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Research report

No role for vitamin D or a moderate fat diet in aging induced cognitive decline and emotional reactivity in C57BL/6 mice

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HIGHLIGHTS

- Aging significantly affects behavioral parameters related to emotion and cognition in mice.
- Vitamin D deficiency or impaired glucose homeostasis did not affect emotion or cognition in old mice.
- There was also no synergistic effect of vitamin D and glucose homeostasis on emotion or cognition.

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ABSTRACT

Background: Epidemiological studies have shown associations between vitamin D, mental health and glucose homeostasis in the elderly. Causal evidence, however, is still lacking.
Objective: The objective of this study was to investigate the importance of vitamin D in the prevention of emotional disturbances and cognitive decline in aging C57BL/6 mice, with pre-diabetes type II as potential effect modifier.
Methods: Mice were exposed to one of four diets from 10 months till 24 months of age: low fat vitamin D adequate (LFD), LF vitamin D deficient (LF), moderate fat vitamin D adequate (MFD), and MF vitamin D deficient (MF). The MFD/MF diet was applied to induce a condition resembling pre-diabetes type II. Behavior was assessed twice in the same group of mice at 6–8 and at 22–23 months of age using the Open Field Test (OFT), Elevated Plus Maze (EPM), Object Recognition Test (ORT) and the Morris Water Maze (MWM).
Results: We successfully induced vitamin D deficiency in the LF/MF mice. Moreover, fasting glucose and fasting insulin levels were significantly higher in MFD/MF mice than in LFD/LF mice. A significant aging effect was observed for most behavioral parameters. A MF(D) diet was shown to delay or prevent the age-related increase in emotional reactivity in the EPM. No effect of vitamin D or vitamin D*fat on behavioral outcomes was measured.
Conclusion: Aging significantly affected emotional reactivity and cognitive performance. Although other studies have shown effects of vitamin D on emotional reactivity and cognitive performance in mice, these findings could not be confirmed in aged C57BL/6 mice in this study.

Abbreviations: LFD, diet containing 9 En% from fat with vitamin D; LF, diet containing 9 En% from fat and no vitamin D; MFD, diet containing 20 En% from fat with vitamin D; MF, diet containing 20 En% from fat and no vitamin D; PT, pre-treatment performance; OGTT, oral glucose tolerance test; 25(OH)D, 25-hydroxyvitamin D, i.e. the inactive metabolite of serum vitamin D.

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1. Introduction

Aging is a universally accepted key risk factor for cognitive decline. Recent studies also suggest that specific nutritional factors may be linked with brain function and as such with cognitive decline [1] and depression [2]. Since pharmacological treatment of cognitive decline and dementia is currently far from effective, knowledge on the effect of modifiable dietary factors may bring us a step closer toward preventive solutions. Vitamin D deficiency is one of the suggested modifiable factors for brain function [3]. Currently, large numbers of mostly elderly individuals suffer from vitamin D deficiency [4]. Therefore, preventing and/or treating vitamin D deficiency may be a relevant, easy and cost-effective strategy to improve long-term mental health. Although several observational studies in humans support the notion of a beneficial link between vitamin D and cognitive performance [5–10] and depression [11–13], other studies do not (for cognition: [14,15], for depression: [10,16,17]). Moreover, evidence for a causal relationship from human intervention trials is still limited (for cognition: [18–22], for depression: [23–27]).

Studies using rodents as subjects offer more opportunities than human studies to investigate causal relationships between vitamin D and brain functions. However, in most studies conducted up to now, mice were already vitamin D deficient prenatally or had an inactivated vitamin D receptor gene [3]. These models do not mimic the most common clinical condition. In humans vitamin D deficiency usually slowly develops over time, mainly because the ability of the human skin to synthesize vitamin D under the influence of ultraviolet-B light exposure decreases while aging. Thus, alike age-related depression and cognitive decline, vitamin D deficiency in humans often just becomes manifest at older age. To our knowledge there has not yet been a study that examined the impact of long-term dietary vitamin D deficiency on brain functions in aging C57BL/6 mice; a model that more closely resembles the clinical condition of vitamin D deficiency in the elderly, and in which the physiological consequences and co-existing pathologies can be studied better.

