevated plus maze (WT 23 ± 14 s vs. PKU 73 ± 38 s, p=0.003 and WT 2 ± 2 vs. PKU 5 ± 2, p=0.024, respectively), and increased immobility times in the forced swim test (WT 133 ± 27 s vs. PKU 194 ± 32 s, p<0.001). These behavioral phenotypes were paralleled by reduced brain concentrations of dopamine, noradrenaline, and serotonin in PKU mice (p<0.001 for all compared to WT mice). In conclusion, C57Bl/6 PKU mice showed behavioral phenotypes reflecting mood and motor function impairments, which were associated with reduced brain concentrations of monoaminergic neurotransmitters. Further studies are needed to investigate the associated biochemical and molecular pathways in further detail, and to assess the effects of intervening in these pathways.
Introduction

Phenylketonuria (PKU; OMIM 261600) is an inborn error of amino acid metabolism, caused by homozygosity for mutations in the gene encoding phenylalanine hydroxylase (PAH; EC 1.14.16.1), which leads to impaired activity of the corresponding enzyme. This impairment results in elevated blood phenylalanine (Phe) concentrations and low to normal blood tyrosine (Tyr) concentrations. Clinically, the main hallmark of untreated PKU is severe mental retardation, which can be prevented by timely diagnosis using neonatal screening followed by early and continuous treatment. PKU treatment is primarily diet-based, aiming to lower blood Phe concentrations by limiting Phe intake. This limitation of Phe intake is achieved by restricting natural protein intake, which is combined with Phe-free amino acid mixtures to prevent nutritional deficiencies (1,2).

The pathophysiology of cognitive dysfunction in PKU is not fully understood. Cognitive outcome in PKU strongly relates to blood Phe concentrations (1). Several studies have provided evidence for a role of altered blood-to-brain transport of large neutral amino acids (LNAAs) in PKU pathophysiology (as reviewed in e.g. 1,3,4). The LNAAs compete for transport across the blood-brain barrier (BBB) mediated by the LNAA type I transporter (5-7). In decreasing order of affinity for this transporter, the LNAAs are Phe, tryptophan, leucine, methionine, isoleucine, Tyr, histidine, valine, and threonine (8). Elevated blood Phe concentrations are considered to concomitantly increase Phe transport and decrease transport of non-Phe LNAAs from blood to brain. The disruption of LNAA BBB transport in PKU is believed to result in both elevated brain Phe concentrations and reduced brain concentrations of non-Phe LNAAs. These brain biochemical alterations likely affect several neurodevelopmental processes, including cerebral protein synthesis, monoaminergic neurotransmitter metabolism (as reflected by reduced brain concentrations of dopamine, noradrenaline, and serotonin), and glutamatergic signaling (3,4,9).

Many insights on PKU pathophysiology have been derived from the genetic Pah-enu2 PKU mouse model (10). In this mouse model, PAH deficiency results from homozygosity for the enu2 missense mutation in the Pah gene. The Pah-enu2 PKU mouse model highly resembles PKU in humans on several biochemical and neurodevelopmental levels (10). The Pah-enu2 mutation was first described on the BTBR background in 1993 (11) and has been most widely studied in this strain. In 2006, the Pah-enu2 mutation was crossed into the C57Bl/6 background, to increase breeding efficacy in the context of gene therapy studies (12). While several studies have investigated behavioral phenotypes of BTBR PKU mice (13-17), such phenotyping studies have only scarcely been reported for C57Bl/6 PKU mice (18). This lack of behavioral phenotyping studies in C57Bl/6 PKU mice is relevant, as the
C57Bl/6 strain is used to study a wide array of behavioral phenotypes, including those reflecting learning and memory (19-22).

Recently, we investigated several behavioral phenotypes reflecting learning and memory in C57Bl/6 PKU mice (under revision). Contrary to our hypotheses, C57Bl/6 PKU mice did not display behavioral learning and memory deficits compared to C57Bl/6 wild type (WT) mice in the studied paradigms. The experiments of the current manuscript aimed to further characterize C57Bl/6 PKU mice on a behavioral level. To this aim, we studied phenotypes reflecting exploration behavior, motor function, anxiety-like behavior, and depression-like behavior (hereafter referred to as “non-learning behavioral phenotypes”), in relation to brain concentrations of monoaminergic neurotransmitter markers. We hypothesized that C57Bl/6 PKU mice would show several non-learning behavioral phenotypes associated with reduced brain concentrations of monoaminergic neurotransmitters compared to C57Bl/6 WT mice.

