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Hippocampal microglia modifications in C57Bl/6 *Pah*^{enu2} and BTBR *Pah*^{enu2} phenylketonuria (PKU) mice depend on the genetic background, irrespective of disturbed sleep patterns

Els van der Goot[a], Vibeke M. Bruinenberg[a], Femke M. Hormann[a], Ulrich L.M. Eisel[a], Francjan J. van Spronsen[b], Eddy A. Van der Zee[a,*]

[a] Groningen Institute for Evolutionary Life Sciences (GELIFES), Molecular Neurobiology, University of Groningen, Groningen, The Netherlands
[b] Division of Metabolic Diseases, Beatrix Children's Hospital, University Medical Center of Groningen, University of Groningen, Groningen, The Netherlands

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ABSTRACT

Toxic levels of phenylalanine in blood and brain is a characteristic of (untreated) phenylketonuria (PKU), leading to cognitive deficits in PKU mice. In addition, our recent findings showed that PKU mice (as well as PKU patients) have a disturbed sleep/wake cycle. As a consequence, sleep loss may contribute to cognitive deficits in PKU. Sleep loss has been linked to increased activation of microglia in the hippocampus. In this study, we set out to examine morphological features of the microglia population in the hippocampus of the mouse PKU model, using both the C57Bl/6 and the BTBR strain and their wild-type controls (age 5.3 ± 0.5 months; n = 16 per group, both males and females; n = 8 each). Microglial activation is reflected by retraction and thickening of the dendritic branches and an increase in cell body size of a microglial cell. Such morphological changes of microglia were studied by way of immunohistochemical staining for Iba-1, a microglia-specific calcium binding protein. We measured the number of microglia in seven subregions of the dorsal hippocampus. The level of microglial activation was determined, based on the ratio between the soma size and total cell size (soma size plus the area covered by the dendritic branches). Results showed subtle but statistical significant activation of hippocampal microglia in the C57Bl6, but not in the BTBR, PKU mice when compared with their wild-type controls. Also the total number of microglia was higher in the C57Bl/6 PKU (compared to the wild-type) mouse, but not in the BTBR PKU mouse. It is concluded that the C57Bl/6 PKU mouse has mildly higher microglia activity, which may support rather than hamper hippocampal homeostasis. The results further indicate that high levels of phenylalanine or disturbed sleep patterns do not consequently cause hippocampal microglial activation in the PKU mouse. It is currently unknown why the two PKU mouse strains show these differences in number and activation level of their hippocampal microglia, and to what extent it influences hippocampal functioning. Further scrutinizing the role of microglia functioning in the context of PKU is therefore warranted.

1. Introduction

PKU is caused by an inborn error of phenylalanine (Phe) metabolism, characterized by an impaired conversion of Phe to tyrosine in the liver due to severely reduced activity of the enzyme phenylalanine hydroxylase (PAH). As a result, Phe levels are very high in blood and brain, leading to disturbed neurotransmitter content and severe neurocognitive dysfunctions, seizures and psychiatric problems in (untreated) PKU patients (Blau, van Spronsen, & Levy, 2010). A *Pah*^{enu2} mouse model, based on a point mutation in the gene encoding for the enzyme PAH, was generated to allow the study of the cellular and molecular mechanisms underlying PKU-specific cognitive problems. Originally, the point mutation was performed in the black and tan brachyury (BTBR) mouse (the BTBR *Pah*^{enu2} mouse (Shedlovsky, McDonald, Symula, & Dove, 1993); hereafter referred to as BTBR PKU mouse). The BTBR PKU mouse was then crossed back on a C57Bl/6JRj background (the C57Bl6 *Pah*^{enu2} mouse; hereafter referred to as B6 PKU mouse). Today, both strains of PKU mice are being used intensively and interchangeably (see Bruinenberg, van der Goot, et al., 2016 and references therein).

