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Biochemical and behavioural profile of NTBC treated Tyrosinemie type 1 mice

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ABSTRACT

Background: Tyrosinemia type 1 (HT1) is a rare metabolic disorder caused by a defect in the tyrosine catabolic pathway. Since HT1 patients are treated with NTBC, outcome improved and life expectancy greatly increased. However extensive neurocognitive and behavioural problems have been described, which might be related to treatment with NTBC, the biochemical changes induced by NTBC, or metabolites accumulating due to the enzymatic defect characterizing the disease.
Objective: To study the possible pathophysiological mechanisms of brain dysfunction in HT1, we assessed blood and brain LNAA, and brain monoamine neurotransmitter metabolite levels in relation to behavioural and cognitive performance of HT1 mice.
Design: C57BL/6 littermates were divided in three different experimental groups: HT1, heterozygous and wild-type mice ($n = 10$; 5 male). All groups were treated with NTBC and underwent cognitive and behavioural testing. One week after behavioural testing, blood and brain material were collected to measure amino acid profiles and brain monoaminergic neurotransmitter levels.
Results: Irrespective of the genetic background, NTBC treatment resulted in a clear increase in brain tyrosine levels, whereas all other brain LNAA levels tended to be lower than their reference values. Despite these changes in blood and brain biochemistry, no significant differences in brain monoamine neurotransmitter (metabolites) were found and all mice showed normal behaviour and learning and memory.
Conclusion: Despite the biochemical changes, NTBC and genotype of the mice were not associated with poorer behavioural and cognitive function of the mice. Further research involving dietary treatment of FAH−/− are warranted to investigate whether this reveals the cognitive impairments that have been seen in treated HT1 patients.

1. Introduction

Tyrosinemia Type 1 (HT1; OMIM 276600, autosomal recessive fumarylacetoacetate deficiency) is clinically characterized by acute liver failure, renal tubulopathy, porphyria like syndrome and the development of hepatocellular carcinoma in early life. In 1992, 2-(2-nitro-4-trifluoromethylbenoyl)-1,3-cyclohexanedione (NTBC) was introduced as a new treatment option [1]. NTBC prevents the accumulation of toxic metabolites by inhibiting tyrosine catabolism upstream from the primary enzymatic defect. This treatment largely improved the clinical outcome, especially when combined with early diagnosis by population based neonatal screening [2–4].

However, behavioural alterations and neurocognitive impairments have been observed in HT1 patients. These impairments among others include a lower IQ, deficits in executive functioning and social cognition and behavioural problems [5,6]. Proposed aetiologies for these problems have included large neutral amino acid (LNAA) imbalances in the central nervous system due to chronically elevated blood and brain

Abbreviations: 5-HIAA, 5-Hydroxyindoleacetic acid; DOPAC, 3,4-Dihydroxyphenylacetic acid; FAH, fumarylacetoacetate hydrolase; FC, fear conditioning; HT1, Tyrosinemia type 1; HVA, homovanillic acid; LNAA, large neutral amino acid; NOR, novel object recognition; NTBC, 2-(2-nitro-4-trifluoromethylbenoyl)-1,3-cyclohexanedione; OF, open field; WM, water maze; WT, wild-type; YM, Y-maze.

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tyrosine, adverse effects of NTBC therapy, and previously unrecognized complications of the disease itself due to accumulating metabolic intermediates [5]. The available literature often only describes group differences between Tyrosinemia patients and control participants, while research into these pathophysiological mechanisms of brain dysfunction has been very limited so far.

Fumarylacetoacetate deficient (*Fah$^{\Delta exon5}$*) mice were created in 1993 and served as a useful model for studies regarding the pathophysiology and treatment of HT1, although research in these mice has primarily focused upon liver associated problems [7]. To study the possible pathophysiological mechanisms of brain dysfunction in HT1, we therefore assessed blood and brain LNAA and brain monoamine neurotransmitter levels in relation to behavioural and cognitive performance of *Fah$^{\Delta exon5}$* mice.

2. Methods

2.1. Animals

C57BL/6 –*Fah$^{\Delta exon5}$* mice were bred in the Harding colony at the Department of Molecular and Medical Genetics at Oregon Health & Science University. C57BL/6 –*Fah$^{\Delta exon5}$* mice harbour an induced deletion of fumarylacetoacetate hydrolase (FAH) exon 5. Mice that are homozygous for this deletion (hereafter designated FAH −/− mice) exhibit complete FAH deficiency, tyrosinemia, and NTBC dependency analogous to that of humans with HT1. C57BL/6 –*Fah$^{\Delta exon5}$* mice were derived from the original Sv129-*Fah$^{\Delta exon5}$* strain previously described [8]. For these experiments, litters with all three mixed genotypes FAH −/−, FAH +/−, and FAH +/+ were generated through breeding of heterozygotes (FAH+/− mice). At three weeks of age, a small ear punch was biopsied for identification and genetic analyses was performed by PCR to confirm genotype. In total, 10 FAH −/− mice (5 male and 5 female), 10 FAH +/− (5 male and 5 female) and 10 FAH +/+ (5 male and 5 female) were included in the study. Mouse were co-reared in a temperature controlled room (21 ± 1 °C) on a 12 h light-dark cycle. Mice were fed standard mouse chow (5LOD food pellets (LabDiet, St. Louis, USA)) and water (supplemented with 8 mg/L NTBC) provided at libitum. The 5LOD food pellets contain 241 g/kg protein with 7.4 g/kg tyrosine and 10.7 g/kg phenylalanine. All procedures were approved by the Institutional Animal Care and Use Committees at OHSU and BNL and were in compliance with all Federal regulations.