Various pathways for a role of vitamin D in brain function have been suggested. It has been shown that vitamin D stimulates the production of several neurotrophins and acetylcholine, reduces oxidative stress, increases phagocytosis and clearance of amyloid β peptide by macrophages [3,28], and interacts with glucocorticoids in hippocampal cell-lines [29]. The exact physiological mechanisms underlying possible effects of vitamin D on brain function, however, are far from clear. Indirect mechanisms have also to be taken into account. There is evidence that vitamin D contributes to the maintenance of glucose homeostasis [30]. In accordance, diabetic patients tend to be more prone to develop cognitive deficits than non-diabetic patients [31]. Therefore, it has been suggested that vitamin D might indirectly affect brain function via a metabolic pathway. Glucose intolerance in mouse models is frequently studied by exposing mice to a high fat load. A 60% fat load has for instance been shown to result in rapid weight gain and increased fasting glucose and insulin levels, and insulin insensitivity as measured in glucose tolerance tests [32]. This extremely high fat load, however, also adversely affects life expectancy of mice and does not reflect the fat load of humans consuming fatty meals. Therefore, the physiological consequences of long-term dietary fat exposure probably can be better studied in a mouse model with a moderate fat load.

The aim of our study was to investigate the effect of long-term *adult* vitamin D deficiency on emotional reactivity and cognitive decline in aging C57BL/6 mice. By studying this relationship in mice exposed to a low fat (LF) diet (9 En% from fat) or a moderate fat (MF) diet (20 En% from fat) also the potential role of glucose homeostasis in the hypothesized relation between vitamin D and brain function was examined. Emotional reactivity and anxiety were assessed in the Open Field Test (OFT) and Elevated Plus Maze (EPM). Cognitive performance and cognitive decline were measured in the Object Recognition Test (ORT) and Morris Water Maze (MWM).

2. Materials and methods

2.1. Ethical approval

The experiment was conducted according to the institutional and national guidelines for the care and use of animals. The Local Committee for Care and Use of Laboratory Animals at Wageningen University approved the experiment (code number: drs-2010123). All effort was taken to minimize the number of animals used and their suffering. By contacting researchers working in the same field of study, effort was taken to optimally use the tissues of the animals subjected to this experiment.

2.2. Animals

Seventy-six male C57BL/6j RJ mice were purchased from Janvier Laboratories (Le Genest Saint Isle, France). At arrival at the animal facility the mice were 5 months of age and did not show signs of illness or behavioral abnormalities. Until 8 months of age all mice received a standard chow diet, containing 9 En% from fat (LF diet). From 8 months of age onwards, succeeding the completion of the baseline behavioral tests, 46 mice were randomly allocated to a 20 En% fat diet (MF diet) to provoke a diet-induced impaired glucose homeostasis. The remaining 30 mice continued on the LF fat diet. At the age of 10 months, mice in the low fat group and the moderate fat group were rank ordered according to body weight and fasting glucose levels, and subsequently randomly assigned to either a vitamin D adequate diet or a vitamin D deficient diet: (1) low fat group receiving a AIN-93W diet, 9 En% fat (LFD) ($n = 15$); (2) low fat group receiving a vitamin D deficient AIN-93 W diet, 9 En% fat (LF) ($n = 15$); (3) moderate fat group receiving a 20 En% fat diet (MFD) ($n = 23$); (4) moderate fat group receiving a vitamin D deficient 20 En% moderate fat diet (MF) ($n = 23$). Mice were weighed once every two weeks. Since sensorimotor function has been shown to be an effective tool to predict health-related drop-out [33], general health was assessed every three months using the Horizontal Wire Test, Balance Rod Test and Open Field Test. At the end of the study, at an age of 24 months, the mice were sacrificed and macroscopically examined for pathologies. A laboratory veterinarian monitored the study. The timeline of the study is depicted in Fig. 1.

2.3. Housing and test conditions

In order to monitor the dietary intake and to prevent large differences in body weight within groups, animals were housed individually. Makrolon® type II cages were cleaned every two weeks and contained standard woodchip bedding material. Tissues served as cage enrichment. Room temperature and humidity were regulated at approx. 21 °C and 50%, respectively. Mice were held on a reversed 12-hour light/dark cycle. Lights were off from 6:00 AM till 6:00 PM. During the mice's active period red lights were turned on and music was played as a background noise to conceal human activities in the lab. During the inactive phase, from 6.00 PM–6.00 AM, there were no activities in the lab and the music was turned off.