Animals, materials and methods

Animals

A breeding colony of C57Bl/6 Pah-enu2 PKU mice was established, using heterozygous (HTZ) founders kindly provided by Prof. B. Thöny (University of Zürich, Zürich, Switzerland). Mice were weaned at age 3-4 weeks and housed in filter top cages, which contained a layer of sawdust and cage enrichment material. At the same age, genotyping was performed by qPCR, as described below. Mice were maintained on a 12 h light/dark cycle (lights on at 8.00 a.m.). Standard mouse chow (RMH-B 2181, AB diets, Woerden, the Netherlands) and water were available ad libitum. Non-learning behavioral phenotypes were investigated in several mouse cohorts, as described in Table 1. The reported experiments were approved by the Animal Ethical Committee.

Genotyping

Mice were genotyped by qPCR, using DNA extracted from tail tissue. The qPCR master mix consisted of 10x PCR buffer, 50 mM MgCl$_2$, 5 mM dNTP-mix, 5 µM FAM, 5 µM Yakima Yellow, and 5 U/µl Hot Goldstar polymerase (all obtained from Eurogentec, Seraing, Belgium) in ddH$_2$O. Forward primer and reverse primer were 5’ CCGTCCTGTTGCTGGCTTAC 3’ and 3’ CTAGATTCGGGTACATGTGTGGAC 5’, respectively (both obtained from Invitrogen, Carlsbad, USA). Samples were analyzed in triplicates.
Behavioral paradigms
Open field test

Exploration behavior was assessed in an open field test (20,23) using a square arena (50 x 50 cm). The arena was divided into a center zone, four border zones, and four corner zones, as depicted in Figure 1A. Open field testing was performed for 10 min. Exploration behavior was quantified as the time spent in different zones, the average exploring velocity, and the total distance moved.

Figure 1 Open field test set-up and results. A) Open field arena (50 x 50 cm), with center zone (30 x 30 cm), border zones (30 x 10 cm), and corner zones (10 x 10 cm). B) Exploration times in each open field area. Data are shown as mean ± SEM. WT: wild type, PKU: phenylketonuric. * WT vs. PKU p<0.05.
**Hanging wire test**

The hanging wire test assesses sustained muscle strength (23). The set-up consisted of a steel bar (length 100 cm, diameter 2 mm), positioned horizontally between two vertical supports, 58 cm above the underlying surface. Paper towels placed underneath the bar served to prevent injuries if mice would fall off the wire. Mice were placed on the wire in a vertical position, with their forepaws containing the wire. Hanging wire performance was scored as the latency time to fall off the wire and behavior during the test, defined as 1) hanging on the wire using both forepaws, 2) hanging on the wire using both forepaws while attempting to climb it, 3) hanging on the wire with both forepaws and one or both hind paws, 4) hanging on the wire with all paws and the tail. For each mouse, two trials were performed consecutively. Latency time to fall was averaged between these two trials. Behavior during the hanging wire test was analyzed as behavior in each trial and as the best behavioral score of both trials.

**Balance beam**

The balance beam assesses coordination and balance (24,25). The balance beam set-up consisted of a wooden beam (length 1 m, cross section 5 x 10 mm), positioned horizontally 50 cm above the underlying surface. One end of the beam was connected to a wooden safe cage (20 x 20 cm) filled with sawdust. Paper towels were placed below the beam, in order to prevent injuries in case mice would fall off. Habituation to the balance beam was done as follows. First, mice were placed inside the safe cage for 60 s. Second, three habituation sessions were performed, in which mice were positioned at 10, 40, and 75 cm from the safe cage, respectively. After these habituation sessions, a read-out trial was performed, in which mice were challenged to reach the safe cage, starting at a 100 cm distance. Balance beam performance was quantified as crossing time, number of steps taken, and number of slips made.

**Elevated Plus Maze**

The Elevated Plus Maze (EPM) was used to assess anxiety-like behavior (26,27). This paradigm consisted of four arms arranged in a plus shape, located 50 cm above the ground. Two of the arms were open, while the other two arms contained plastic boxes, which were closed from all sides except the front side. Exploratory behavior in this maze was scored independently by two observers during 8 min. The following parameters were recorded: the number of open arm entries and closed arm entries, the time spent in the open arms and closed arms, and the number of crossings from one arm type to another. An increase in the time spent in the closed
arms and/or an increased number of entries in the closed arms are consistent with more anxiety-like behavior (26,27).