2.2. Experimental design

Since FAH −/− mice need continuous treatment with NTBC starting already before birth, all heterozygous dams were treated with 8 mg/L NTBC dissolved in the drinking water, from pregnancy until weaning, similarly as in earlier studies [9–11]. In this way, all experimental mice, including the FAH −/−, received NTBC through the mother pre- and postnatal, up to weaning age. At 4 weeks of age, mice were weaned and assigned to one of the three experimental groups, FAH −/− (or HT1) mice, FAH +/− (or heterozygous) mice and FAH +/+ (or wild-type) mice. Body weight was measured weekly. The mean age (±SD) of the mice was 106 ± 6 days at the start of the first behavioural test. One week after the testing, the mice were euthanized by heart puncture and exsanguination under inhalation-anesthetics with isoflurane after 3–5 h fasting to eliminate prandial effects on amino acid levels. Subsequently, tissues were harvested. The blood was centrifuged at 12.000 RPM in an Eppendorf microcentrifuge for 10 min to obtain serum. The animals were perfused with 15 mL saline (0.9%) via the left cardiac ventricle to clear blood from the cerebral circulation. Brain material was collected, split sagitally, and immediately submerged in liquid nitrogen. Serum and brain material were stored at −80 °C until further analyses.

2.3. Behavioural and cognitive testing

All behavioural and cognitive testing was conducted in the Raber laboratory at the Department of Behavioural Neuroscience at OHSU. All mice were housed individually during the testing period. The experimental paradigm consisted of the following tests: spontaneous alterations in the Y-maze, exploratory behaviour and measures of anxiety in the open field (OF), novel object recognition (NOR), spatial learning and memory in the water maze (WM), and emotional learning and memory in the fear conditioning (FC) test. An overview and timeline of the different behavioural and cognitive tests is shown in Fig. 1.

Y-maze.

Mice were placed in the center of the Y-maze with three equal arms (O'Hara & Co, Tokyo, Japan) and were able to freely explore the Y-maze for 5 min. All trials were video recorded and the number of arm entries and the percentage of alternation was analysed manually, as described [12,13].

2.3.1. Open field and object recognition

A square open field (41 × 41 cm) was used during two simultaneous days to assess activity and anxiety-like behaviour. At the start of the trial, the animal was placed in the center of the field and left to freely explore the OF for 10 min. All tests were video recorded and total distance (as measure of activity) and time spent in the center of the field (as measure of anxiety-like behaviour) were analysed using Noldus Ethovision 15 XT video tracking (Wageningen, The Netherlands). Arenas were cleaned with 0.5% acetic acid between trials.

The NOR test was used to examine memory retention by the ability of the mice to recall a familiar object after 24 h. After habituation (done with the OF test), mice were allowed to freely explore 2 identical objects for 15 min during the familiarization phase. Twenty-four hours later, mice were exposed to one of the identical and one new object during the test phase for 15 min. The exploratory behaviour of the mice was analysed manually based on the videos acquired using Buttonbox 5 (Behavioural Research Solutions, LLC).

2.3.2. Water maze

The ability to locate a visible or a hidden platform was evaluated using the Morris water maze and Ethovision *15 XT* video tracking software. The maze (circular tub, 122 cm diameter) contained a platform which was visible and marked with a beacon or hidden just below the surface of opaque (using chalk) water. The water maze test design involved five consecutive days with 2 trials per day (am and pm). Each session contained two trials for each mouse with a 10–15 min inter-trial interval. The mice had 60 s in each trial to locate the platform. If the mice did not locate the platform within 60 s, they were led to the

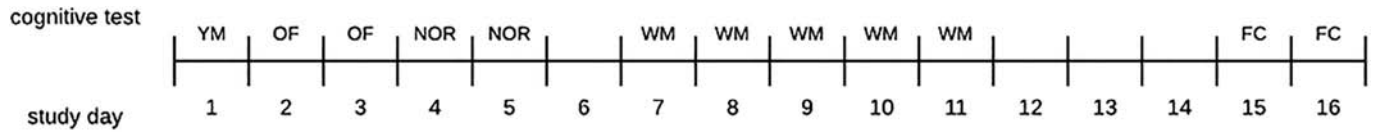

Fig. 1. Overview of behavioural tests. A timeline of the cognitive-behavioural tests performed during the study, including the following tests: Y-maze (YM), open field (OF), novel object recognition (NOR), Morris water maze (WM) and fear conditioning (FC).

platform by the experimenter and allowed to remain on the platform for 3 s. All mice were trained to find the hidden platform during the first 6 sessions (12 trials) using different drop locations around the pool. Session 7–9 were carried out with a new hidden platform location and during session 10 the platform was made visible with a beacon to assess task learning. Time to find the platform (latency), cumulative distance to the platform, and swim speed were analysed.

2.3.3. Fear conditioning

Hippocampus-dependent (contextual) memory and hippocampus-independent (cued) fear learning and memory were assessed using the FC task using near-infrared video and automated analysis and Video Freeze SOF 843 automated scoring software (Med Associates Inc. St. Albans, VT, USA). During fear conditioning, mice learn to associate an environmental contact or cue (tone, conditioned stimulus) with a mild foot shock (unconditioned stimulus). Associative learning is assessed based on freezing behaviour. During the first session, mice were placed inside a white LED lit (100 lx) chamber with metal grid floor for a total duration of 6 min. After 2 and 4 min a tone (80 dB, 2800 Hz) was presented for 28 s followed by a 2 s lasting 0.35 mA foot shock. Average motion (cm/s) and percentage of time freezing at baseline (before the tone and shock) were analysed to determine whether there were potential pre-conditioning group differences in behaviour such as immobility. Potential group differences in the motion during the two shocks on day 1 were also analysed. Chambers were cleaned with 0.5% acetic acid solution. The following day, mice were reintroduced to the same chamber for a total duration of 5 min without tone and shock and freezing behaviour was assessed as measure of contextual memory. Four hours later, mice were placed into a novel context (containing a smooth white plastic covering the wire grid floor, a "tented" black plastic ceiling, and scented with hidden vanilla extract soaked nestlets) for a total duration of 4.5 min. After 90 s, a tone (80 dB, 2800 Hz) was presented for the remaining 3 min and freezing behaviour before and during the tone was analysed as indicator of cued memory. The chambers were cleaned between trials with a 10% isopropanol solution.

2.4. Biochemical analyses

Frozen brain was first pulverized to a powder using a cryoPREP Dry Impactor (Covaris, Masschusetts, USA), and brain powder was divided into aliquots. Frozen brain powder for amino acid measurements was processed to 20% (weight: volume (w:v)) homogenates in phosphate-buffered saline (pH 7.4). Brain homogenates were sonified on ice at 10 W. Next, samples were centrifuged at 12.800 RPM in an Eppendorf microcentrifuge for 10 min (4 °C), and the supernatant was stored maximally 2 days at −80 °C until further analyses.