2.4. Diet

Food and tap water were available ad libitum. Food was weighed and refreshed every two weeks. Low fat groups were fed a Wageningen variant (AIN-93W) of the AIN-93 M diet until they were

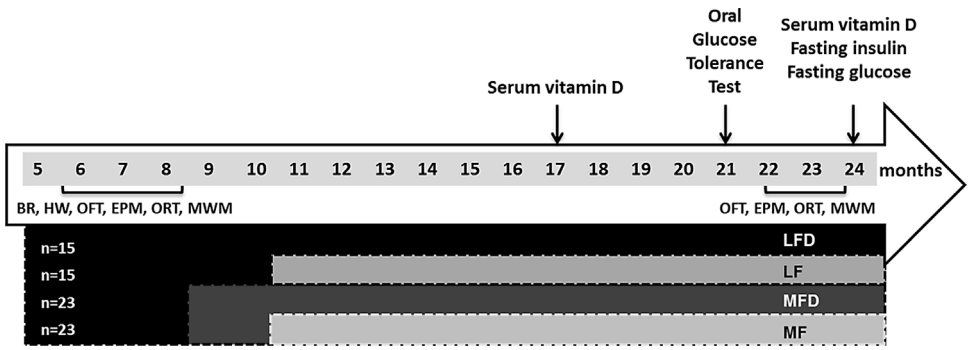

Fig. 1. Study design. Mice arrived at the laboratory at an age of 5 months and were fed a standard low fat diet. From 8 months of age onwards 46 mice were randomly allocated to a moderate fat diet to provoke a diet-induced impaired glucose homeostasis. At the age of 10 months, mice in the low fat group and the moderate fat group were randomly assigned to either a vitamin D adequate diet or a vitamin D deficient diet. LFD: low fat diet with vitamin D. LF: low fat diet without vitamin D. MFD: moderate fat diet with vitamin D. MF: moderate fat diet without vitamin D.

10-month-old. The AIN-93M diet is generally accepted as the 'golden standard' for adult maintenance [34]. AIN-93W is almost similar to AIN-93M diet, with the exception that the soy bean oil in AIN-93M diet has been partially replaced by palm oil, in order to create a diet that is more equivalent to a Western style diet. MF(D) animals were fed an AIN-93W diet until they were 8-month-old; from 8 to 10 months of age, all MF animal were switched to an AIN-93W with 20 En% from fat. At the age of 10 months, half of the mice in the low-fat group (LFD) and moderate fat group (MFD) were switched to a vitamin D deficient regimen (LF and MF) containing 0 IU vitamin D_3 per gram (Fig. 1). The exact diet composition of the different diets can be found in the supplementary material (eTable 1).

2.5. Biochemical parameters

Glucose homeostasis was assessed via fasting blood glucose levels, fasting plasma insulin levels and an Oral Glucose Tolerance Test (OGTT). Fasting glucose and insulin levels prior to the OGTT were determined in samples obtained after a 5- to 6-h fast. Blood samples were collected by cutting 1–2 mm of tissue from the tail tip and then gently massaging the tail. Glucose concentrations were obtained from whole blood samples and measured using Accu-Chek hand-held whole-blood glucose devices (Roche Diagnostics, Almere, The Netherlands). Plasma insulin was determined using a mouse insulin ELISA kit (ALPCO Diagnostics, Salem NH, United States). Following the fasting glucose measurement mice received 5 μl of 20% glucose solution per gram body weight via a feeding needle in order to determine the glucose response in the OGTT. Blood samples were taken at 15, 30, 45, 60, 90 and 150 min following the glucose load. Fasting glucose and fasting insulin levels at the day of sacrifice were determined in arterial blood collected by heart puncture using a syringe with EDTA, after 11 ± 1 h of fasting. During the procedure mice were anesthetized with a mixture of isoflurane (1.5%), nitrous oxide (70%), and oxygen (30%). Serum 25(OH)D was measured by isotope dilution–online solid phase extraction liquid chromatography–tandem mass spectrometry (ID–XLC–MS/MS), which was performed at the Endocrine Laboratory of the VU University Medical Center (Amsterdam, the Netherlands). Serum was incubated with a deuterated internal standard (IS: 25(OH)D3-d6), where after 25(OH)D was released from its binding protein(s). Samples were extracted and analyzed by XLC–MS/MS [a Symbiosis online Solid Phase Extraction system (Spark Holland, Emmen, the Netherlands) coupled to a Quattro Premier XE tandem mass spectrometer (Waters Corp, Milford, MA)]]. Serum 25(OH)D measurements require relatively large sample volumes, specifically 150 μl per sample. Therefore, the 25(OH)D levels in blood from 17-month-old mice had to be estimated in pooled samples, which consisted of equal volumes (25 μl) of 6 randomly selected mice per group. At the moment of the sacrifice, larger blood samples could be obtained from each mouse, and as such the 25(OH)D levels could be determined for each individual mouse.

