ARE PHENYLKETONURIA MICE ALREADY DIFFERENT DURING EARLY POSTNATAL LIFE?

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Content

Abstract

Introduction: Phenylketonuria (PKU) is a recessive inherited metabolic disorder which is characterized by a dysfunction in the conversion of phenylalanine (Phe) into tyrosine, due to inactivity of the enzyme phenylalanine hydroxylase. This leads to increased concentrations of Phe in the blood and brain. If untreated, patients can suffer symptoms such as mental retardation, delayed development and motor dysfunctions. This study investigated the early development of the genetic mouse model of PKU, Pah^{ENU2} BTBR mice.

Methods: 66 fourteen day-old homozygous (HMZ), heterozygous (HTZ) and wild-type (WT) mice of the background strain BTBR were used. Length, weight and behavioral assessments were used to detect differences in early development. Immunohistochemistry was used to detect differences in neurogenesis (DCX) and pathways involved in learning and memory (pCREB).

Results: In accordance with literature, WT and HTZ did not differ on any parameter. Weight and length measurements were significantly different for the HMZ group compared with both WT and HTZ (Repeated measures, p-value < 0.0001). Righting reflex on postnatal day (PND) 5 is significantly different between HMZ and both WT and HTZ animals (Mann-Whitney U Test, p-value 0.009). Righting reflex on PND 8 showed no difference for HMZ compared to WT and HTZ. In the homing test (PND 10), distance moved, velocity, mobility and immobility were not significantly different between groups. Finally, the responses to the auditory startle test were also not different between groups.

Conclusion: HMZ animals show differences as early as PND 5 in weight, length and righting reflex. After PND 8 it seems that the HMZ animals can keep up with the WT and HTZ in the behavioral tests, but not in development of weight and length.

Introduction

In 1934 Dr Asbjørn Følling found increased levels of phenylpyruvic acids in the urine of a specific group of mentally retarded patients. Later it was found that these patients have elevated levels of phenylalanine (Phe) in their blood, supporting the hypothesis that these patients have a deficiency in Phe metabolism (Christ, 2003; Camp, 2014). The disease is inherited in an autosomal recessive manner and nowadays known as phenylketonuria (PKU). PKU is a metabolic disorder characterized by a loss of function of the liver enzyme phenylalanine hydroxylase (PAH). PAH is responsible for the conversion of Phe to tyrosine.

The symptoms seen in untreated patients are associated with the high blood concentration of Phe. Untreated patients display a wide range of neurological, behavioral and physical problems. Neurological symptoms include severe mental retardation and epilepsy. Physical difficulties consist of growth impairments, microcephaly, motor dysfunction, and pigmentation abnormalities, due to decreased melanin synthesis which is produces out of tyrosine. Behavioral deficits include aggressive behavior and self-injury (Walter, 2006).

Treatment of PKU aims at a reduction of Phe levels and an increase of non-Phe large neutral amino acids (LNAA's). This is done by a strict low-Phe diet with supplementation of non-Phe LNAA's (van Spronsen, 2010). Phe can be found in common consumed protein-rich food including milk, eggs and nuts. Unfortunately, treated patients with bad dietary control during infancy still show several behavioral deficiencies such as increased anxiety, social withdrawal, hyperactivity, and concentration problems. Besides, they often still suffer some intellectual impairment. On the other hand, well-treated patients might display a higher risk of depressive symptoms and low self-esteem (Walter, 2006). The clinical outcome for a treated patient is dependent upon several variables of which the most important one is the Phe levels in the blood during infancy and childhood. Other factors include the age at which treatment is initiated, blood Phe levels at different periods of age, duration of periods of Phe deficiency, and the gradient for Phe transport across the blood brain barrier (Walter, 2006).

In 1968 Western countries started to use a simple screening test to detect PKU in newborns (Camp, 2014). Early detection of the disease is important, because when treatment is initiated early, severe mental retardation can be prevented. Longitudinal studies revealed that treatment initiation within the first 3 weeks after birth with average blood Phe levels ≤ 400 µmol/l during infancy and childhood result in close to normal intelligence. Furthermore, the IQ score at 4 years of age is decreased by 0,25 standard deviation (SD) for every 4 weeks of delay of treatment initiation (Walter, 2006). Unfortunately, until now the exact and most optimal period of treatment induction is still unknown.

Due to the dysfunctional PAH, patients fail to convert Phe to tyrosine. Tyrosine is a precursor for the monoamine neurotransmitters dopamine and norepinephrine in the brain. A decreased level of tyrosine can, therefore, result in decreased levels of these neurotransmitters (Pascucci, 2002; Pascucci, 2008; Pascucci, 2012). Moreover, PKU is associated with reduced brain serotonin levels (Pascucci, 2002). This is due to diminished conversion from tryptophan to serotonin (Walter, 2006).

Several mechanisms are proposed by which excess phenylalanine can lead to decreased monoamine neurotransmitters. The first hypothesis concerns the blood-brain transfer of Phe. Phe, together with other large neutral amino acids (LNAA's) such as tyrosine and tryptophan, is transported over the blood brain barrier by a large neutral amino acid transporter (LAT1). Due to the high affinity of

this transporter for Phe, this amino acid competes with the other LNAA's at the LAT1 when peripheral Phe levels are high, like in PKU. This results in an increased transport of Phe and an interference with the transport of other LNAA's (Shulkin, 1995). Therefore, this hypothesis suggests that the major symptoms of PKU are caused by decreased neurotransmitter availability and protein synthesis due to diminished transport of non-Phe LNAA's (de Groot, 2010). Second, Phe could interfere with the activity of tyrosine hydroxylase (TH) and tryptophan hydroxylase (TRH) by competitive inhibition. These enzymes are the rate-limiting steps in dopamine and serotonin synthesis, respectively (Harding, 2014). The third mechanism that possibly contributes to the pathophysiology of PKU is impaired glutamatergic transmission. Phe does so by competitive binding to the glutamate receptors, NMDA, AMPA and kainate, and by reduction of glutamate release. Glutamate is the major excitatory neurotransmitter of the brain. Reduced glutamate release and signaling leads to decreased synaptic plasticity and thereby less cognitive flexibility (Martynyuk, 2005).

Neurotransmitters play an important role in brain development. Monoamine neurotransmitter concentrations increase during the critical period of brain development (Pascucci, 2002). It is suggested that symptoms such as mental retardation, which are found in untreated PKU patients, are caused by delayed brain development. This is a consequence of deficiencies in monoamine neurotransmitters together with toxic concentrations of phenylalanine. During pregnancy the fetus is protected against high levels of Phe, because the transport of Phe across the placenta is determined by the blood Phe level of the mother. When this level is normal, normal levels of Phe will reach the fetus (Rastogi, 1998). Therefore, Phe levels will not increase to a toxic level until after birth. Especially late developing brain structures, such as the prefrontal cortex, are vulnerable for the high levels of Phe (Huttenlocher, 2000). Morphologic analysis performed on brains of adult untreated PKU patients revealed a cell density pattern of the pyramidal neurons of the prefrontal area similar to that of a child between 6 months and 2 years of age, suggesting a delayed development of these pyramidal neurons. Moreover, the pyramidal neurons had smaller cell bodies than normal and contained immature dendrites (Bauman, 1962).