Amino acid levels in serum and in brain homogenates were analysed by pre-column derivitization with 6-aminoquinolyl-N-hydroxy-succinimidyl carbamate (AQC, Waters AccQ Tag™ derivitization system) and separation by ultra-high performance liquid chromatography and UV absorbance detection (Waters Acquity™ UPLC, Milford, MA) using the Waters Masstrak Amino Acid Analysis method. Serum samples were de-proteinized by adding an equal volume of 10% sulfosalicylic acid containing the non-physiologic amino acid norvaline as an internal recovery standard (final concentration of norvaline 125 μM). Serum amino acid levels were corrected for dilution and reported as μM. Brain tissue homogenates were treated similarly by deproteinizing with and equal volume of 10% sulfosalicylic acid containing norvaline as internal standard. After correcting for dilution, the measured amino acid levels in the brain homogenates were corrected for the wet weight of the brain powder and expressed as nmol/g wet weight.

For brain monoamine neurotransmitter metabolite measurements, mouse hemibrains were pulverized using a CyroPrep system (Covaris, Massachusetts, USA) according to the manufacturer's instructions, and brain powder was divided into aliquots. Lysates were obtained by adding to ice-cold homogenizing buffer (50 mM Tris-HCl, pH 7.5, 0.1 M KCl, 1 mM EDTA, 1 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride, 1 μM leupeptin, and 1 μM pepstatin), according to the amount of powder in a 2 mL tube with a metal bead inside (QIAGEN, Hilden, Germany). Samples were lysed in a TissueLyser (QIAGEN) two times at 20 Hz for 90 s. After, samples were centrifuged for 20 min at maximum speed and supernatants were aliquoted in several tubes and kept at −80 °C until further use. The whole procedure was performed at 4 °C. Protein concentration was measured using the ABBOTT Alinity C System, a turbidimetric method (Abbot, Abbotpark, Illinois, USA, kit-no. 7P5920). For further preparation, 10% 1 M HCl was added to brain lysates and samples were centrifuged through a Amicon Ultra-0.5 mL filter (Merck-Millipore S.A., Darmstadt, Germany) for 30 min at maximum speed and 4 °C. Analysis of brain monoamine neurotransmitter metabolites in the filtered lysate was performed on a modified Thermo Fisher UltiMate 3000 High Sensitivity HPLC Electrochemical System (Thermo Fisher Scientific, Waltham, Massachusetts, USA). Injection of sample volume was 20 μL and separation of the compounds was achieved using an YMC-Hydrosphere UHPLC column (C18 12 nm, S-2.0 μm, 150 × 30 mm, YMC Inc., Wilmington, NC, USA). As mobile phase, a 56.7 mM sodium phosphate buffer, containing 5 mM octanesulphonic acid, 50 μM EDTA, 0.28% phosphoric acid (85%), and 23% methanol (pH 3.1, adjust with concentrated 10 M NaOH), was used with an isocratic flow rate of 490 μL/min. The column is maintained at 27 °C by a surrounding TCC-3000SD column-thermostat. The analytical cell (Coulometric Cell Model 6011RS, Thermo Fisher Scientific), within the electrochemical detector ECD-3000RS (Thermo Fisher Scientific), was adjusted to a 10 mV potential and 100 μA gain range for the upstream electrode, and +400 mV potential, plus 500 nA gain range for the downstream electrode, with a response time of 1 s. Data was analysed using the Chromeleon Chromatography Data System (CDS) Software 7.1 (Thermo Fisher) and corrected for the protein concentration of the homogenate.

2.5. Statistics

All data are expressed as mean ± SEM unless otherwise specified. For all biochemical and behavioural analyses, the same approach has been used. All analyses were done using pooled data including male and female mice with the different genotypes. Main effects of both sex and genotype (FAH −/−, FAH +/− and FAH +/+, and possible interactions between them were studied using Two-way ANOVA and/or repeated measures ANOVA. In case of non-normal distributions, Ln transformation has been performed.

The body weights at the start and end of the experiment and brain weights at the end of the study were analysed by Two-way ANOVA with sex and genotype as factors. The weight change during the experiment was analysed with repeated measures ANOVA with weight at start and end of the experiment as within-subject factor and sex and genotype as between-subject factors.

All analyses of serum and brain biochemistry were performed using Two-way ANOVA analyses with sex and genotype as factors and Tukey post hoc tests when appropriate. As all mice were treated with NTBC, which could influence serum and brain biochemistry, serum and brain levels of C57Bl/6 wild-type (WT) mice treated without NTBC are included in the graphs as reference values. The mice used for the reference values were WT mice of the same age, who received the same food, were handled in the same room by the same researchers and were euthanized in the same manner. Regarding, serum and brain LNAA levels, 18 C57Bl/6 WT mice (9 male, 9 female) were used for reference values, whereas for neurotransmitter levels 10 C57Bl/6 WT mice (5 male, 5 female) were used.

The percentage of alternation in the Y-maze test and discrimination index in the NOR test were analysed using two-way ANOVA analyses with sex and genotype as factors and Tukey post hoc tests when appropriate. Repeated measures ANOVA was performed to assess the activity

and anxiety in the open field with total distance as the within-subject factor or time spent in the center of the open field as the within-subject factor (both 2 levels, day 1 and 2) and sex and genotype as the between-subject factor in both analyses. Main effects of genotype and sex on the water maze were assessed with two-way ANOVA (average velocity) and repeated measures ANOVA with latency time or cumulative distance to the target as within-subject factors. Results on the fear conditioning test were analysed with two-way ANOVA (baseline motion, motion during shocks, baseline freezing and contextual freezing) with sex and genotype as factors. Repeated measurements ANOVA were performed to study differences between the pre-tone freezing and freezing during the tone (within subject factor) in the cued fear memory test with genotype and sex as between subject factors. All statistical analyses were carried out using SPSS23 software (Chicago, IL) and a $p < 0.05$ was considered significant. All figures were generated using Graphpad Prism 9 (San Diego CA).