2.6. Behavioral testing

2.6.1. Setting

To reduce stress due to handling animals were regularly handled after arrival in the lab. One month after arrival, at that time mice were 6 months of age, behavioral parameters were measured pre-treatment. The final behavioral measurements started when the mice were 22-month-old. Behavioral tests were performed according to the expected burden of the specific tests, starting with the least stressful tasks: OFT, EPM, ORT and finishing with the MWM. The day before testing started mice were transported to the test room, which was about 5 m away from their 'home' room. Mice were allowed to habituate to their new environment for approximately 14 h before behavioral testing started. The 'home' and 'test' room were similar with respect to the conditions, i.e. lighting, temperature and humidity. During the testing phase, mice were housed in the test room. Testing occurred in randomized order, started 1 h after the beginning of the dark period and was performed under red light conditions. The test equipment was cleaned with a damp tissue and dried with a towel after each mouse.

2.6.2. Open Field Test

The circular OFT, which is widely used to assess both sensorimotor functioning and emotional reactivity [35], was made of gray polyvinyl chloride (PVC) with a 78 cm diameter. The surface of the OFT was subdivided in three zones (periphery, middle and center) with a width of 13 cm each. Mice always started from the same point along the wall of the open field with their head pointing in the direction of the wall. Behavior during a 5-min trial was recorded by a video camera. Total distance moved and the number of times mice entered the center were quantified using the software system Ethovision 3.1. (Noldus IT, Wageningen, the Netherlands).

2.6.3. Elevated Plus Maze

The EPM is another validated tool to measure the degree of emotionality [35]. The EPM was made of gray PVC and consisted of two open and two closed arms; length 28 cm, width 5 cm, height closed arms 15 cm. In the center of the four arms there was a squared platform of 5 cm × 5 cm. The animal was placed in the center of the EPM facing an open arm, having the experimenter in the back. Subsequently behavior was recorded during 5 min and analyzed using Ethovision 3.1. The number of open arm entries, the time spent in

the open arms, the percentage of open and closed arm entries and the total number of arm entries were key measures in this task.

2.6.4. Object Recognition Test

Recognition memory was assessed by the ORT [36]. The arena of the ORT consisted of a circular field – diameter 48 cm – made of transparent PVC. Objects were placed in a symmetrical position about 12 cm from the wall, at a distance of 24 cm from each other. Mice were placed in the arena at a standardized point along the wall of the arena always facing the wall in front of the experimenter. The first part of the procedure consisted of two adaptation days, in which the mice were allowed to explore the empty apparatus twice a day for 5 min per session. The second part of the ORT included the test day. On the test day, the animal underwent two sessions, separated by a retention interval of 1 h. In the first session the animal was exposed to two identical objects during 5 min. In the second session the animal was again placed in the arena for 5 min with a familiar object from the first session and a novel object. The time spent exploring the two objects during the first and second session was manually registered using a non-commercial software program "Object Recognition Task" version 2. Two sets of objects were used, specifically small white stones and yellow Lego bricks. Objects met the following requirements: they had a comparable height and volume, were too heavy to be replaced by a mouse, were easy to clean and there were no possibilities for mice to hide under or climb on the objects. Habituation, exploration and discrimination were the main parameters measured in this task [36]. Exploration was defined as the time spent exploring both the familiar and new object, i.e. exploration of object 1 plus exploration of object 2. Habituation was defined as the difference in exploration time between the two sessions on the same day, i.e. exploration time in trial 1 minus exploration time in trial 2. Discrimination was defined as the discrimination between the new and familiar object, i.e. time spent in exploring the novel object minus the time spent in exploring the familiar object. Based upon these measures we finally calculated the discrimination index in which we adjusted for total exploration time, i.e. discrimination divided by the total exploration time at both objects.