In mice, body weight and brain weight increase proportionally during the first two postnatal weeks. However, around postnatal day (PND) 14 there is a critical point where only the body weight keeps increasing in the same line and the brain weight increase slows down (Kobayashi, 1963). Retarded growth in body weight correlates with a retarded growth in brain weight. The ratio that is found in these retarded mice at a certain time point is comparable with a normal animal of several days younger (Kobayashi, 1963). It is known that the critical period for brain neuronal maturation in mice is between 10 and 15 days after birth (Fuller, 1966). For example, the neurons of the dentate gyrus, situated in the hippocampus, are produced short before birth and development of these neurons continues until at least 14 days after birth (Rodier, 1980).

To study PKU, a transgenic mouse model mimicking the human disease was developed. The Pah^{ENU2} mouse model shows, like patients, no PAH enzyme activity and consequently high Phe and phenyl pyruvic acid levels (Shedlovsky, 1993). In this genetic mouse model of PKU reduced dopamine, serotonin and norepinephrine levels together with reduced metabolites of these neurotransmitters are found in different brain regions. In addition, it shows decreased performance on cognitive tests (Zagreda, 1999; Puglisi-Allegra, 2000; Cabib, 2002; Pascucci, 2013). The Pah^{ENU2} mouse model is therefore a good representation of the human situation in case of untreated PKU.

Preliminary research showed that until PND 14 is a critical period, indicated by the finding that

at this age the differences between PKU and control mice on several behavioral tests were the most pronounced. These tests include righting reflex, homing test, auditory startle response, bar grasping, cliff avoidance and negative geotaxis (Pascucci, unpublished). This study examined the early development of physical landmarks and developmental milestones in the genetic mouse model of PKU, Pah^{ENU2} BTBR mice.

Material and methods

Animals

66 fourteen day-old mice were obtained by heterozygous breeding of Pah^{ENU2} BTBR mice. The breeding resulted in 13 homozygous (HMZ), 35 heterozygous (HTZ) Pah^{ENU2} mice and 18 wild-type (WT) mice. Only litters within the range of between 7 and 11 animals per litter were used. As a consequence, 2 litters were excluded for analysis, resulting in 9 HMZ, 25 HTZ, and 15 WT mice (total of 49 animals). All animals were used for physical, behavioral and immunohistochemistry assessments, except for 1 WT, which was an outlier in the righting reflex on PND 5 and therefore not used in the behavioral analysis. Animals were housed with the mother in a standard breeding cage with food (AM II 2141, ABdiets) and water *ad libitum* on a 12:12 h light: dark cycle (lights on 07.00-19.00 h) and a controlled temperature of 21^{o}C ± 1^{o}C. The first morning appearance of a litter was considered as PND 0. Genetic categorization was performed on DNA prepared from tail tissue, attained after transcardial perfusion of the animals. All experiments were conducted in accordance with the DierExperimentenCommissie of the University of Groningen (DEC-RUG) (6731B). First a pilot study was performed with 30 additional, naïve, fourteen day-old mice to create a perfusion protocol. Afterwards the experiment outlined below started.

Physical landmarks

Before every procedure, the subjects, together with their nest material, were placed in a heated holding cage covered with wood-chip bedding. To maintain normal body temperature, half of the cage was located on a waterproof heating pad. The temperature of the heating pad was set between 32-34^{o}C, resulting in an ambient temperature of the holding cage between 22-24^{o}C. From PND 5 on, between 11.00-12.00 h, every subject was weighted on a scale and length was measured from snout till base of the tail. The subject was also checked for opening of both eyes and the detachment of the pinnae from the head.

Behavioral assessment

All behavioral assessments were videotaped to be analyzed afterwards. The sequence of testing of the mice progressed from the least to most stressful procedure (Crawley, 2008).

Surface righting reflex: The surface righting reflex was performed on PND 5 and 8. The subject was placed on a heating pad on its back with its paws pointing upwards. The time (in sec) needed to turn over onto its abdomen with all four paws touching the surface was recorded. In case the subject took longer than 60 sec the test was terminated and scored as 60 sec.

Homing test: At age 10 days the subject was placed in a heated cage with 3 compartments. One part was covered with home cage bedding and the other with clean wood-chip bedding subdivided by an empty center. The pup was placed in the empty center, facing the wall, and habituated to the center for 1 minute. Afterwards the subject could enter all compartments and was videotaped for 5 additional minutes. Video-based Ethovision was used to analyze the data and measure distance moved, mobility and velocity of the animals. For some animals a heating lamp in addition to the heating pad was used to maintain normal body temperature as a pilot experiment.

Auditory startle: On PND 14 every subject was placed in a small heated box for 1 minute habituation and afterwards exposed to a single tone of 7435 Hz at between 85-90 dB for 200 msec. (Shnerson, 1980). After 30 sec the animal was exposed again to the same tone. The mice were videotaped for the presence of a startle response by scoring either yes, little or no. The ambient noise of the room was between 33-36 dB.

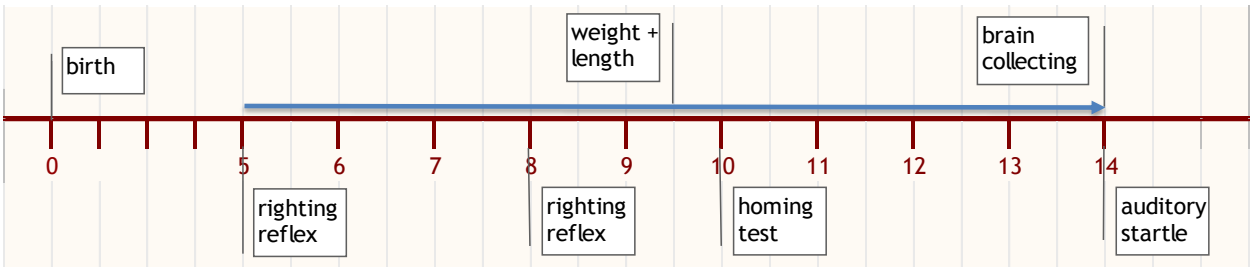

Fig. 1: **Timeline of the experimental procedures performed**. Day of birth was considered as postnatal (PND) 0. From PND 5 until PND 14 weight and length of the animals were measured daily. On PND 5 and PND 8 the righting reflex was tested. Homing test was performed on PND 10. 2 hours before sacrifice on PND 14 auditory startle was measured.

Genotyping

To determine genotype, tail tissue was obtained during the perfusion of the mice. First 0.5 ml lysis buffer containing proteinase K (100:1) is added to the tail tissue and incubated overnight at 55°C to digest the tissue. After centrifuging, the supernatant of the tissue is added to 0.5 ml isopropanol to precipitate DNA. After centrifuging the samples, isopropanol is removed and DNA is dried. Next 200 µl TE buffer is added and incubated at 55°C for 30 minutes to dissolve the DNA (Laird, 1991). The DNA concentration is measured using the nanodrop device. Dilutions are prepared with TE buffer to reach a DNA concentration between 5 and 10 ng/µl. PCR mastermix is prepared with TE buffer, PCR-mix, a forward and a reverse primer, and a probe for WT and a probe for the Enu2 mutation. A 96-well plate is loaded with 5 µl mastermix and 2 µl DNA sample in threefold. Real-time PCR is used to distinguish between the three possible genotypes, HMZ, HTZ, and WT. The used qPCR protocol consists of preheating: 95°C for 10 min, 95°C for 15 min and 65°C for 1 min, this is repeated another 39 times.