3. Results

3.1. General health

At the start of the experiment, males exhibited a higher body weight than females ($p = 0.019$), but no significant differences between the different experimental groups were found ($p = 0.543$). The weight of all mice increased during the study ($p < 0.001$), although males gained more weight than females ($p < 0.001$). Furthermore, FAH −/− mice grew more during the experiment than the other groups of mice ($p < 0.05$). Post-hoc analyses showed that FAH −/− males were heavier at the end of the experiment than FAH +/− and FAH +/+ mice ($p = 0.002$ and $p = 0.008$ respectively) (Fig. 2). Brain weights of the mice, measured at the end of the experiment, were not significantly different between FAH −/−, FAH +/− and FAH +/+ mice ($p = 0.295$) nor between males and females ($p = 0.984$).

3.2. Biochemistry

Serum phenylalanine, tyrosine and tryptophan levels are displayed in Fig. 3A, whereas all other LNAA levels are listed in Table 1. When serum LNAA are compared to the reference levels, NTBC treatment resulted in a great increase in serum tyrosine levels as expected, whereas especially serum phenylalanine levels tended to be slightly lower in all mice. When the different experimental groups were compared to each other, serum tyrosine levels were significantly lower in FAH−/− mice (582 ± 26 μmol/L) when compared to FAH+/− mice (763 ± 31 μmol/L; $p = 0.001$) and FAH +/+ mice (808 ± 30 μmol/L; $p < 0.001$). No significant differences among all other serum LNAA levels were found. Female mice showed significantly higher serum tryptophan ($p < 0.001$), isoleucine ($p = 0.013$), methionine ($p = 0.001$) and threonine ($p = 0.007$) levels than male mice. Serum phenylalanine and tyrosine levels were not significantly different between males and females ($p = 0.601$ and $p = 0.839$ respectively). There were no significant interactions between sex and genotype affecting the serum concentration of any large neutral amino acid. Differences in serum tryptophan levels between males and females were also seen in the untreated C57Bl/6 mice used for reference values. Sex based differences in serum LNAA levels are displayed in Supplementary Fig. 1.

Brain phenylalanine, tyrosine and tryptophan contents are displayed in Fig. 3B, whereas all other LNAA are listed in Table 1. When brain LNAA are compared to reference levels, all mice showed increased tyrosine levels, whereas brain levels of the other LNAA (especially phenylalanine and tryptophan) tended to be decreased. When the different experimental groups were compared to each other, brain tyrosine levels were significantly lower in FAH−/− mice (511 ± 24 μmol/L) compared to both FAH+/− (690 ± 23 μmol/L; $p = 0.001$) and FAH +/+ mice (747 ± 35 μmol/L; $p < 0.001$). All other brain LNAA levels were not significantly different between the different experimental groups. Female mice exhibited significantly higher brain phenylalanine ($p = 0.021$), tryptophan ($p < 0.001$), isoleucine ($p = 0.006$), leucine ($p = 0.019$) and methionine ($p = 0.001$) levels. Sex based differences in brain LNAA levels are displayed in Supplementary Fig. 1.

Brain monoaminergic neurotransmitters and associated metabolite levels are displayed in Table 2. Although brain 3,4-Dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) levels seem to be slightly higher in all NTBC treated animals (without reaching statistical significance), all neurotransmitters and associated metabolites are generally in accordance with reference values and no significant differences between the different experimental groups were found.

Table 1 shows serum and brain amino acid concentrations in the different experimental groups. Lysine is included since this is an essential amino acid that does not use the same transporters as all LNAA. Data are expressed as mean ± SEM. *FAH−/− mice have significantly lower serum and brain tyrosine levels when compared to FAH +/− and FAH +/+ mice.

Table 2 shows levels of brain dopamine, homovanillic acid (HVA), 3,4-Dihydroxyphenylacetic acid (DOPAC), serotonin and 5-Hydroxyindoleacetic acid (5-HIAA) neurotransmitters and associated metabolites in the different experimental groups. No significant differences between the different experimental groups were found. Data are expressed as mean ± SEM.

3.3. Behavioural and cognitive performance

The total distance ("activity") in the open field decreased with a lower distance on the second day ($p < 0.001$) (Supplementary Fig. 2), indicating habituation to the OF. No significant differences based on genotype or sex were found ($p = 0.311$ and $p = 0.541$ respectively). However, since the activity does not seem to decrease in the FAH +/− mice (Supplementary Fig. 2), repeated measures analyses were performed again for each genotype separately, with sex as between-subjects factor. These analyses indeed showed significant habituation in FAH−/− and

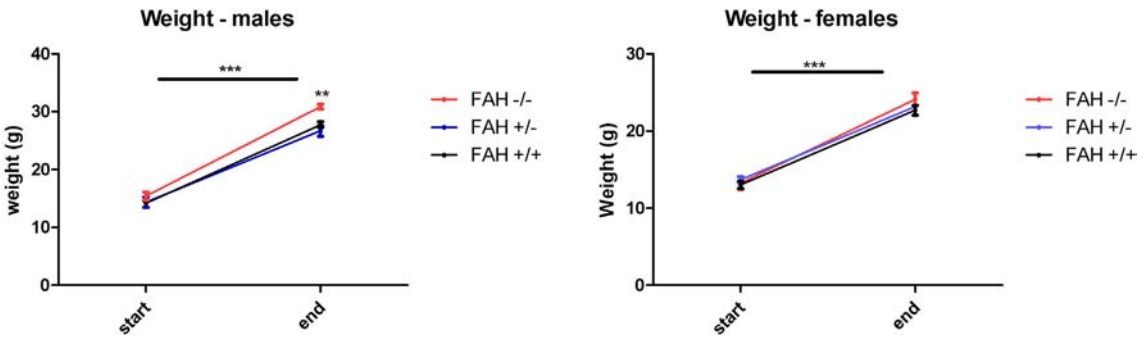

Fig. 2. Body weights at the start and end of the study shown as mean ± SEM.