We recently demonstrated that PKU patients, compared to healthy first-degree relatives, suffer more from sleep disorders, reduced sleep quality, an increased latency to fall asleep, and experience more sleepiness during the day (Bruinenberg, Gordijn, MacDonald, van Spronsen,

* Corresponding author at: Groningen Institute for Evolutionary Life Sciences (GELIFES), University of Groningen, 9747 AG Groningen, The Netherlands.
E-mail address: e.a.van.der.zee@rug.nl (E.A. Van der Zee).

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& Van der Zee, 2017). Soon thereafter, Bilder et al. (2017) also reported a sleep disturbance prevalence in adult PKU patients. In line with these findings, we showed that PKU mice of both strains have a disturbed sleep/wake cycle. PKU mice switch more often between active and non-active behavior and shift a part of their resting behavior into the active period (Bruinenberg et al., 2017). This disturbance was equally severe in both strains. Next to sleep problems, PKU mice have hippocampus-dependent cognitive deficits, (Cabib, Pascucci, Ventura, Romano, & Puglisi-Allegra, 2003; Zagreda, Goodman, Druin, McDonald, & Diamond, 1999; Pascucci, Andolina, Ventura, Puglisi-Allegra, & Cabib, 2008; Martynyuk, van Spronsen, & Van der Zee, 2010; Pascucci et al., 2013; Bruinenberg, van der Goot, et al., 2016; Bruinenberg, van Vliet, et al.,2016; Fiori et al., 2017; Winn et al., 2018). It is currently unknown to what extent the sleep problems contribute to hippocampus-dependent cognitive problems.

Hippocampal microglia seem to play a role in sleep-related dysfunction of the hippocampus. It has been postulated that microglia regulate the sleep-wake cycle dependent changes in synaptic strength through the extension and retraction of their processes (Hayashi, Koyanagi, Kusunose, Okada, et al., 2013; Hayashi, Koyanagi, Kusunose, Takayama, et al., 2013). Ample studies have linked strongly enhanced hippocampal microglia activation (neuroinflammation) to cognitive and behavioral deficits (see Calcia et al., 2016, for a recent systematic review), whereas mildly activated microglia play a crucial role in the maintenance of brain homeostasis (de Miranda et al., 2017 for review, and references therein). To study a putative role of microglia in PKU-specific cognitive deficits, we determined the degree of hippocampal microglia activation by way of morphological alterations based on immunostaining for Iba-1 in both B6 and BTBR PKU mice and their respective WT controls. It is a first step in deciphering the complex functional relationship between memory deficits and disturbed sleep patterns in the context of PKU. We hypothesized that PKU mice would show higher levels of hippocampal microglia activation than WT controls. Highest levels were expected in the BTBR mice as we previously showed that, in our hands, the BTBR PKU mouse has more PKU-specific behavioral and cognitive problems than the B6 PKU mouse (Bruinenberg, van der Goot, et al., 2016).

2. Materials and methods

2.1. Animals

A breeding colony of heterozygous mating pairs bred PKU and WT littermates from both genetic backgrounds. On postnatal day (PND) 28 the animals were weaned. After weaning, all littermates were kept in the initial cage without the mother. The cages had sawdust bedding and cage enrichment in the form of nesting material and paper rolls. The climate condition of the housing facility was kept constant at a temperature of 21 ± 1 °C, 51% ± 5 humidity, and a 12/12 light/dark cycle (lights on at 7:00 a.m.). The animals had *ad libitum* access to food (AM-II *Arie Block BV, Woerden, The Netherlands*) and water. All experimental procedures were approved by an independent ethics committee for animal experimentation (Groningen, the Netherlands) and complied with the principles of good laboratory animal care following the European Directive for the protection of animals used for scientific purposes.

2.2. Genotyping

Animals were genotyped at PND 28 (Bruinenberg, van der Goot, et al., 2016). The genotype of all individuals was established by way of quantitative PCR (forward primer: 5′ CCGTCCTGTTGCTGGCTTAC 3′, reverse primer: 3′ CAGGTGTGTACATGGGCTTAGATC 5, WT probe: CCGAGTCZZLCALTGCA, PKU probe: CCGAGTCZLLCACTGCA, aimed at exon 7 of the PAH gene (Eurogentec, Fremont, USA). DNA was extracted from ear tissue. Hence, it was assured that all Pah^{enu2} mice used in this study were homozygous for the mutation in the phenylalanine hydroxylase gene, and that WT mice did not have the mutation.