Immunohistochemistry

Due to the limited availability of literature on perfusion in young mice, a pilot study to establish a perfusion protocol was executed. The pilot study was performed with 30 additional, naïve, animals. In this pilot three different variables were varied. The first variable was speed of perfusion; two different speeds were used, 4 (Cheng, 2009) and 8 ml/min. The second variable was duration of post-fixation; three different durations were applied, 24, 48 and 72 hours. The last variable was sucrose solution concentration; two different concentrations were used, 10 and 30 %. After an analysis of the brain tissue, we decided to use the variables 8 ml/min for perfusion speed, 48 hours post-fixation and 10 % sucrose followed by 30 %.

Within the main experiment 2 hours after stimulation, using the auditory startle test, the 14 day old mice were anesthetized with a 0.1 ml intraperitoneal injection of pentobarbital. When no hind paw reflexes were present anymore, they were transcardially perfused first with a 0.9% sodium chloride solution with heparin, followed by 4% paraformaldehyde in 0.1 M phosphate buffer. Brains were removed from the skull and post fixated in 4% paraformaldehyde for 48 h and dehydrated first 24h in 10% sucrose followed by 24 h in 30% sucrose. All these procedures were carried out by room temperature. Liquid nitrogen was used for freezing the brains and afterwards they were stored at -80^{o}C until use. 25 µm thick coronal sections were cut serially and stored at 4^{o}C until staining. Immunohistochemistry was performed on free-floating slices. First endogenous peroxidase activity was inactivated with 0.3% hydrogen peroxide. Afterwards the slices were incubated in primary antibody C-Fos AB-5 (Vector, 1:8000), monoclonal rabbit IgG pCREB (1:1000) or DCX (1:1000) in 0.01M PBS with 4% BSA and 0.1% Triton-X-100 (TX) for 48 h for C-Fos; in 0.01M PBS with 2% BSA and 0.1% TX for 48 h for pCREB; and in 0.01M PBS containing 3% NRS and 0.1% TX for 72 h for DCX. Slices were rinsed eight times with 0.01M PBS, DCX slices were rinsed three times, before 2 h incubation in secondary antibody biotinylated goat anti Rabbit IgG BA (Jackson, 1:400) for C-Fos; biotinylated goat anti rabbit IgG (1:500) for pCREB; and biotinylated rabbit anti goat (1:500) for DCX. Slices were rinsed several times with 0.01M PBS and then incubated 2 h in avidin-biotin complex (Vectastain elite ABC kit; Vector laboratories, 1:500) in 0.01M PBS. After several times rinsing with 0.01M PBS the diaminobenzidine (DAB) reaction was activated by 100 µl 0.1% hydrogen peroxide. For a stronger and more visible cell labeling, 1.2 mg NAS per 10 ml DAB solution was added for the C-Fos and DCX staining**.** The DAB reaction was terminated by rinsing with 0.01M PBS. Sliced were mounted on glass with 1% gelatin and cover slipped after a xylol chain. Xylol is used in tissue fixation since it removes lipid components which allow the hydrophilic structures to adhere to the glass.

Statistical analysis

Every mouse tested for each given response was treated as an individual even though the individuals in the same litter are not independent from each other, since each individual of a litter is subject to the same genetic, nutritional and maternal variables.

First a test for normality was performed on all data. The ANOVA repeated measures was used when comparing multiple samples followed by a Bonferroni post-hoc test. The Kruskal-Wallis was performed as non-parametric test to compare multiple groups, when there were significant differences found we performed a Mann-Whitney U test. The Mann-Whitney U test was performed as non-

parametric test to compare two groups. For nominal data a crosstabs was used to perform a Chi-square test. Statistical significance was determined at P <0.05. All data is displayed as mean ± SEM.

Results

Physical landmarks

No gender differences were found for weight and length measurements. Therefore, gender is not mentioned in the analysis reported for the physical landmarks. The weight of the HMZ animals was significantly lower over the complete time course compared to the WT and HTZ animals (ANOVA repeated measures, p=0.000). All groups increased in weight over time (Fig. 2). A significant weight x genotype x litter size interaction effect was found. After exclusion of the biggest and the smallest litters, of 12 and 5 animals, respectively, the interaction effect disappeared. Therefore, we decided to exclude these two litters of all further analysis.

Length measurements showed the same pattern as the weight measurements, the HMZ animals are significantly smaller, except for PND 6, compared to the WT and HTZ animals (ANOVA repeated measures, p=0.000). The length of the animals increased over time (Fig. 3).

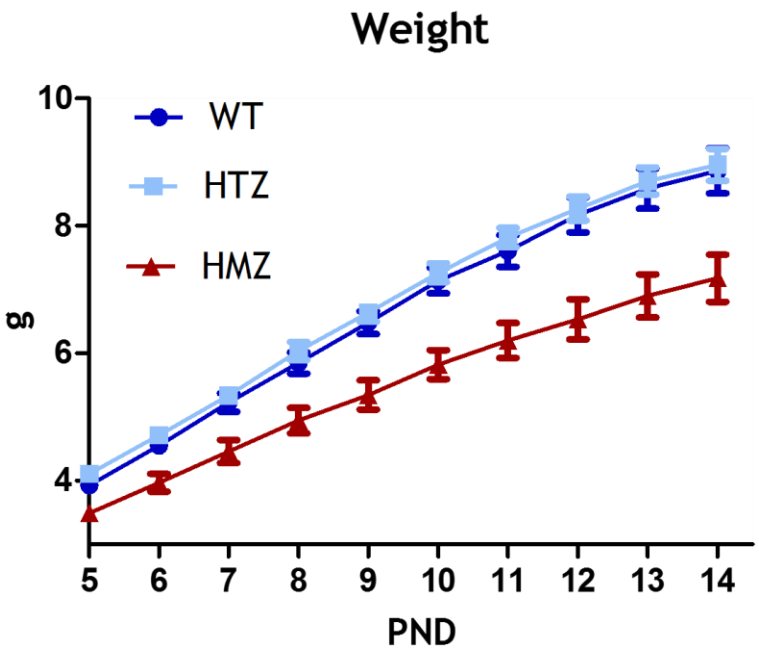

Fig. 2: **Weight measurements.** All groups increased in weight over the course of time. The HMZ animals weighted significantly less compared to the HTZ and WT animals (p=0.000).