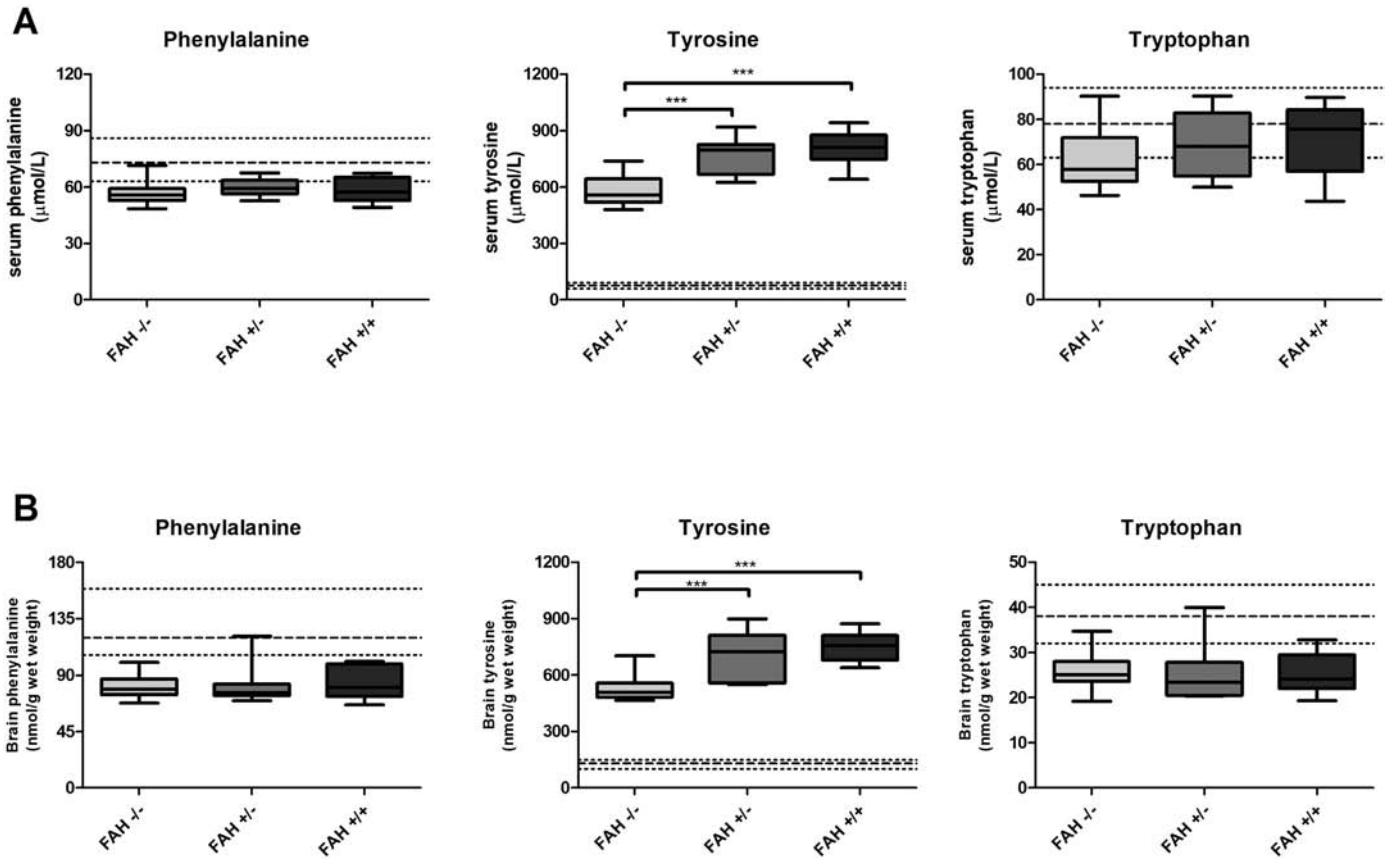

Fig. 3. Serum (A) and brain (B) phenylalanine, tyrosine and tryptophan levels in the different experimental groups ($n = 10$ in each group) presented as boxplots with min-max whiskers. The dotted lines represent the median and IQR of a reference group of C57Bl/6 WT mice treated without NTBC.

Table 1
Serum and brain amino acids.

	Reference values (µmol/L)	FAH −/− (µmol/L)	FAH +/− (µmol/L)	FAH +/+ (µmol/L)	*p*-value
Serum amino acids					
Phenylalanine	82 ± 7	57 ± 6	60 ± 5	59 ± 7	0.540
Tyrosine	78 ± 5	582 ± 83*	763 ± 98	808 ± 90	<0.001
Tryptophan	79 ± 4	62 ± 14	69 ± 15	70 ± 16	0.126
Valine	243 ± 7	219 ± 24	217 ± 19	230 ± 33	0.579
Isoleucine	118 ± 5	114 ± 16	112 ± 12	118 ± 21	0.785
Leucine	182 ± 7	177 ± 27	171 ± 16	176 ± 27	0.834
Methionine	55 ± 2	51 ± 7	55 ± 6	55 ± 8	0.220
Histidine	75 ± 2	71 ± 9	68 ± 6	68 ± 7	0.744
Threonine	149 ± 7	117 ± 24	125 ± 18	135 ± 26	0.252
Lysine	222 ± 9	230 ± 14	230 ± 12	229 ± 15	0.985
	Reference values (nmol/g wet weight)	FAH −/− (nmol/g wet weight)	FAH +/− (nmol/g wet weight)	FAH +/+ (nmol/g wet weight)	p-value
Brain amino acids					
Phenylalanine	131 ± 7	81 ± 10	81 ± 15	83 ± 13	0.932
Tyrosine	129 ± 5	532 ± 69*	702 ± 129	754 ± 75	<0.001
Tryptophan	38 ± 2	26 ± 4	25 ± 6	25 ± 5	0.860
Valine	128 ± 4	96 ± 10	92 ± 13	95 ± 14	0.710
Isoleucine	75 ± 3	58 ± 8	55 ± 9	57 ± 10	0.542
Leucine	183 ± 6	132 ± 21	125 ± 24	132 ± 23	0.612
Methionine	83 ± 3	59 ± 10	60 ± 9	60 ± 9	0.921
Histidine	104 ± 5	88 ± 5	87 ± 11	90 ± 9	0.699
Threonine	259 ± 9	207 ± 27	203 ± 33	203 ± 27	0.915
Lysine	200 ± 6	185 ± 5	187 ± 10	184 ± 7	0.960

Table 2
Brain neurotransmitters and associated metabolites.