2.3. Experimental design

In total, 64 mice were used in this study, of which 32 had a BTBR background and another 32 had a C57Bl/6 (B6) background. Each group existed of 16 wt and 16 PKU mice. We used both males and females, resulting in 8 groups of 8 animals per gender and genetic background. Brains were collected at the age of 5.3 ± 0.5 months.

2.4. Immunohistochemistry

To obtain brain material, animals were transcardially perfused between Zeitgeber Time 5 and 9 with 0.1 M PBS, followed by 4% PFA in 0.1 M PBS. After dissection, brains were post-fixed in PFA for 24 h at 4 °C and stored in 0.1 M PBS, containing 1% sodium-azide, at 4 °C. For cutting sections (at a thickness of 20 μm), brains were cryoprotected with a 30% buffered sucrose solution and sections were stored in PBS with 1% sodium azide, at 4 °C. Five sections containing the dorsal hippocampus were selected for immunohistochemistry. The sections were pretreated with 0.3% H_2O_2 for 30 min. The sections were incubated for 72 h at 4 °C with 1:2500 rabbit anti-Iba-1 (Wako, Osaka, Japan) with 1% BSA and 0.1% TX, followed by 2 h incubation with 1:500 goat-anti rabbit antibodies (Jackson, West Grove, USA) in 0.01 M PBS with 1% BSA at room temperature. The sections were then incubated with avidin-biotin peroxidase complex 1:500 for 1 h at room temperature. The sections were treated with 1 mg/ml diaminobenzidine (DAB) tablets (Sigma Aldrich MDL: MFCD00007725), activated by adding 100 μL 0.1% H_2O_2 for seven minutes.

2.5. Quantification of Iba-1 immunostaining

Microglia, identified by way of Iba-1 immunostaining, represent the resident immune cells in the brain. Ramified microglia continuously scan the environment under resting conditions. When the environmental conditions change, these microglia cells can be activated and undergo morphological changes (Ulvestad et al., 1994). The branched processes or dendrites retract, causing the processes to thicken, and the size of the cell body increases. As a consequence, the ratio of cell body to cell size increases (Kreutzberg, 1996). To determine these morphological alterations, pictures of the hippocampus were taken at a 200 times magnification with a Leica DM IRB microscope and a Leica DFC320 camera (Leica, Rijswijk, the Netherlands). Three sections of the dorsal hippocampus were selected and pictures of the CA1 radiatum and oriens, Dentate gyrus inner blade and outer blade, CA3 radiatum and oriens and the Hilus were taken (see Fig. 1A) on both the left and right side of the brain, resulting in six pictures of each hippocampal subregion of each mouse. The pictures were analyzed using Image Pro Plus 6.0.0.26 for Windows software (Media Cybernetics Inc., Rockville, USA). Either the averaged number of microglia per area of interest (AOI) (Fig. 1) or the relative number was expressed, with the value of the WT controls set at 1.0 (Fig. 2). During this analysis, the researcher was blinded for the strain, genotype, and gender of each mouse. The analysis was based on the protocol described by Hovens and coworkers (Hovens, Nyakas, & Schoemaker, 2014). The AOI for each hippocampal subregion was manually set. After assessing the automated dark objects, the intensity threshold was manually set between 80 and 180 standardized gray values and the area threshold was at least 600 pixels to measure the area of the cell bodies. Each measurement was corrected for the size of the AOI. The ratio was calculated using the formula: *ratio = (area of cell bodies)/(total dark objects area) * 100%.*