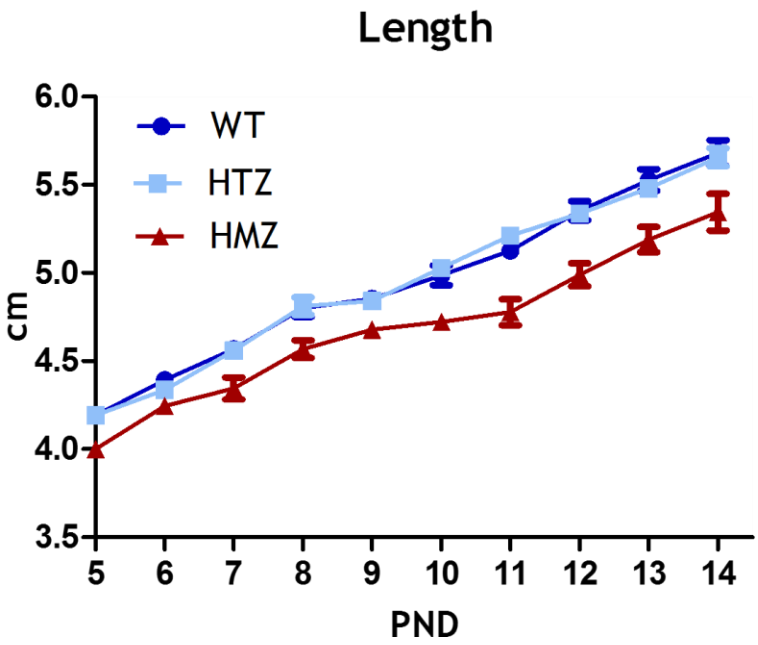

Fig. 3: **Length measurements.** All groups increased in length over the course of time. The HMZ animals were significantly smaller, except for PND 6, compared to the HTZ and WT animals (p=0.000).

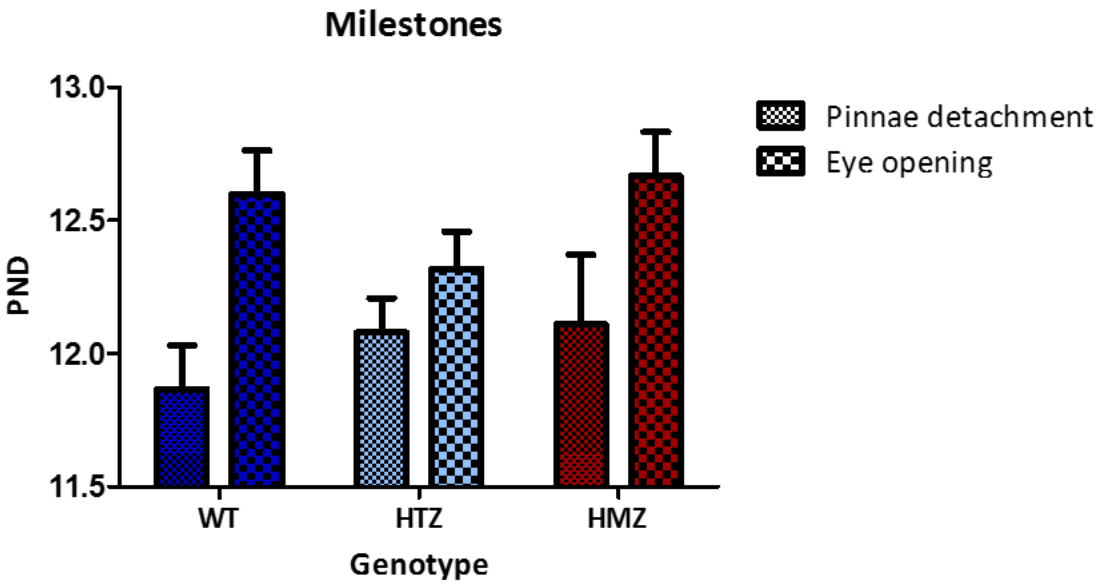

Fig. 4: **Developmental milestones of physical landmarks.** Pinnae detachment and opening of both eyes occurred at comparable time points for each genotype. Pinnae detachment took place around PND 12. Eye opening took place around PND 12,5.

The pinnae of all genotypes were detached around PND 12. WT (11,87 ± 0,17), HTZ (12,08 ± 0,13) and HMZ (12,11 ± 0,26). The eyes of all animals in the different groups opened around PND 12,5. WT (12,60 ± 0,16), HTZ (12,32 ± 0,14), HMZ (12,67 ± 0,17) (Fig. 4). There was no gender difference in pinnae detachment and eye opening found.

Behavioral assessment

Righting reflex on PND 5 differed significantly for the HMZ group (11,67 ± 4,24) compared to both WT (4,10 ± 1,04) (Mann-Whitney U, p=0.011) and HTZ (3,72 ± 1,13) (Mann-Whitney U, p=0.000). On PND 8 all groups show comparable performance in their righting reflex (WT (1,43 ± 0,23), HTZ (1,56 ± 0,40) and HMZ (1,39 ± 0,14)). Only the HMZ (ANOVA repeated measures, p=0.044) and the WT (ANOVA repeated measures, p=0.026) performed significanlty better on PND 8 compared to PND 5 (Fig. 5). No gender and litter size differences were found in the righting reflex.

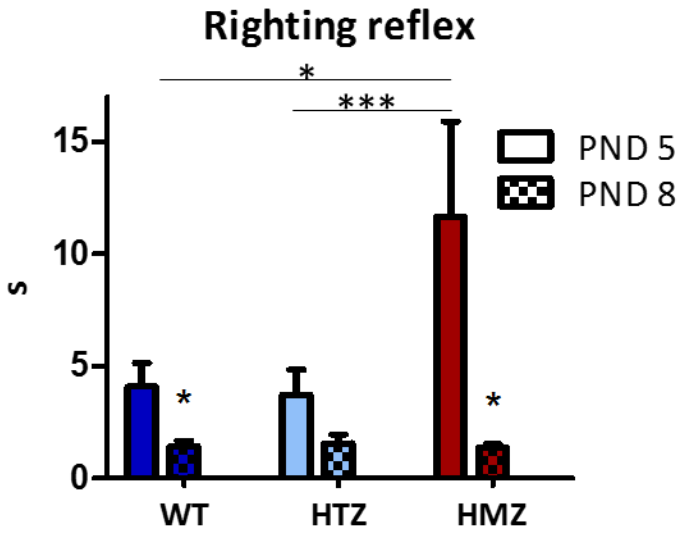

Fig. 5: **Righting reflex.** Righting reflex on PND 5 is significantly slower for HMZ animals compared to both WT (p=0.011) and HTZ animals (p=0.000). On PND 8 this difference between the groups has disappeared. Both WT (p=0.026) and HMZ animals (p=0.044) improved significantly on PND 8 compared to PND 5. *p<0.05, ***p<0.000

The homing test showed no significant differences between all groups. However, distance moved shows a tendency to be lower in the HMZ (473,20 ± 70,00) animals compared to the WT (651,09 ± 55,75) and HTZ (686,48 ± 60,37) animals (Kruskall Wallis, p=0.105) (Fig. 6). Moreover, velocity shows a tendency to be lower in the HMZ (1,75 ±0,26) animals compared to WT (2,43 ±0,21) and HTZ (2,58 ± 0,23) animals (Kruskall Wallis, p=0.106) (Fig. 7). No significant differences were found in the ability to discriminate between the home cage sawdust and clean sawdust between the groups (Pearson chi-

square = 0.255) (not shown). All groups spent equal amounts of time being immobile, WT (256,79 ± 2,74), HTZ (251,16 ± 5,47), and HMZ (256,15 ± 5,62). The same pattern was found for being mobile, WT (11,22 ± 2,41), HTZ (15,55 ± 3,84), and HMZ (12,28 ± 4,78) (Fig. 8). There were no gender differences for distance moved, velocity, mobile and immobile. Moreover, no differences for heating lamp were found (not shown).