**Forced Swim Test**

The Forced Swim Test (FST) paradigm was used to assess depression-like behavior (28,29). In this paradigm, mice were placed in a 2000 ml cylinder containing 1700 ml of 24 °C tap water for 6 min. Mice typically start swimming in response to this challenge. During the test, swimming behavior gradually diminishes, and mice remain immobile for certain periods of time. An increase in immobility time is consistent with more depression-like behavior (28,29). FST performance was scored independently by two observers.

**Biochemical measurements**

**Blood and brain LNAA concentrations**

Blood and brain tissue were obtained for cohort 3 only (described in Table 1). For blood and brain tissue collection, mice were first anesthetized with isoflurane. Next, full blood samples were obtained by cardiac puncture, transferred to heparinized tubes, and centrifuged (14,000 rpm x 10 min). After centrifugation, plasma supernatants were transferred to new vials, and stored at -80 °C. Cerebral tissue was extracted after rapid decapitation directly after cardiac puncture, snap frozen in liquid nitrogen, and stored at -80 °C. Shortly before brain amino acid concentration measurements, brain tissue was grinded in liquid nitrogen, using a mortar and pestle. Brain homogenates were obtained by first adding phosphate-buffered saline (PBS, pH 7.4) to brain tissue samples, using a 1:4 weight to volume ratio (mg:µl). Next, brain tissue samples were sonified (30 s per sample at 11-12 W).

**Table 1 Mouse cohorts and corresponding behavioral paradigms and biochemical analyses.**

<table>
<thead>
<tr>
<th>Cohort</th>
<th>Composition</th>
<th>Gender</th>
<th>Age (months)</th>
<th>Parameter</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>16 WT</td>
<td>8 M, 8 F</td>
<td>4 – 5</td>
<td>Open field test</td>
</tr>
<tr>
<td></td>
<td>16 PKU</td>
<td>8 M, 8 F</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>5 WT</td>
<td>1 M, 4 F</td>
<td>2 – 13</td>
<td>Hanging wire</td>
</tr>
<tr>
<td></td>
<td>18 HTZ</td>
<td>14 M, 4 F</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>8 PKU</td>
<td>1 M, 7 F</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>8 WT</td>
<td>2 M, 6 F</td>
<td>5 – 6</td>
<td>Balance beam</td>
</tr>
<tr>
<td></td>
<td>8 PKU</td>
<td>2 M, 6 F</td>
<td></td>
<td>Blood and brain LNAAas</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>Brain NTs</td>
</tr>
<tr>
<td>4</td>
<td>8 WT</td>
<td>4 M, 4 F</td>
<td>8 – 14</td>
<td>Elevated Plus Maze</td>
</tr>
<tr>
<td></td>
<td>8 PKU</td>
<td>4 M, 4 F</td>
<td></td>
<td>Forced Swim Test</td>
</tr>
</tbody>
</table>

and centrifuged (12,800 rpm x 10 min), after which brain homogenates were transferred to new vials. Blood and brain amino acid concentrations were determined using high-performance liquid chromatography (HPLC) coupled to derivatization with ninhydrin, according to the manufacturer’s protocol (Pharmacia Biotech, Cambridge, UK).

**Brain monoaminergic neurotransmitter concentrations**

Brain dopaminergic and serotonergic pathways were analyzed by determining brain homogenate concentrations of dopamine (DA), noradrenaline (NA), serotonin (5-HT), and 5-hydroxyindoleacetic acid (5-HIAA), hereafter collectively referred to as brain monoaminergic neurotransmitter markers. These neurotransmitter markers were measured in brain homogenates obtained as described above, with the following modifications. For dopaminergic pathway markers, glutathione (80 g/L in 0.08 M acetic acid, volume ratio 1:20) rather than PBS was used as the homogenization buffer. 2,3-dihydroxybenzoic acid served as an internal standard. DA and NA concentrations were determined using HPLC coupled to electrochemical detection (Thermo Scientific, Waltham, MA, USA). For serotonergic pathway markers, 0.08 M acetic acid rather than PBS was used as the homogenization buffer, at the same weight:volume ratio as used for PBS. A solution of ascorbic acid (250 g/L), EDTA (104 g/L), and sodium metabisulphite (104 g/L) in ddH₂O was used to suppress oxidation (volume ratio 2:1:1:6). 5-methyltryptophan served as an internal standard. 5-HT and 5-HIAA concentrations were determined by HPLC coupled to fluorometric detection (Waters, Milford, MA, USA).