2.6. Statistical analysis

Statistical analyses were performed separately for each strain using

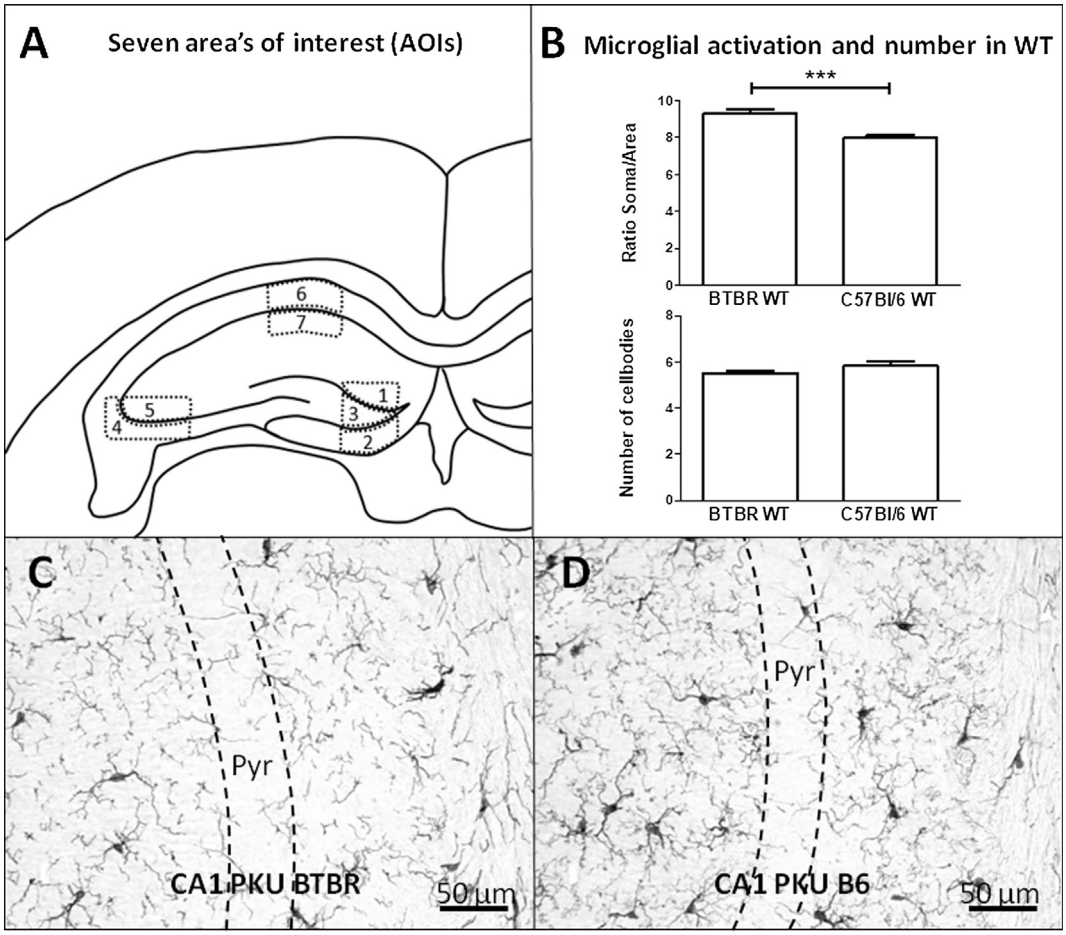

Fig. 1. The seven AOIs in the dorsal hippocampus which were sampled are schematically delineated (A; 1 = DGi, 2 = DGo, 3 = Hilus, 4 = CA3 Oriens, 5 = CA3 Radiatum, 6 = CA1 Oriens, 7 = CA1 Radiatum). Six pictures of each AOI were analyzed per animal. A comparison between WT controls of the two strains revealed a significantly higher level of microglial activation in the BTBR mice (B). Representative pictures of Iba-1 immunostaining in the BTBR and B6 PKU mouse are shown in panel C and D, respectively. Pyr = Stratum Pyramidale. Bars represent the mean ± SEM. Number of animals in each bar: BTBR: 14; B6: 13. $^{**}p \leq 0.01$.