Auditory startle response revealed no significant differences between the different genotypes. Neither at the tone, around 1 minute after habituation (Pearson chi-square = 0.149) nor at 1.30 minutes after habituation (Pearson chi-square = 0.650) (not shown).

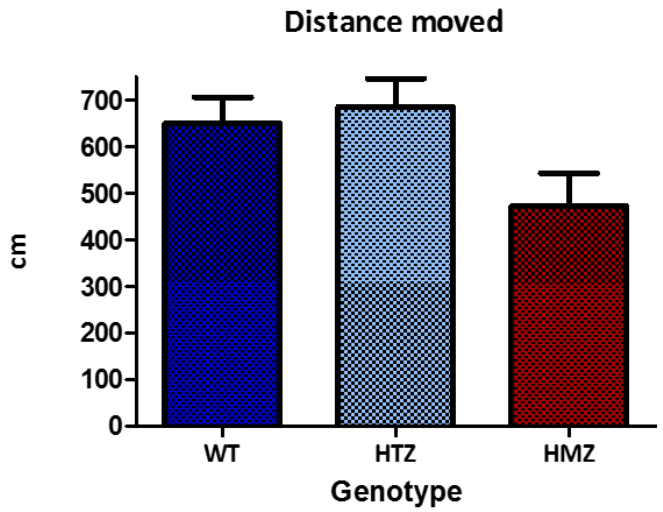

Fig. 6: **Distance moved during homing test**. The HMZ animals show a tendency towards less distance covered compared to the WT and HTZ animals (p=0.105). The overall average of distance moved is 603,59 cm.

Immunohistochemistry

3 pilot studies for immunohistochemistry were executed. A staining for C-Fos, pCREB and DCX was performed to check whether these antibodies would bind to the fairly young tissue used in this study. It was visible by eye that pCREB and DCX were staining our tissue properly. Unfortunately, due to technical problems a detailed analysis was not possible. Therefore we were not able to detect differences between the different genotypes. C-Fos expression was very low, and the accuracy of the staining hence needs to be analyzed in more detail (not shown).

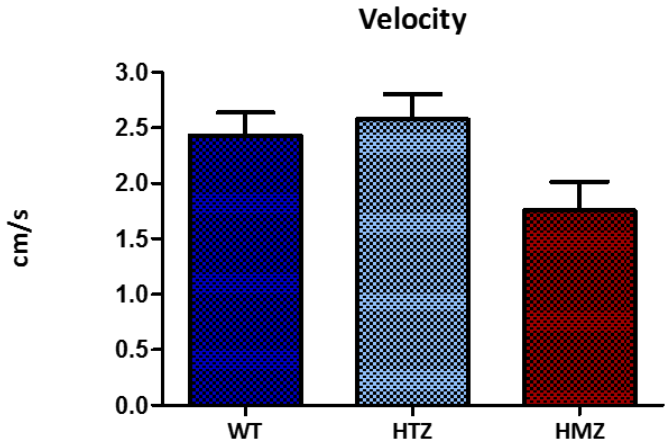

Fig. 7: **Velocity during homing test.** The HMZ animals show a tendency towards a slower velocity compared to the WT and HTZ animals (p-0.106). The overall average velocity is 2,25 cm/s

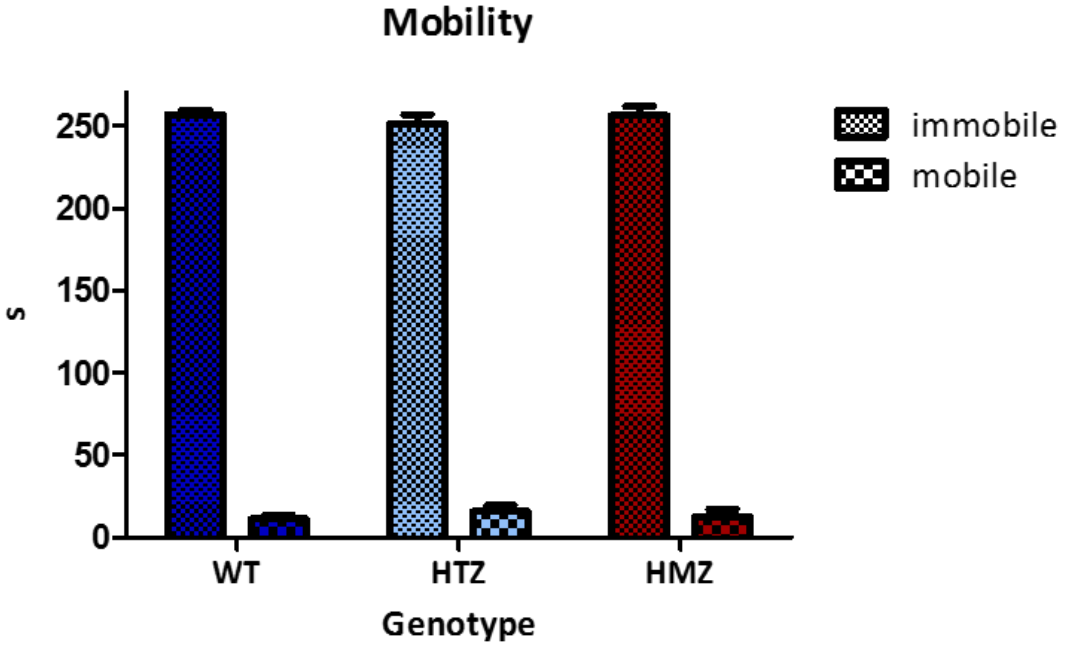

Fig. 8: **Mobility during homing test.** Time spent immobile is comparable across all genotypes. The overall average time spent immobile is 254,70 s. Time spent mobile is comparable across all genotypes. The overall average time spent mobile is 13,02 s. Total time analyzed is 270 s.

Discussion

PKU is associated with a retarded development of the brain, body and behavior. This study showed that HMZ PKU mice are delayed in their early development measured until two weeks of age. This is evident trough weight and length measurements and behavioral assessment of the animals. In the first, HMZ animals show reduced weight and length compared to WT and HTZ animals. The behavioral assessment showed that the righting reflex on PND 5 is delayed. However, the righting reflex on PND 8 is comparable between the three different genotypes. The homing test on PND 10 and the auditory startle response on PND 14 reveal no differences between the genotypes. These results indicate that the HMZ animals are able to keep up with their littermates after PND 5 in the behavioral assessments performed. However, the weight and length measurements of the HMZ animals still indicated a retarded growth. This retarded body growth could correlate with a retarded brain development (Kobayashi, 1963). However, the brains of all genotypes are not examined yet. Therefore, no conclusions can be drawn concerning this statement.