2.6.5. Morris Water Maze

The MWM test was conducted to measure spatial learning [37]. The MWM was a circular pool made of gray PVC with a diameter of 110 cm. The escape platform consisted of gray PVC, had a diameter of 9 cm and was invisibly hidden at approximately 1 cm beyond the water surface at the north quadrant of the pool. Extra maze cues were placed at two of the nearby walls of the testing room. To avoid the negative effects of a decrease in body temperature, the water was kept at a constant temperature of 24–26 °C. Water was colored with non-toxic white paint in order to obtain a clear contrast between the water and the mice and to mask the platform position. Mice performed 4 trials during 5 daily acquisition sessions. In every trial the mouse started from a different starting position, which was pre-determined by randomization. As soon as an animal succeeded to reach the escape-platform within the cut-off time of 60 s it was allowed to stay on it for 15 s. Thereafter, it was removed from the pool and put back in its cage were it was allowed to rest for 15 s, after which the animal was again released into the pool. This procedure was repeated four times on each test day. When an animal did not find the escape-platform within the cut-off time, it was placed on the escape platform by hand and stayed on it for 15 s. To avoid possible olfactory tracks the water was stirred after a mouse completed the test session. Moreover, in order to prevent a decrease in body temperature during testing, the cage of the animals was placed on a rodent warming pad with a temperature of approximately 37 °C for 10 min. In addition, all mice received a new, dry tissue. MWM performance was recorded and analyzed using Ethovision 3.1.

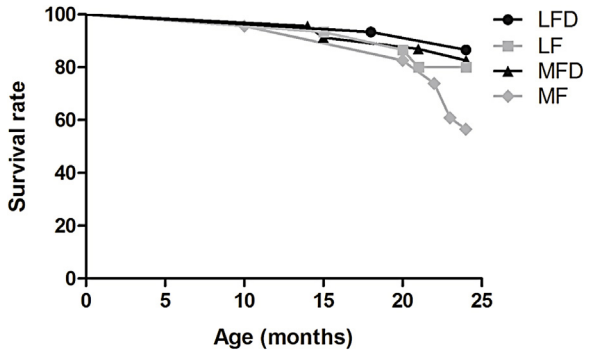

Fig. 2. Survival per month. Survival rates were 87%, 80%, 83% and 59% in the LFD, LF, MFD and MF groups, respectively. MF mice showed a substantial lower survival rate than the LFD, LF and MFD mice. Though, survival in MF mice did not significantly differ from the LFD ($X^2 = 3.0$, $P = 0.08$), LF ($X^2 = 1.2$, $P = 0.27$) or MFD mice ($X^2 = 2.6$, $P = 0.11$).

2.6.6. Morris Water Maze – probe trial

After completing the fifth testing day in the MWM, a probe trial was conducted in which we assessed reference memory [37]. Mice were released into the pool, without the escape platform, starting in the south quadrant. During 60 s we measured the time spent in each quadrant using Ethovision 3.1. Subsequently, we calculated the percentage of time spent in the quadrant that initially contained the escape platform.

2.7. Statistical analyses

Analysis of variance (ANOVA) was used to test whether vitamin D adequate and vitamin D deficient mice differed with respect to their serum 25(OH)D concentrations. Similarly, ANOVA was performed to test for differences in blood glucose concentrations or plasma insulin concentrations between LF(D) and MF(D) mice. Aging effects were tested using a paired sample *t*-test, comparing the results of all mice tested pre-treatment with the results of all mice after treatment. Kaplan–Meier survival analysis and Mantel–Cox log-rank test were used for survival comparisons between the four treatment groups. ANOVA was used to study potential main effects of vitamin D and the type of fat diet and its interaction on the absolute change in behavior in the OFT, EPM, ORT and the MWM. Linear Mixed Models were used to assess whether there was a learning curve over test days in the MWM. A *P*-value of <0.05 was used to determine statistical significance. All statistical analyses were performed using SPSS Statistics v19 (SPSS Inc. Chicago, IL).

3. Results

3.1. Survival and drop-out

With survival rates of 87%, 80% and 83% in the LFD, LF and MFD groups respectively, no obvious survival differences were observed between these groups. With a survival rate of 59%, MF mice did notably though not significantly differ from the LFD ($X^2 = 3.0$, $P = 0.08$), LF ($X^2 = 1.2$, $P = 0.27$) and MFD ($X^2 = 2.6$, $P = 0.11$) mice (Fig. 2). When reviewing the data of the mice that dropped-out during the study and the mice that showed pathologies at necropsy, the most frequently observed abnormalities were liver pathologies, followed by abnormal coagulating glands and kidney abnormalities (eFigures 1 and 2). Since the pathologies were expected – and