IBM SPSS Statistics 23 for windows. The distribution of all parameters was checked with the Shapiro-Wilk normality test. Normally distributed data were tested by a two-way ANOVA with genotype and sex as fixed factors. Non-parametric data were analyzed using the Kruskal-Wallis test with Mann-Whitney U as *post hoc* test. All tests were performed two-sided at a significance level of $p \leq 0.05$. Values two standard deviations outside the mean of a parameter within an AOI were considered outliers and in that case all parameters obtained within the AOI for that individual were discarded from the data analysis. In the bar graphs data are expressed as the mean ± standard error of mean (SEM).

3. Results

3.1. Iba-1 immunostaining

The appearance of the Iba-1 positive microglia (see Fig. 1C and 1D, with representative examples in the CA1 areas of the BTBR and B6 PKU mice) in the PKU and control mice resembled the previously reported appearance in different species (see, for example, Ahmed et al., 2007; Cogut, Bruintjes, Eggen, van der Zee, & Henning, 2017; Hovens et al., 2014, 2015). In brief, cell bodies were distributed over all hippocampal subregions, but were mainly absent in the white matter. Dendritic branches, originating from the cell body, penetrated randomly into the neighboring cell tissue. No gross anatomical differences in the appearance or distribution of the microglial cells were found between the two mouse strains. No differences in the number of microglia cells were found between the BTBR and B6 WT mice (Fig. 1B). However, the BTBR WT mice have a slightly higher level of microglial activation than B6 WT mice (BTBR WT 9.2941 ± 0.263 versus B6 WT 7.982 ± 0.159 $p \leq 0.000$; Fig. 1B).

3.2. Microglial activation in the C57Bl/6 Pah^{enu2} and BTBR Pah^{enu2} mice

The microglial activation was determined based on morphological analysis as described above, and expressed relative to their WT controls. No clear and consistent differences were found between male and female mice and we therefore combined their data. The results showed that when all hippocampal subregions were combined (column "Tot." in Fig. 2A and C), no changes in activity state were found in the BTBR PKU mice (0.975 ± 0.036, $p = 0.620$) whereas in the B6 PKU mice a significant increase was found as compared to their respective WT controls (1.085 ± 0.025, $p = 0.014$). Analysis per hippocampal AOI revealed that except for the Hilus, all AOIs showed increased activity, although this only reached statistical significance in the DG inner blade and the CA3 Stratum radiatum (DG: 1.171 ± 0.045, $p = 0.005$; CA3: 1.217 ± 0.051, $p = 0.002$).

3.3. Number of microglia in the C57Bl/6 Pah^{enu2} and BTBR Pah^{enu2} mice

The number of microglia in the hippocampal AOI was also determined, and the values of the PKU mice were expressed relative to their WT controls. No clear and consistent differences were found between male and female mice and we therefore combined their data.