The results found in this study differ from earlier findings by Pascucci. Whereas our findings indicate significant differences for both weight and length for the complete course of time measured, Pascucci did not find significant differences in weight and length in the period of PND 5 until PND 14. They found a reduced performance of the HMZ animals in the righting reflex on both PND 5 and PND 8, time spent in zone and number of entries during the homing test on PND 10, and auditory startle response on PND 14 (Pascucci, unpublished). Our study only found a significant difference of performance in righting reflex on PND 5, whereas all other behavioral tests performed showed equal accomplishments.

The divergence in results on the same behavioral tests performed in two different labs, demonstrates the difficulty of repeating behavioral assessments. Many factors can influence the animals, thereby leading to differences in the results. For example, the researcher performing the tests has a big influence on the response of the animals tested. Handling of the animals is different between researchers and the consequence of that is often unnoticeable. Handling on its own could already have an effect on the performance of the animals. Other factors influencing the animals include the housing conditions, but also differences in the testing device can lead to different outcomes. Moreover, most measurements are subjective observations and are hence unlikely to be identical between researchers and different labs. Videotaping all procedures is a way to deal with this discrepancy. In this way several researchers can score the behavior, which can be averaged.

The HMZ animals displayed a delayed response in righting reflex on PND 5, but on PND 8 it was comparable with the control. This is not in line with preliminary research performed with mice of the same background. So, in some way the HMZ animals were able to compensate for their earlier delayed development. Mice should be able to right themselves from PND 3 on and become really fast around PND 7 (Fox, 1965; Heyser, 2003). The HMZ animals on PND 8 are possibly comparable to WT and HTZ animals on PND 6 or 7. This is at least the case for the weight and length and a retarded body development correlates with a retarded brain development (Kobayashi, 1963). To test this possibility, the behavioral assessment should be performed every day. It is important to note that the absence of differences in behavioral data between the genotypes does not exclude the presence of differences in the brain development.

During the homing test it was noticed that the animals were often immobile and sometimes even fell asleep. Therefore, we performed the test in one litter with a heating lamp besides the heating pad, to better maintain normal body temperature. Still no differences in behavior could be observed and it complicated analysis by ethovision, so we decided to not use the lamp. From literature on children it is known that HMZ children show motor impairments, therefore one would expect differences between HMZ animals and control groups (Pietz, 1994; Nazi, 2014). In our findings distance moved and velocity only showed tendencies to be lower in the HMZ group compared to the other groups. The group of the HMZ animals only contains 9 subjects, so maybe with a bigger number there will be significant differences. Another explanation could be that only 4,5 minutes of the homing test were analyzed, because in the first trials we already started measuring time when the researcher was still near the device. Maybe when the complete 5 minutes would be analyzed, the tendencies for distance moved and velocity would become significant differences. The results do not follow the same pattern as the results by Pascucci. Our animals did not change between the three different compartments once they entered one part, as observed by the lab of Pascucci. Besides, our results do not show a difference in discrimination of home odor between the genotypes, as seen by Pascucci. To be able to explain these differences a detailed protocol from Pascucci is necessary. In this way the execution of the homing test can be compared and this can lead to an explanation for the discrepancy.

No differences were found in the auditory startle response on PND 14, whereas Pascucci did find significant differences. A reason for the absence of difference in our results could be that the setup used was not sensitive enough to detect small differences in response. Analysis was done by subjective scoring of the videotape and therefore only big startle responses are detectable. This method of scoring a response is very subjective. It would be better to use an oscillator that can convert pressure produced by the startle response of a mouse into voltages which can be analyzed for changes (Shnerson, 1980). Furthermore, it could be that the ears of the PKU mice are not fully developed yet and therefore could not hear the tone. The cochlea, the auditory part of the inner ear, is still developing until 14 days after birth (Fuller, 1966) and it is known that PKU mice have a retarded growth and it is likely that other parts of the body have therefore also a retarded development. However, whether this affects the applicability of the auditory startle response as a test for development is unknown.

There are several important points to keep in mind during the execution of behavioral tests with young animals. First of all it is very important to provide the animals with a heated environment, because mice of this young age are unable to maintain normal body temperature on their own. In the auditory startle response test we removed one animal from the nest and placed it back after the test to continue with the next animal. Otherwise the animals would be separated from the mother for about two hours, the time it took to test the whole litter. The potential effects of maternal separation and the need to maintain normal body temperature by an external source, limit the time available for testing individuals. However, constant opening of the cage and removal of one animal is stressful for the mother and the exact consequences of this for our data are unknown. It is known that after replacement of a removed litter, the mother will immediately start grooming the animals. Therefore it is best to return animals from a litter all in the same time to prevent interindividual differences (Heyser, 2003).

Data from individuals of the same litter are not independent from each other due to similarity in environmental factors. The best way to deal with this is to measure all animals of a litter and average these data to get a litter mean (Heyser, 2003). However, in our case this would lead to a very small

number of subjects. More importantly, the litters are usually composed out of all three possible genotypes, which is our main variable. By using a litter mean, the genotype variable would be lost. Therefore, it was decided to take every subject of a litter as an individual and keep this effect of environment in our mind by testing litter as a covariate.

Two litters that fell outside the set range of 7 to 11 animals per litter were excluded from analysis. It is known that litters with fewer pups will on average weigh more compared to litters with more pups. The relationship between litter size and bodyweight, on day 32 post conception, is best described by an inverse linear regression, where the smallest litters have the highest bodyweights (Wainwright, 1989). A higher bodyweight correlates well with a higher brain weight as well as with better behavioral scores. Litter size or sex ratio were not corrected beforehand, because there is a chance of removing HMZ or WT animals and it is difficult to reach sufficient group sizes for these genotypes. It is known from literature that sex ratio in the litter as well as the sex of an adjacent littermate in the uterus can affect a subject's behavior. When a female is located between two males in the uterus it shows more masculine behavior compared to a female not placed besides males (Vom Saal, 1980). Furthermore, the position of the mother in the uterus has an effect on the sex ratio of her litter; a mother with two adjacent males has more males in her litter compared to a mother between two females (Vandenbergh, 1994). Moreover, even the ratio of the different genotypes in the litter has an effect on behavior (Crews, 2009). However, in this research the number of animals is too small to take all these variables into account and therefore this study only focused on the main variable which is genotype.

During the pilot study three parameters in the tissue fixation step that are well established in adult mice but not yet in 14 day-old mice were varied. Fixation is important in immunohistochemistry via four different actions; stabilization of cell and tissue morphology, immobilization of the antigen, maintenance of immune reactivity of the antigen, and enabling penetration of the tissue by the reagent against the antigen (Berod, 1981). The varied parameters were speed of perfusion, duration of post fixation and concentration of sucrose solution. Speed of perfusion determines the pressure that is exerted on the vascular system of the animal. A smaller animal has smaller blood vessels and can therefore handle high pressure less well. Post-fixation improves fixation of the isolated tissue. Sucrose is used to dehydrate the brain and thereby prevents the formation of ice crystal artefacts in frozen tissue. Starting with 10% sucrose will prevent an osmotic shock to the cerebral cortex cells near the surface of the brain. These cells are particularly vulnerable when the tissue is not that well fixed.