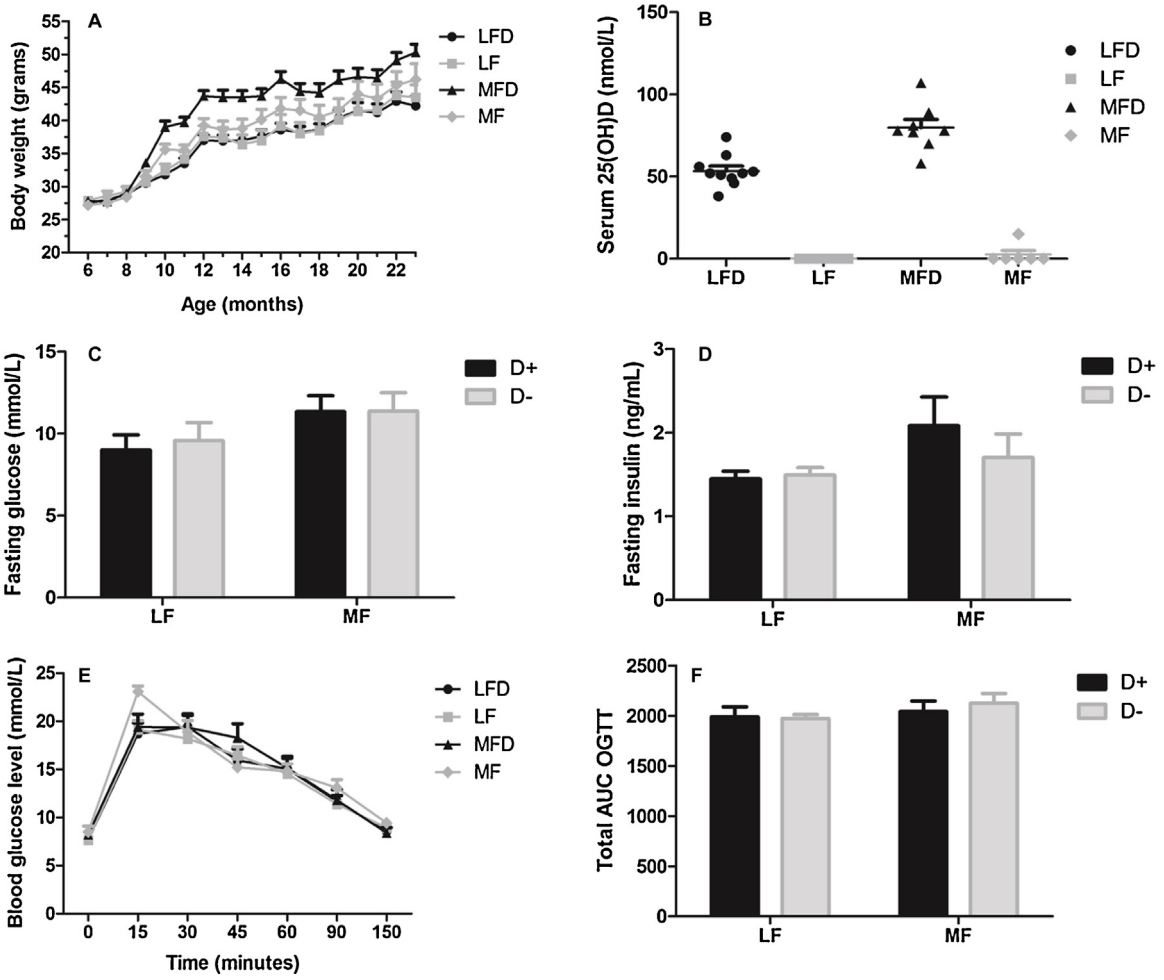

Fig. 3. Body weight development and biochemical characteristics at 21–24 months. (A) Body weight development of the mice during the study, as shown by the mean with SEM. (B) Serum 25(OH)D levels at 24 months old. (C) Fasting plasma glucose levels at 24 months old. (D) Fasting plasma insulin levels at 24 months old. (E) Oral glucose tolerance test (OGTT) results at 21 months old; blood samples were taken at 15, 30, 45, 60, 90 and 150 min following the glucose load of 5 μl of 20% glucose solution per gram body weight. (F) Area under the curve for OGTT data.

in many cases also observed – to interfere with the performance in the behavioral tests, the results presented below are based upon the analyses conducted with the data of the mice without any sign of underlying illness ($n = 33$), specifically LFD ($n = 10$), LF ($n = 9$), MFD ($n = 8$) and MF ($n = 6$).