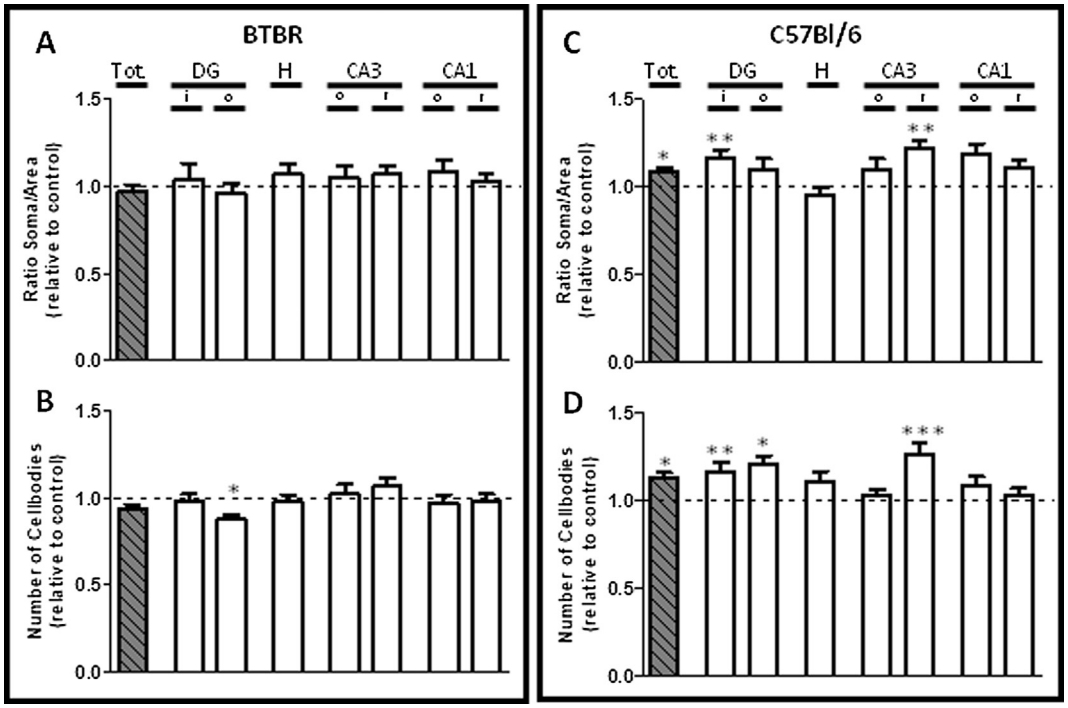

Fig. 2. Level of microglia activation (ratio soma/area; panels A and C) and relative number of microglia (panels B and D) for the BTBR (left panel) and the B6 (right panel) PKU mouse. Total values (Tot.; pooled values of all seven AOIs) are shown in the gray/hatched bars. Values are expressed relative to controls (dashed lines). DG = Dentate Gyrus (I = inner blade; O = outer blade); H = Hilus; CA3 = Cornu Ammonis 3 (O = Oriens; R = Radiatum); CA1 = Cornu Ammonis 1 (O = Oriens; R = Radiatum). Bars represent the mean ± SEM. Number of animals in each bar: BTBR: Tot.: 14; DGi: 15; DGo: 16; H: 15; CA3o: 16; CA3r: 16; CA1o: 16; CA1r: 15. B6: Tot.: 15; DGi: 15; DGo: 16; H: 16; CA3o: 14; CA3r: 16; CA1o: 16; CA1r: 16. $^{*}p \leq 0.05$; $^{**}p \leq 0.01$; $^{***}p \leq 0.001$.

When all hippocampal subregions were combined (column "Tot." in Fig. 2B and 2D), a significant increase was found in the B6 PKU mice (1.128 ± 0.033, $p = 0.014$). No change was found in the BTBR mice (0.938 ± 0.023, $p = 0.072$). Analysis per hippocampal AOI revealed a significant reduction in the outer blade of the DG in the BTBR mice (0.875 ± 0.029, $p = 0.015$), and significant increases in the inner and outer blade of the DG and the CA3 Stratum radiatum in the B6 PKU mouse (DGi: 1.168 ± 0.049, $p = 0.007$; DGo: 1.203 ± 0.054, $p = 0.015$; CA3: 1.268 ± 0.062, $p = 0.001$).