**Statistical analyses**

Normality of variable distribution and homogeneity of variance were tested using the Shapiro-Wilk test and Levene’s test, respectively. Normally distributed variables with homogeneity of variance were analyzed using Student’s t-test, ANOVA testing, and MANOVA testing as indicated. Continuous variables not meeting these criteria were analyzed using Mann-Whitney U testing and Kruskal-Wallis testing as indicated. Categorical variables were analyzed by Fisher’s exact test. A two-sided p-value <0.05 was considered to reflect statistical significance.
Results

**Behavioral paradigms**

**Open field test**

Figure 1B shows exploration behavior results in the open field test. Two-way independent MANOVA, using time spent in the center zone, time spent in the border zones, and time spent in the corner zones as outcome parameters, with genotype and gender as factors, showed a main effect of genotype (F=11.866, p<0.001), with a statistical trend towards a main effect of gender (F=2.834, p=0.058). There was no interaction effect between genotype and gender (F=0.804, p=0.503). Follow-up ANOVA testing revealed that genotype affected exploration times in the center zone, in the border zones, and in the corner zones (p<0.001, p=0.008 and p<0.001, respectively). Both the average exploring velocity and the total distance moved were not different between genotype groups (F=2.398, p=0.110 in two-way MANOVA, data not shown).

**Hanging wire test**

Latency times to fall were 56 ± 25 s for WT mice, 44 ± 34 s for HTZ mice, and 43 ± 28 s for PKU mice. These data were analyzed by three-way independent ANOVA, with mean latency time as outcome variable, and genotype, gender and age (categorized as <30 weeks or ≥30 weeks) as factors. The observed ANOVA model showed that none of the factors influenced mean latency time (F=1.170, p=0.149). Behavioral scores during trial 1, behavioral scores during trial 2, and best behavioral scores of both trials did not differ between WT, HTZ, and PKU mice (p=0.553, p=0.321, and p=0.589 respectively, using Fisher’s exact test).

**Balance beam**

Two-way independent MANOVA, using crossing time, number of steps, and number of slips as outcome parameters, with genotype and gender as factors, showed a main effect of genotype (F=16.512, p<0.001). Gender did not have a main effect (F=0.114, p=0.950). There was no interaction effect between genotype and gender (F=0.064, p=0.978). Post-hoc testing revealed that mean crossing times of PKU mice and WT mice did not differ (WT 20 ± 10 s vs. PKU 17 ± 4 s, p=0.482). Figure 2 shows the number of steps and the number of slips during balance beam testing in each genotype group. Mean values of both parameters were higher in PKU mice compared to WT mice (p<0.001 for both).
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Figure 2 Balance beam performance for number of steps (left panel) and number of slips (right panel). Data are shown as mean ± SEM. WT: wild type, PKU: phenylketonuric. *** WT vs. PKU p<0.001.

Elevated Plus Maze

Table 2 shows EPM behavior in WT and PKU mice. Compared to WT mice, PKU mice spent more time in the open arms and showed a higher number of open arm entries (p=0.003 and p=0.024 using Student’s t-test, respectively). Additional EPM parameters did not differ significantly between genotype groups.

Table 2 Elevated Plus Maze behavior in wild type mice and phenylketonuric mice.

<table>
<thead>
<tr>
<th></th>
<th>WT (n=8)</th>
<th>PKU (n=8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Open arm time (s)</td>
<td>23 ± 14</td>
<td>73 ± 38*</td>
</tr>
<tr>
<td>Closed arm time (s)</td>
<td>395 ± 29</td>
<td>357 ± 53</td>
</tr>
<tr>
<td>Center time (s)</td>
<td>63 ± 39</td>
<td>50 ± 27</td>
</tr>
<tr>
<td>Open arm entries</td>
<td>2 ± 2</td>
<td>5 ± 2*</td>
</tr>
<tr>
<td>Closed arm entries</td>
<td>17 ± 4</td>
<td>19 ± 4</td>
</tr>
<tr>
<td>Total arm entries</td>
<td>19 ± 5</td>
<td>23 ± 5</td>
</tr>
</tbody>
</table>

Exploration times and number of arm entries are given as mean ± SD. WT: wild type, PKU: phenylketonuric. * p<0.05 vs. WT.