Three pilot studies for immunohistochemistry were executed during the main study. C-Fos, pCREB and DCX staining were used in this study. The pilot immunohistochemistry executed until so far, only used tissue from the pilot, and therefore naïve, animals, this could be an explanation for the low C-Fos expression observed by eye. C-Fos is an early gene transcription factor and is often used as an indirect marker for neuronal activity, because it is expressed during firing of action potentials (Bullitt, 1990). However, when there are few action potentials, C-fos expression will be low. The pCREB and DCX staining were well visible by eye. pCREB is a staining for structures involved in learning and memory. DCX will generally stain immature neurons. However, the tissue needs to be analyzed under the microscope before making statements about the quality of the staining. Unfortunately, due to technical problems we were not able to perform the detailed analysis.

The neuropathology of PKU consists of delayed cortical neuronal development and a decreased

number of cortical neurons together with a poor maintenance of the myelin of the cortex (Huttenlocher, 2000). The first two can probably be detected with the DCX and pCREB staining. The hypo myelination can be due to a toxic effect of phenylalanine on the cells producing myelin, named oligodendrocytes, or because of the delayed and retarded development of neurons and thereby less outgrowth of mature axons that can be myelinated. Only active axons will be myelinated, because oligodendrocytes need factors, such as neurotransmitters, growth factors and adhesive signals from active neurons to produce the myelin that covers the axons. Furthermore, especially late developing structures show hypomyelination and reactive gliosis indicating that hyperphenylalanemia only occurs after birth (Huttenlocher, 2000). Therefore, it would be interesting to stain our tissue with a marker for myelin, such as myelin basic protein (MBP) or myelin proteolipid protein (PLP). In this way all aspects of the neuropathology will be covered.

This report discusses the first data concerning the early development of PKU mice. The focus of the complete study is to compare the early development of BTBR PKU mice to the C57B/l6 PKU mice, which have the same point mutation in the PAH gene but have a different background. Furthermore, this research will include naïve animals, because, especially in the C57B/l6 background our research group found that differences between HMZ and WT animals will disappear after handling (unpublished).

This study found a retarded physical development of HMZ animals the first two weeks after birth. Brain analysis is necessary to relate this to a similar pattern in brain development. Future studies are required to bring research closer to the establishment of the exact critical period and hence, the optimal period of treatment initiation.

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Supplementary protocols

Perfusion protocol for 14 day-old mice

Checklist of materials for perfusion of 20 mice

- ☐ 1000 ml 0.9% NaCl + Heparin
- ☐ 1000 ml bufferd Paraformaldehyde
- ☐ 1000 ml Milli Q H_2O
- ☐ 2 Erlenmeyer's with a 100 ml scale marking
- ☐ 2 beakers
- ☐ For post fixation 20 labelled cups filled with 10 ml 4% PFA
- ☐ For storage 20 labelled cups filled with 0.01 M PBS + 0.1% Sodium Azide

PBS = Phosphate Buffer (buffer for PH7.4) + 0.9% Saline for osmotic stability.
No NaAz if stored for 1 or 2 days; 0.01% for half a year, 0.1% if stored for over a year

- ☐ Instruments: scissors, tweezers, ect.
- ☐ Nitril Gloves (blue)
- ☐ Role of Paper

Checklist for freezing the tissue

- ☐ 500 ml 10% sucrose
- ☐ 500 ml 30% sucrose
- ☐ 20 cups filled with the 10% sucrose solution
- ☐ 5 liter Liquid Nitrogen
- ☐ Wooden slat for protecting the table
- ☐ Small Metal Container with a metal tissue holder for freezing
- ☐ Syphon with Milli Q H_2O for rinsing the brains
- ☐ Tweezers
- ☐ Role of Paper
- ☐ 20 cups filled with aluminium foil for storage of the brains in the -80 freezer

Solutions:

Rinsing Solution *(100% = 1000g/L (afgeleid van water), dus 1%=10g/L=1g/100ml)*

0.9% NaCl solution (9 gr NaCl in 900 ml H_2O) + heparin 400 U (0.25-1 ml per 100 ml NaCl solution is used.→ 0.5 ml heparine per 100 ml total solution). = 5 ml heparine
→Aanvullen tot 1 L.

Fixation Solution

4% PFA in 0.1 M PB (1 liter 4% PFA: 500 ml 0.2 M PB + 400 ml 10% PFA + 100 ml Milli Q H_2O pH 7.4)
Amount for 14 day old mice: 25 ml

Rinsing/fixation solution speed

For 14 day old mice: 8 ml/min (15,3 on small pump of Wanda)

Anesthetic

Pentobarbital, ip (stock solution stored at 4ºC or Avertin).
Amount for 14 day old mice: 0.1 ml= 100 µl

- Clean the pumps tubes with Milli Q H_2O and fill the system with rinsing solution.
- Anesthetize the animal with Pentobarbital i.p. and open the body to visualize the heart.
- Insert the needle into the left ventricle and cut the right atrium of the heart (the solution goes into the blood vessels but not in the lungs.).
- Rinse the animal (1 min.) and change to the fixation solution; pay attention to the needle when the fixation solution flowing (animal movement could take off the needle.).
- After the animal is perfused, remove the brain.
- Post-fixate the brains in 4% PFA in 0.01 M PB for ~48 hr
- Rinse brains twice with 0.01 M PBS and place on shaker overnight.
- Rinse brains again the next morning and at the end of the day change PBS with 10% sucrose in 0.01 M PB.
- Keep the brains in sucrose solution until they are at the bottom of the tube. By **room temperature** it takes ~ 18 hr. (in the coldroom, this process goes very slow!) Mouse brains: 10 ml greiner cups.
- Change 10% sucrose with 30% sucrose and keep the brains in sucrose solution until they are at the bottom of the tube.
- Rinse sucrose off the brains with MilliQ. Cut cerebellum/olfactory bulb (not after freezing! Brain will break!). Dry brain on a paper (rotate over paper), freeze with liquid nitrogen. Store temporarily in -20 until all brains are frozen, then immediately to -80.

Sucrose solution: 1 liter 10 % sucrose solution

- Dissolve 100 gr sucrose in 600 ml 0.01 M PBS
- Complete to 1 liter with 0.01 M PBS

Sucrose solution: 1 liter 30 % sucrose solution

- Dissolve 300 gr sucrose in 600 ml 0.01 M PBS
- Complete to 1 liter with 0.01 M PBS