4. Discussion

Taken together, the data revealed a subtle difference in microglial morphology in the PKU mouse, however in a strain-dependent way and differently from what we hypothesized. An increased level of microglial activation was found in the B6 PKU mouse (both in activity state and in cell number), but not in the BTBR PKU mouse. We therefore conclude that the B6 PKU mouse, but not the BTBR PKU mouse, has a mildly elevated level of hippocampal microglia activation. This level of activation likely favors neuroprotection rather than indicating neurodegeneration. Also, this level of microglial change in the B6 PKU mouse (circa 10% increase) is comparable to the early stages of microglia activation seen before the onset of neurodegenerative conditions in pathological disorders as described by Kreutzberg (1996), which suggests that such low levels of microglial activation reflect rather a compensatory (neuroprotective) mechanism. A comparable level of microglia activation (20% increase) was found in rat hippocampus of individuals who had experienced and survived a viral infection in the past. In contrast, much higher levels of microglial activation based on Iba-1 staining and the morphological indication of activation as we used here have been reported, for example, in the hippocampus of old rats (Hovens et al., 2015) or in the hippocampus of hibernating hamsters (Cogut et al., 2017). In these studies, the level of microglial activation was approximately 200%, illustrating that the hippocampal microglia in the PKU mouse are far from maximal activation. Finally, it is noteworthy to mention that our observed strain differences in the WT controls replicate the findings of Heo, Zhang, Gao, Miller, and Lawrence (2011), who also reported that the BTBR mouse has a higher level of microglial activation than the B6 mouse.

A role of microglia in hippocampal synaptic pruning in B6 PKU mice has been suggested by Horling et al. (2015). At the age of two weeks the staining intensity of Iba-1did not differ between the PKU and control mice. At the age of twelve weeks, Horling and coworkers found a significant reduction in Iba-1 staining intensity in the hippocampal CA1 and CA3 regions (n = 3 per group). They suggest that the reduced staining intensity reflects reduced microglial activation which is functionally linked to reduced synaptic pruning and reduced hippocampal functioning. Our findings revealed a significant increase in microglial activity in the B6 PKU mouse (n = 16 per group). The cause of this discrepancy is unknown and warrants further studies.

If the increased levels of microglia activation found in the B6 PKU mouse are indeed neuroprotective, it could help explaining why, in our hands, the B6 PKU mouse has less hippocampus-dependent deficits than the BTBR PKU mouse. Given the role of hippocampal microglia in synaptic pruning, there are two ideas how reduced synaptic pruning by microglia can affect homeostasis. Reduced microglial activity may cause a failure to prune the excitatory synapses, or enhanced microglial activity may prune inhibitory synapses (Koyama et al., 2015). The slightly higher level of microglial activation in B6 PKU mice may help to maintain a balance in excitatory and inhibitory synapses which may support hippocampal functioning. This could be a factor by which the B6 PKU mouse, in contrast to the BTBR PKU mouse, is able to keep performing proper hippocampal functioning in the spatial object recognition task (Bruinenberg, van der Goot, et al., 2016). Overall, the DG, next to the CA3 region, seems to be the most sensitive area in the B6 PKU mouse. Microglial activation could influence adult hippocampal neurogenesis in the DG, critically involved in hippocampus-dependent learning. Although hippocampal neurogenesis does not

depend on microglial activation (Van der Borght et al., 2005; Olah et al., 2009), a supportive role of microglia in the DG-CA3 axis via the stimulation of adult hippocampal neurogenesis could make a difference (Winn et al., 2018).

Combining previous literature concerning these two genetic backgrounds in the PKU mouse model, the results further indicate that high levels of phenylalanine or disturbed sleep patterns do not consequently cause hippocampal microglial activation in the PKU mouse, because both strains are characterized by these features but do not both reveal enhanced microglial activation. Also in hippocampal slice cultures of five-day old C57Bl6 mice, no dose-dependent effect of Phe on Iba1-immunostaining was found, indicating that Phe does not directly influence the state of microglial activation, despite a general tendency to reduce microglial activation (Schlegel et al., 2016). It is worthwhile to further investigate the impact of hippocampal microglial activation in the PKU brain, and to compare these findings between laboratories. A next step would be to determine the cytokine profiles of the PKU mice, to measure other markers at the protein or mRNA level specifically linked to microglia functioning, and to examine whether dietary interventions (van Vliet et al., 2015, 2016) or exercise (Mazzola et al., 2016) can influence hippocampal microglia activation in the PKU mouse. Next to the hippocampus, other brain regions as well as different age groups (e.g. juveniles and old individuals) might provide novel insights in the role of microglia, PKU and sleep.

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Conflict of interest

The authors declare no conflict of interest.

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