3.2. Body weight development

Mean body weight of the mice in all treatment arms increased until the time of sacrifice at 24 months of age. As intended, the mice on the MF diet gained more weight than mice receiving a LF diet.

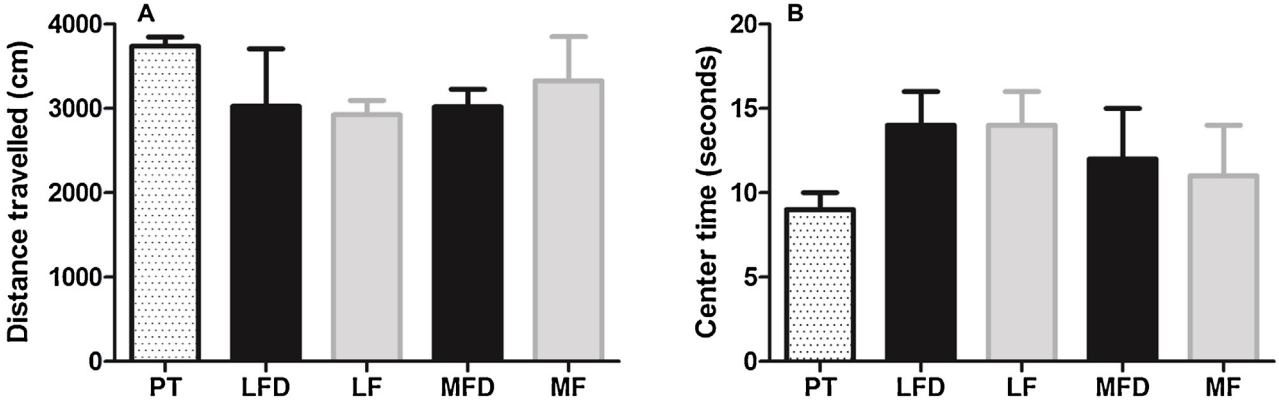

Fig. 4. Open Field Test (OFT) performance during a 5-min trial at 6 (PT) and 22 months old (LFD, LF, MFD, MF). Specifically, the total distance traveled and the time spend in the center of the OFT, displayed by the mean with SEM. LFD: low fat diet with vitamin D. PT: pre-treatment. LF: low fat diet without vitamin D. MFD: moderate fat diet with vitamin D. MF: moderate fat diet without vitamin D.

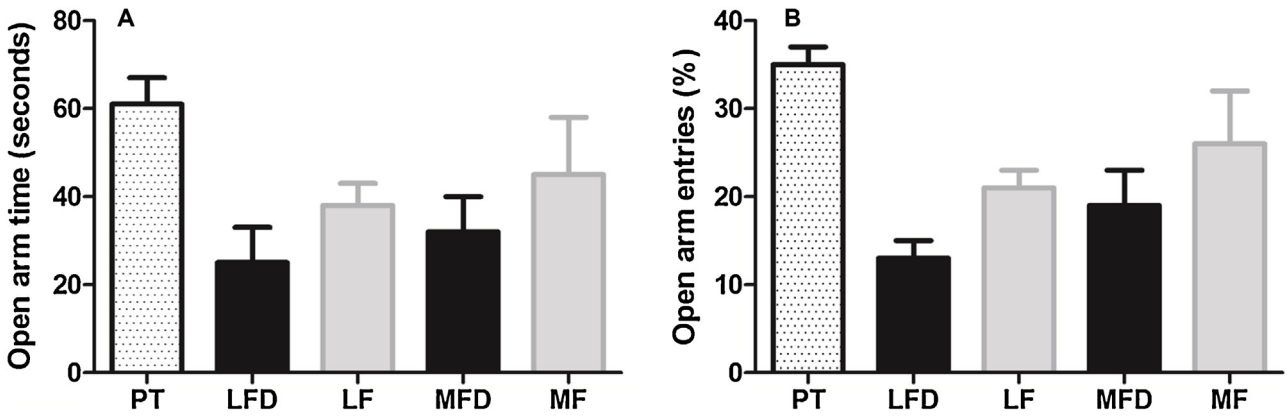

Fig. 5. Performance in the Elevated Plus Maze (EPM) during a 5-min trial at 6 (PT) and 22 (LFD, LF, MFD, MF) months old. Specifically, the total time spent in the open arm and the percentage of open arm entries, displayed by the mean with SEM. PT: pre-treatment. LF: low fat diet without vitamin D. MFD: moderate fat diet with vitamin D. MF: moderate fat diet without vitamin D.