Forced Swim Test

Figure 3 shows the immobility times in the FST. Data from both genders were
pooled, as gender did not significantly influence immobility time. PKU mice had higher immobility times than WT mice (p<0.001 using Student’s t-test).

**Figure 3** Immobility times in the forced swim test. Dotted lines represent group mean. WT: wild type, PKU: phenylketonuric. * WT vs. PKU p<0.05.

**Biochemical measurements**

**Blood and brain LNAA concentrations**

Table 3 shows blood and brain LNAA concentrations of WT and PKU mice (determined for cohort 3 only, i.e. the cohort in which balance beam testing was done). Compared to WT mice, PKU mice had higher blood Phe concentrations, lower blood Tyr concentrations, and lower blood methionine concentrations (p=0.001, p=0.020, and p=0.031, respectively). Mean blood concentrations of the additional LNAAAs did not differ between WT and PKU mice (p-values shown in Table 3). Brain LNAA concentration measurements showed that, compared to WT mice, PKU mice had higher brain Phe concentrations (p<0.001). These higher brain Phe concentrations were paralleled by reduced brain concentrations of threonine,
valine, methionine, isoleucine, leucine, and tyrosine (p<0.001, p<0.001, p<0.001, p=0.003, p=0.002, and p<0.001, respectively). Mean brain concentrations of these non-Phe LNAAs in PKU mice were decreased by ~10-40% compared to mean WT concentrations. Mean brain histidine concentrations were higher in PKU mice than in WT mice (p<0.001), while mean brain tryptophan concentrations did not differ between genotype groups (p=0.194).

| Table 3 | Blood LNAA concentrations (µmol/L), brain LNAA concentrations (nmol/g wet weight) and brain neurotransmitter marker concentrations (nmol/g wet weight) per experimental group. |
|------------------|------------------|------------------|------------------|
|                  | WT (n=8)         | PKU (n=8)        | p                |
| **Blood LNAA**   |                  |                  |                  |
| Threonine        | 249 ± 116        | 215 ± 34         | 0.505            |
| Valine           | 216 ± 71         | 220 ± 64         | 0.920            |
| Methionine       | 25 ± 14          | 56 ± 27          | 0.031            |
| Isoleucine       | 86 ± 28          | 76 ± 12          | 0.436            |
| Leucine          | 163 ± 63         | 144 ± 25         | 0.483            |
| Tyrosine         | 107 ± 35         | 64 ± 36          | 0.020            |
| Histidine        | 95 ± 33          | 101 ± 19         | 0.240            |
| Tryptophan       | n.m.             | n.m.             | n.a.             |
| Phenylalanine    | 84 ± 28          | 1663 ± 133       | 0.001            |
| **Brain LNAA**   |                  |                  |                  |
| Threonine        | 86 ± 5           | 64 ± 16          | <0.001           |
| Valine           | 19 ± 2           | 13 ± 2           | <0.001           |
| Methionine       | 19 ± 1           | 14 ± 1           | <0.001           |
| Isoleucine       | 10 ± 2           | 8 ± 1            | 0.003            |
| Leucine          | 42 ± 2           | 38 ± 3           | 0.002            |
| Tyrosine         | 30 ± 3           | 19 ± 4           | <0.001           |
| Histidine        | 22 ± 1           | 30 ± 2           | <0.001           |
| Tryptophan       | 36 ±10           | 28 ± 13          | 0.194            |
| Phenylalanine    | 25 ± 1           | 181 ± 21         | <0.001           |
| **Brain NT markers** |              |                  |                  |
| Dopamine         | 12.14 ± 1.45     | 7.81 ± 0.87      | <0.001           |
| Noradrenaline    | 2.782 ± 0.223    | 1.425 ± 0.188    | <0.001           |
| Serotonin        | 4.312 ± 0.403    | 2.003 ± 0.282    | <0.001           |
| 5-HIAA           | 1.451 ± 0.251    | 0.369 ± 0.116    | <0.001           |

Data were obtained in test cohort 3. Results are shown as mean ± standard deviation. Blood LNAA samples could not be obtained in 2/8 PKU mice. Blood methionine concentrations were below the threshold for reliable detection in 1/8 WT mouse and 2/6 PKU mice. WT: wild type, PKU: phenylketonuric, LNAA: large neutral amino acid, NT: neurotransmitter. n.m.: not measured, n.a.: not applicable.
Brain monoaminergic neurotransmitter concentrations