	Reference values (nmol/g protein)	FAH −/− (nmol/g protein)	FAH +/− (nmol/g protein)	FAH +/+ (nmol/g protein)	p-value
Dopamine	280 ± 18	281 ± 94	343 ± 116	265 ± 57	0.227
HVA	24 ± 2	30 ± 7	30 ± 12	28 ± 5	0.753
DOPAC	18 ± 2	26 ± 11	29 ± 8	27 ± 7	0.767
Serotonin	106 ± 13	92 ± 19	103 ± 18	90 ± 16	0.311
5-HIAA	55 ± 9	63 ± 26	62 ± 15	53 ± 14	0.348

WT mice ($p = 0.048$ and $p = 0.018$ respectively), without a significant effect of sex ($p = 0.64$ and $p = 0.407$ respectively). However, no habituation was seen in FAH +/− mice ($p = 0.129$), which is mainly caused by poorer habituation of the FAH+/− females ($p = 0.03$). The reason for these differences is unknown.

The time spent in the center of the open field, as measure of anxiety, was not significantly different between the first and second day ($p = 0.516$), nor were there any differences based on genotype or sex ($p = 0.317$ and $p = 0.079$ respectively).

The results of Y-maze and NOR testing are displayed in Supplementary Fig. 3. For both assessments, the percentage of alternation (Y-maze) and the discrimination index (NOR), no differences were found based on genotype ($p = 0.417$ and $p = 0.542$ respectively), nor differences based on sex ($p = 0.571$ and $p = 0.365$ respectively). In the NOR, all groups of mice (FAH−/−, FAH+/− and FAH +/+) performed significantly above change level (all $p < 0.01$).

The results of the WM testing are displayed in Fig. 4. Average swim speed was analysed first to rule out that possible differences between groups might contribute to performance differences in this test. The average swim speed was analysed during session 10, when the platform was made visible with a beacon. There were no effects of either genotype ($p = 0.683$) nor sex ($p = 0.220$) upon the average swim speed. During the training sessions (session 1–6), the latency time and cumulative distance to the target decreased significantly (both $p < 0.001$). Post hoc tests showed that both latency and cumulative distance to target were significantly lower at session 6 compared to session 1–4 ($p < 0.05$). After the platform location changed (session 7–9), the latency time and cumulative distance to the target decreased significantly as well (both $p < 0.001$), with lower latency and cumulative distance to the target values in session 9 when compared to session 7 and 8 ($p < 0.05$). There were no effects of either genotype or sex on latency or cumulative distance to the target during both training sessions (session 1–6) and after the platform location changed (session 7–9).

The results of the fear conditioning test are displayed in Fig. 5. During the training session, baseline motion, baseline freezing and the motion during the shocks were not statistically significant between the different genotypes of mice, nor between the different sexes (Fig. 5A). The percentage of time freezing increased during the contextual fear memory test when compared to baseline freezing levels. Although no main effects of genotype or sex on the percentage of time freezing during the contextual fear memory test were found ($p = 0.308$ and $p = 0.346$ respectively), there was a significant interaction between genotype and sex ($p = 0.004$). This interaction has been made visible in Fig. 5B, showing that especially male FAH−/− and female FAH+/+ mice show higher percentage of freezing than the other groups of mice. During the cued fear memory test, freezing levels during the tone were significantly higher when compared to freezing levels