Behavioral protocols

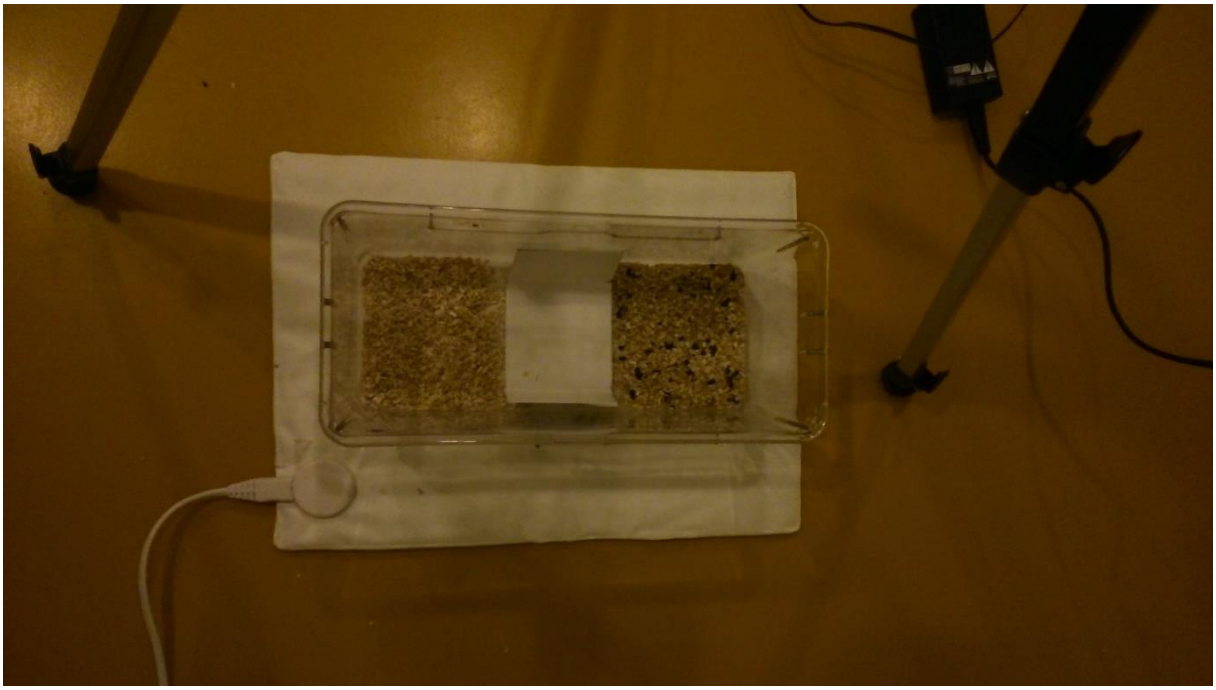
Protocol Righting reflex

- Place the litter in a heated holding cage (set the temperature of the heating pad at 32-34 ^{o}C), this will result in an ambient temperature of the holding cage between 22-24 ^{o}C to maintain normal body temperature.
- Grab an animal from the litter and place it on another heating pad set at 22-24 ^{o}C.
- Turn on the camera
- Keep the number of the animal in front of the camera
- Roll the animal on its back
- Keep the animal on its back until it does not move anymore by holding all paws
- Release the animal by letting go the paws
- Videotape until animal has gained its normal position on 4 paws again
- Turn off the camera
- Place the animal back into another heated holding cage
- Repeat for all the animals of the litter
- Analyze the videos by using a stopwatch (repeat several times for the same animal)

Protocol homing test

- Place the litter in a heated holding cage (set the temperature of the heating pad at 32-34 ^{o}C), this will result in an ambient temperature of the holding cage between 22-24 ^{o}C to maintain normal body temperature.
- Separate litter at least 15 minutes from the mother
- Grab an animal from the litter
- Turn on the camera
- Keep the number of the animal in front of the camera
- Place the animal in the center of the homing test device with the walls closed
- Habituate to the center for 1 minute
- After 1 minute remove the walls, so the animal can enter the complete homing test device
- Record for 5 minutes the behavior
- After 5 minutes turn of the camera
- Remove the animal from the homing test device and put it back into another heated holding cage or with the mother
- Clean the homing test device (center) with 70% alcohol, followed by water and dry it
- Repeat for all the animals of the litter
- Analyze the videos by Ethovision XT
- Determine the arena and the different zones (Left, Center, Right)

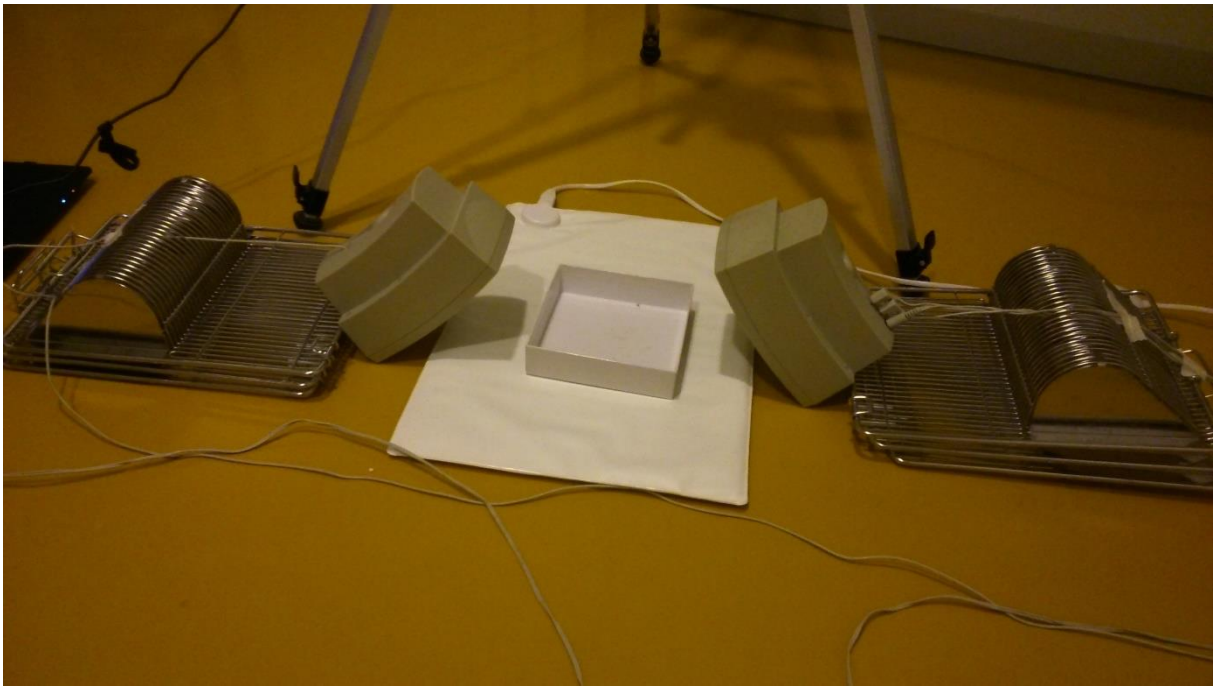
- Determine detection setting where there is a good representation of the animal with no background
- Acquire data for all animals
- Determine which parameters you want to use (Distance Moved, Velocity, Mobility…)
- Export output analysis into an excel file



Supp Fig. 1: **Homing test device.** On the left side is clean sawdust and on the right side is home cage sawdust. The device is placed on a heating pad to maintain normal body temperature.

Protocol Auditory startle

- Grab an animal from the litter in the home cage
- Turn on the camera
- Keep the number of the animal in front of the camera
- Place the animal in the heated testing box
- Turn on the stopwatch
- After 1 minute play the tone (single triangular tone of 7435 Hz; 85-90 dB; 200 msec, NCH tone generator)
- After 30 minutes play the same tone again
- Turn of the camera and the stopwatch
- Remove the animal from the box and return it to the home cage
- Repeat for all the animals of the litter
- Analyze the videos by scoring whether there is a startle response to the tone (yes, little, no)



Supp Fig. 2: **Set up for recording auditory startle response**. The holding box is placed on a heating pad. The speakers are placed in an angle of approximately 45 degrees.