Table 3 shows brain concentrations of monoaminergic neurotransmitter markers. Compared to WT mice, PKU displayed reduced brain concentrations of all investigated markers (p<0.001 for all). Specifically, compared to mean concentrations in WT mice, mean concentrations in PKU mice were decreased by ~35% and ~45% for DA and NA, respectively. For 5-HT and 5-HIAA, these decreases were ~50% and ~75, respectively. Mean ratios of brain DA concentration to brain Tyr concentration were comparable between WT mice and PKU mice (WT 0.41 ± 0.05 vs. PKU 0.43 ± 0.06, p=0.584). The mean ratio of brain 5-HT concentration to brain Trp concentration was lower in PKU mice than in WT mice (WT 12.69 ± 2.79 vs. PKU 8.17 ± 2.55, p=0.004).

Discussion

This study investigated non-learning behavioral phenotypes of C57Bl/6 Pahenu2 PKU mice, in relation to brain concentrations of monoaminergic neurotransmitter markers. The main findings of this manuscript are that, as hypothesized, C57Bl/6 PKU mice showed non-learning behavioral phenotypes in several paradigms, which were paralleled by reduced brain concentrations of monoaminergic neurotransmitters. Specifically, the observed behavioral phenotypes consisted of increased time spent in the corner zones of the open field test, increases of the number of steps and number of slips in the balance beam paradigm, increases of open arm entries and open arm time in the EPM, and increased immobility times in the FST.

The observed biochemical findings in the current manuscript are in line with previously published data in C57Bl/6 PKU mice, both in terms of which parameters are affected and the relative changes of these parameters (18,30-33). Specifically, this similarity concerns elevated blood and brain Phe concentrations, reduced brain concentrations of most non-Phe LNAAs, and reduced brain concentrations of monoaminergic neurotransmitter markers in C57Bl/6 PKU mice. In addition, as previously reported by us and others (18,31,32), the relative reduction of mean brain 5-HT concentrations in C57Bl/6 PKU mice compared to mean WT concentrations exceeded the reduction of mean brain DA concentrations. Together, these findings suggest that several biochemical phenotypes of C57Bl/6 PKU mice are robust across different testing conditions and scientific groups.

Most of the non-learning behavioral phenotypes of C57Bl/6 PKU mice described in this study have not been investigated previously. To our knowledge, of these phenotypes, only locomotor activity has been reported by others (18). In that study, C57Bl/6 PKU mice showed increased locomotor activity, which contrasts with the similar locomotor activity parameters in PKU mice and WT mice currently
observed. Possibly, the apparent phenotype difference between both studies relates to the relatively long testing period in the recently published study. If so, the translational value of those locomotor activity data may be limited.

Our findings underscore the association between behavioral phenotypes of disturbed mood and impaired motor function with reduced serotonergic and/or dopaminergic signaling, as reported previously (28,29,34-38). In the open field test, C57Bl/6 PKU mice spent more time in the corner zones than WT mice. This phenotype is consistent with increased anxiety-like behavior (19,20), which is likely associated with reduced brain serotonergic signaling (39). In the balance beam test, PKU mice displayed both a higher number of steps and a higher number of slips. These phenotypes are consistent with impairments of coordination and balance (thus reflecting motor deficits) (34,35,37), as reported in mice with reduced dopaminergic signaling in the basal ganglia (35,37). In the EPM, both the number of open arm entries and the open arm time were higher in PKU mice. These observations contradicted our expectations, as this behavior is consistent with decreased (rather than increased) anxiety-like behavior (19,20,34,40). Possibly, the observed EPM phenotype may relate to the simultaneous reductions of brain 5-HT and brain DA concentrations. As DA is involved in reward-mediated signaling (41,42), it may be hypothesized that such signaling is impaired in C57Bl/6 PKU mice. If this holds true, the observed EPM phenotype may reflect insufficient reward perception induced by visiting the EPM closed arms. Contrary to this possible role of reward in the EPM, reward-related signaling may not be as important for the behavioral phenotypes observed in the open field test. This interpretation could explain why an anxiety-like phenotype was observed in the open field, but not in the EPM. In the FST, PKU mice showed higher mean immobility times than WT mice. This phenotype may reflect increased depression-like behavior (23,28,29) and/or reflect motor deficits (28), and may thus relate to reduced signaling of the serotonergic and/or dopaminergic systems in the brain (23,28,29,35,37).