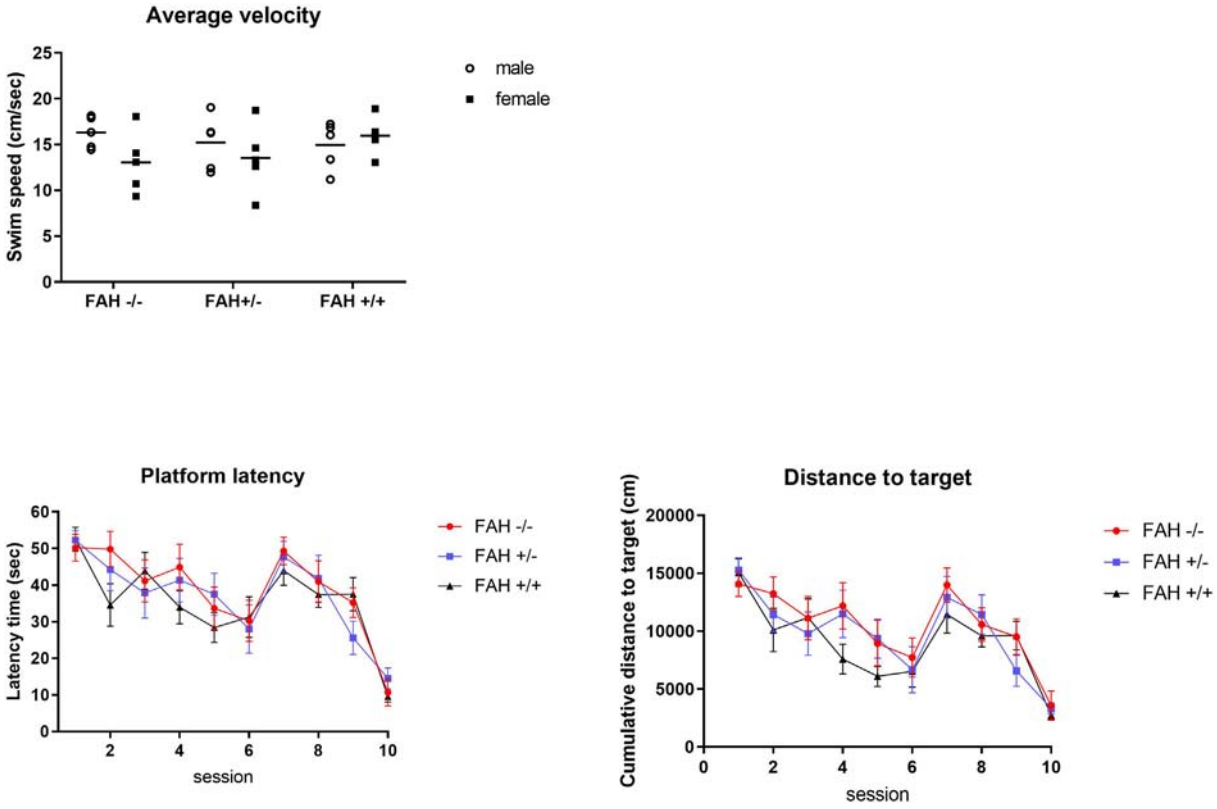

Fig. 4. The latency time and cumulative distance to the target of each session is calculated by the average of the two trials performed each part of the day. During session 1–6, the location of the hidden platform remained constant. Session 7–9 were carried out with a new platform location. During session 10 the platform was made visible with a beacon. The swim speed of the mice was calculated by the average speed at session 10. Data on swim speed is presented as individual dots with the median and latency time and cumulative distance to the target are presented with the mean ± SEM. The swim speed did not differ between the different groups of mice and apart from a significant learning effect, no significant differences on latency time and distance to target were seen between the different groups of mice.

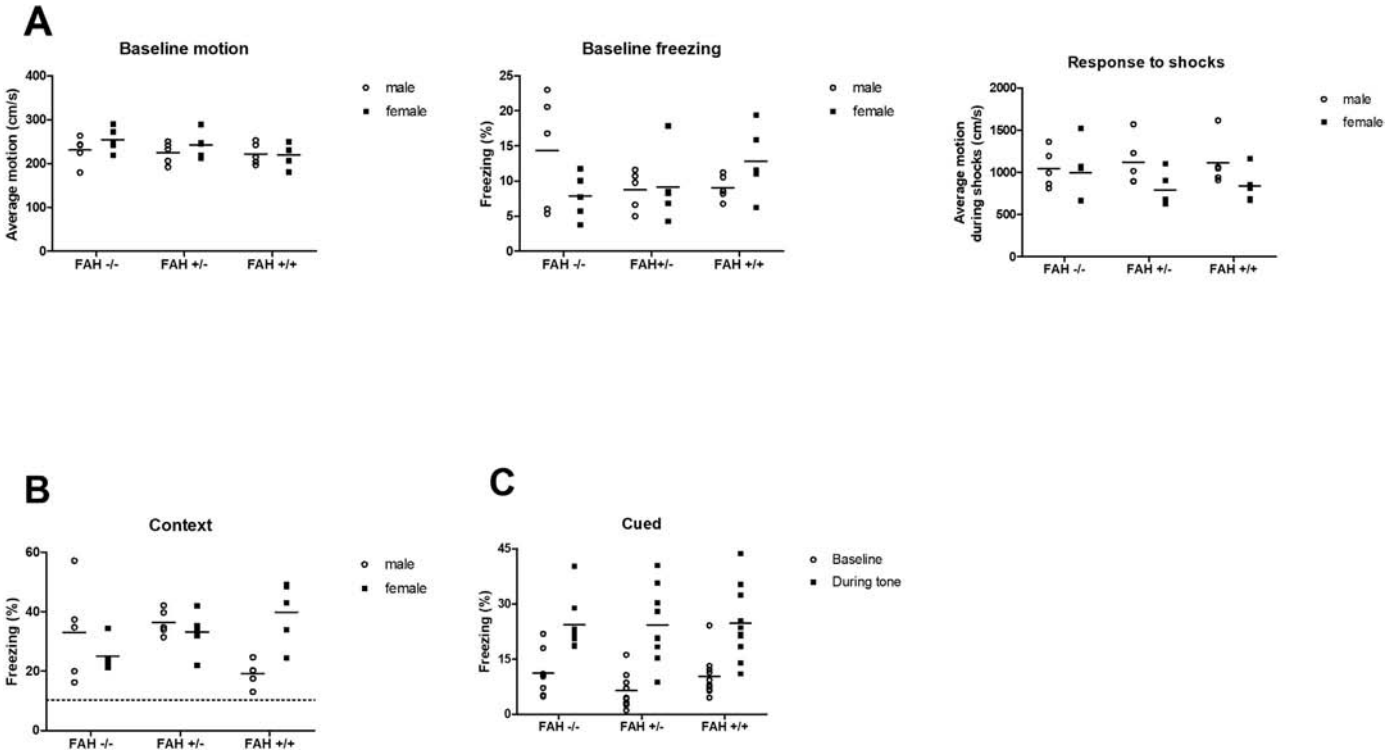

Fig. 5. Results on fear conditioning showed as individual dots with means. During the training session (A) baseline motion, motion during shocks and % of freezing were calculated. There were no effects of either genotype nor sex on the different variables. B shows freezing levels during the contextual fear memory test. The % of time freezing increased compared to freezing levels at baseline (dotted line reflects the overall mean of baseline freezing). C shows the percentage of time freezing during the pre-tone period and during the tone in the cued fear memory test (male and female together). Although the percentage of time freezing increased significantly during the tone ($p < 0.001$), no effects of genotype and sex were seen.

during the pre-tone period ($p < 0.001$). However, there were no effects of genotype and sex on freezing levels ($p = 0.287$ and $p = 0.137$ respectively).

4. Discussion

In this study, we investigated serum and brain LNAA levels, brain neurotransmitter levels and cognitive-behavioural performance of FAH −/− mice. The main findings of this study are that independent of the genetic background, NTBC treated animals show 1) high serum tyrosine levels, whereas serum phenylalanine levels tend to be lower than normal, 2) high brain tyrosine levels, with low to normal levels of all other brain LNAA, including low brain phenylalanine, and 3) monoaminergic neurotransmitter and associated metabolite levels within the normal range. Despite these biochemical changes induced by NTBC and the differences in genotype, the behavioural and cognitive performance of the FAH−/− mice remained within normal range.