Contrary to the aforementioned behavioral phenotype differences between C57Bl/6 PKU mice and C57Bl/6 WT mice, hanging wire performance and balance beam crossing time did not differ between PKU mice and WT mice. These parameters both relate to brain dopaminergic signaling (23,35,37,43,44). Possibly, in order to additionally observe such phenotypes, brain dopamine concentrations should be reduced to an even greater extent. This idea is supported by previous studies showing that brain dopaminergic signaling reductions of at least 60% may be needed to increase balance beam crossing time (35,37).

When comparing behavioral phenotypes of learning and memory in C57Bl/6 PKU mice to non-learning behavioral phenotypes in these mice, the question arises
why C57Bl/6 PKU mice are spared from behavioral learning and memory deficits, but not from several non-learning behavioral impairments. Recently, we found that C57Bl/6 PKU mice do not show behavioral phenotypes reflecting learning and memory deficits. In behaviorally tested C57Bl/6 PKU mice, these intact behavioral phenotypes are associated with normalized pCREB/CREB ratios compared to home cage control C57Bl/6 PKU mice (under revision). These findings suggest that the arousal associated with behavioral testing, may lead to increased phosphorylation of CREB, a neuronal marker critically involved in learning and memory formation. In this context, it should be noted that the biochemical phenotypes currently observed in behaviorally tested C57Bl/6 PKU mice are highly similar to those of home cage control C57Bl/6 PKU in our recent study. Together, these findings suggest that C57Bl/6 PKU mice maintain learning and memory performance by upregulating neuronal signaling pathways, rather than by restoring brain concentrations of amino acids and/or neurotransmitters. In non-learning behavioral challenges, such an upregulation of associated neuronal signaling pathways may not sufficiently occur, thus leading to the behavioral phenotypes reported in this study.

The current observations may have relevance for both fundamental research and clinical practice in PKU for several reasons. First, the finding that certain behavioral parameters are affected in C57Bl/6 PKU mice whereas others are not, suggests that different biochemical and/or molecular pathways may be involved in mediating the observed phenotypes. Increased knowledge of these pathways may contribute to understanding the pathophysiology of cognitive dysfunction in PKU. Second, this manuscript identified various non-learning behavioral phenotypes of C57Bl/6 PKU mice, which may serve as read-out parameters in future treatment-oriented studies in this mouse model. Third, the consideration that the currently observed phenotypes seem to be particularly related to impaired monoaminergic neurotransmitter signaling is relevant from a translational perspective. In early and continuously treated PKU patients, impaired neurotransmitter signaling appears to affect cerebral functioning, as reflected by executive function deficits and mood disturbances (45-47). Thus, the C57Bl/6 PKU mouse model may be well-suited for studying therapeutic interventions aiming to improve mental outcome in early and continuously treated PKU patients.

Future studies should validate the currently reported behavioral findings. This validation appears to be indicated, particularly considering the variations in group size, gender, and age between the cohorts in this manuscript. If the observed behavioral phenotypes prove to be robust, follow-up studies may aim to identify the neuroanatomical, neurochemical, and neuromolecular parameters associated with the currently observed behavioral phenotypes in C57Bl/6 PKU mice. Moreover,
comparative studies between C57Bl/6 PKU mice and BTBR PKU mice, both regarding learning and memory phenotypes and non-learning phenotypes, may increase pathophysiological understanding. Finally, future intervention studies should attempt to restore neurological phenotypes of PKU mice at several levels.

In summary, we investigated non-learning behavioral phenotypes in C57Bl/6 PKU mice in relation to brain concentrations of monoaminergic neurotransmitters. In line with our hypothesis, C57Bl/6 PKU mice displayed behavioral phenotypes in most paradigms, paralleled by reduced brain concentrations of monoaminergic neurotransmitters, which are known to induce such behavioral phenotypes. The observed non-learning behavioral phenotype differences between C57Bl/6 PKU mice and C57Bl/6 WT mice contrast with behavioral phenotypes reflecting learning and memory in C57Bl/6 PKU mice, which we recently showed to be comparable to those of WT mice (under revision). Future studies are indicated to investigate which processes underlie the observed non-learning behavioral phenotypes, and how these processes may be targeted by interventional studies. Thus, the current manuscript may provide a stepping stone for the development of treatments aimed at improving therapeutic outcome and quality of life in PKU patients.

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