Before discussing these findings in more detail, some methodological issues need to be addressed. In the current study, the mice were singly housed during the behavioural testing. Therefore, we cannot exclude that stress associated with singly housing might have contributed to some of the behavioural data. This experiment did not include a group of WT mice not receiving NTBC (nor heterozygous mice without NTBC). As NTBC could influence both blood and brain biochemistry and behavioural and cognitive performance, it would be interesting to compare NTBC treated animals to WT animals not receiving NTBC. Therefore, we included biochemical data of WT mice not receiving NTBC as reference values. These “reference” mice were of the same age, underwent the same procedure but did not receive NTBC. Furthermore, previous studies did not show an effect of NTBC on cognitive-behavioural performance of mice [10,11,14] and during this experiment, all mice showed appropriate learning and memory during the cognitive tests, indicating no clear effect of NTBC (and its associated biochemical changes) on behavioural or cognitive performance.

During this study, FAH−/− grew more than the other groups of mice, especially FAH−/− males were heavier than heterozygous and WT mice at the end of the study. Although, weight gain of NTBC treated FAH−/− mice haven't been studied often to date, current literature mostly indicates normal growth of NTBC treated animals [11]. On the other hand, it is known that obesity is frequently seen in HT1 patients [15,16]. Since food intake might be related to the weight gain found in this study and/or obesity in HT1 patients, future research should include information about food intake as well, which could not be studied adequately in this study.

To date, only one other published study has investigated brain LNAA and neurotransmitter levels in HT1 mice [11]. In agreement with that study, our current data also demonstrate that, irrespective of the genetic background, NTBC treated mice show greatly increased serum and brain tyrosine levels. However, in contrast to previous studies with HT1 mice, tyrosine levels in blood and brain are higher in NTBC treated heterozygous and WT mice when compared to FAH−/− mice. This effect might be seen in men as well, since a small amount of NTBC seem to largely increase tyrosine levels in healthy volunteers [17], but a clear explanation for this finding is missing so far. The difference in tyrosine levels between FAH−/− and WT mice could theoretically be related to (recent) food intake. However, all mice showed appropriate growth and all other serum LNAA were similar.

FAH−/− mice tend to have low serum phenylalanine levels even without a phenylalanine-tyrosine restricted diet. This is in accordance with low phenylalanine levels that are frequently seen in HT1 patients and the lowering effect of NTBC treatment on serum phenylalanine levels in phenylalanine hydroxylase-deficient mice [18–21].

Irrespective of the genetic background, the high serum tyrosine levels caused by NTBC resulted in high brain tyrosine levels in all

experimental groups, which have been hypothesized to be neurotoxic, perhaps by promoting oxidative stress [22]. However, apart from that, the changes in serum biochemistry also led to modestly lower brain levels of all other LNAA. As all LNAA are competitively transported across the blood brain barrier by the almost fully saturated L-type amino acid transporter, the high serum tyrosine levels most likely outcompete the transport of other LNAA across the blood brain barrier. This is made more likely by measuring normal blood and brain lysine levels, since lysine is transported by a different transporter. This competitive effect at the blood brain barrier has also been seen in other diseases that affect LNAA metabolism, such as phenylketonuria and maple syrup urine disease [23,24]. In these diseases it is hypothesized that the low brain LNAA levels might affect brain protein synthesis and thereby outcome [25].

Despite the changes in brain LNAA levels, brain neurotransmitter levels are in general within the reference range and did not differ between the different experimental groups. It is noteworthy that a 6 fold increase in brain tyrosine levels did not result in major differences in dopamine levels in any of the mice. This is in agreement with earlier studies in mice and men [11,26] and might be related to the highly regulated enzymes within this pathway aiming to keep dopamine levels within range [27,28].

Behavioural and cognitive performance have only been investigated three times in FAH−/− mice, with conflicting results. *FAH*^{5961SB} mice, mice with a point mutation between exon 7 and intron 8 creating a protein null allele, showed slower learning, poor cognitive flexibility and abnormal social behaviour; this was not associated with treatment with NTBC but with the genotype of the mice [10,14]. However, in another study using FAH$^{\Delta exon5}$ (which also have a complete null mutation) no deficits in explorative and anxiety-like behaviour or memory retention were seen [11]. Therefore, this study aimed to investigate behavioural and cognitive performance of FAH −/− mice using different tests investigating distinct aspects of learning and memory and to associate this with the genotype of the mice and with the treatment. Despite the biochemical changes found, all the mice showed appropriate learning and memory during the cognitive tasks and no clear differences in behavioural or cognitive performance based on genotype were found. The reported differences in behavioural and cognitive performance of FAH−/− mice across different studies might be caused by the different FAH−/− mouse models (*Fah*$^{\Delta exon5}$ or *Fah*^{5961SB}) that were used and/or differences in the genetic background of the mice (C57Bl/6 or BALB/c). However, all FAH−/− mouse models used in the different studies have a complete null mutation that results in the same biochemical phenotype [7] and both C57BL/6 and BALB/c mice are frequently used for behavioural and cognitive studies [29–31].

5. Conclusion

To conclude, this study investigated blood and brain biochemistry, and behavioural and cognitive performance of NTBC treated FAH−/− mice. Irrespective of the genetic background (FAH−/−, FAH +/− and FAH+/+), NTBC treatment resulted in a clear increase in brain tyrosine levels, whereas all other brain LNAA levels were somewhat lower than their reference values. Despite these changes in blood and brain biochemistry, no significant differences in brain neurotransmitter (metabolites) were found and all mice showed normal behaviour and learning and memory. In contrast to earlier studies, the disease itself or genotype of the mice was not associated with poorer behavioural and cognitive function of the mice. Further research involving different levels of NTBC, analyses of succinylacetone or delta aminolevulinic acid and dietary treatment of FAH−/− are warranted to investigate whether this reveals the cognitive impairments that have been seen in treated HT1 patients.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ymgme.2022.07.001.

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Declaration of Competing Interest

WG van Ginkel received speakers honoraria from SOBI. CO Harding has received grants from StrideBio, Biomarin, royalties or licenses from Ultragenyx Pharmaceuticals and advisory board fees and/or speaker honoraria from BioMarin, Ultragenyx, Synlogic, Sanofi-Genzyme, LogicBio and Pfizer. The present study and manuscript are supported by a grant from the National Institutes of Health (US) (R01 NS080866) obtained by CO Harding. All other authors have indicated that they have no other conflicts of interest to declare.

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