MF mice were on average less heavy when compared to MFD mice (Fig. 3A).

3.3. Serum 25(OH)D levels and glucose homeostasis

Serum 25(OH)D analyses at the age of 17 months (data not shown) and at 24 months (Fig. 3B) showed a clear vitamin D deficiency in mice receiving vitamin D deficient diets ($F_{32} = 388.1$, $P < 0.0001$). Moreover, a significant interaction between vitamin D and a moderate fat diet in relation to serum 25(OH)D was observed, indicating that mice on a moderate fat diet had higher serum 25(OH)D concentrations than mice on a low fat diet ($F_{32} = 14.1$, $P = 0.001$). In addition, serum 25(OH)D of mice receiving the vitamin D adequate diet reached in most cases levels of at least 50 nmol/L. At sacrifice, mice fed a moderate fat diet exhibited higher fasting glucose levels (11.4 ± 0.71 mmol/L vs. 9.3 ± 0.70 mmol/L, $F_{32} = 4.1$, $P = 0.05$) and higher fasting plasma insulin levels (1.92 ± 0.23 ng/mL vs. 1.47 ± 0.06 ng/mL, $F_{31} = 4.4$, $P < 0.05$) than their low fat counterparts (Fig. 3C and D). Oral Glucose Tolerance Tests conducted three months before sacrifice did not show a difference in glucose response between the four groups (Fig. 3E and F).

3.4. Aging in relation to behavior

When comparing the overall mean performance at young age with the overall mean performance at old age, it is concluded that aging significantly affected behavioral parameters related to exploration and emotional reactivity. At old age, mice traveled significantly less far during a 5 min trial in the OFT ($t_{32} = 4.5$, $P < 0.0001$), and spent significantly more time in the center of the OFT ($t_{32} = -2.7$, $P = 0.01$) (Fig. 4A and B and Table 1). In the EPM, aging significantly decreased the time spent in the open arms ($t_{32} = 3.7$, $P = 0.001$), the percentage of open arm entries ($t_{32} = 6.0$, $P < 0.0001$) and the number of open arm entries ($t_{32} = 6.4$, $P < 0.0001$) (Fig. 5A and B and Table 1). In addition, recognition memory, as assessed in the ORT, significantly decreased when comparing mice's performance at young age with the performance at old age ($t_{32} = 3.5$, $P = 0.001$; Fig. 6 and Table 1). With respect to the MWM, when comparing the area under the curve (AUC) for the escape latency and distance moved to platform at young age with the AUC at old age, a significant effect was observed for the distance moved to the platform ($t_{32} = -2.1$, $P = 0.05$), but not for the escape latency ($t_{32} = -0.31$, $P = 0.76$) (Fig. 7 A–D and Table 1). At old age, mice spent significantly more time in the target quadrant during the probe trial when compared to their performance at young age ($t_{32} = -6.1$, $P < 0.0001$) (Fig. 8 and Table 1).

3.5. Vitamin D and impaired glucose homeostasis in relation to behavior

3.5.1. Exploration and emotional reactivity

In the OFT, there was no significant effect of vitamin D, the type of fat diet or the interaction between the two on the total distance moved or the time spent in the center (Fig. 4A and B and Table 1). There was also no significant effect of vitamin D or an interacting effect of vitamin D with fat on EPM performance.

Fat did significantly affect emotional reactivity as assessed with the EPM, showing a larger decrease in the percentage of open arm entries in LF(D) than MF(D) mice (absolute change ± SEM in MF(D) vs. LF(D) mice: -8 ± 5 vs. -23 ± 2, $F_{1,29} = 9.72$, $P = 0.004$) (Fig. 5A and B and Table 1). Accordingly, the total number of arm entries decreased less in the MF(D) than LF(D) mice (absolute change ± SEM in MF(D) vs. LF(D) mice: -5 ± 2 vs. -11 ± 2, $F_{1,29} = 5.31$, $P = 0.03$), which was mainly attributable to the MFD group that showed a decrease of -2 ± 2 vs. -8 ± 3 in MF mice. Moreover, the decrease in the percentage of open arm entries tended to be lower in vitamin D deficient mice than in mice receiving a vitamin D adequate diet (absolute change ± SEM in LF/MF vs. LFD/MFD mice: -25 ± 11 vs. -41 ± 24, $F_{1,29} = 3.21$, $P = 0.08$).

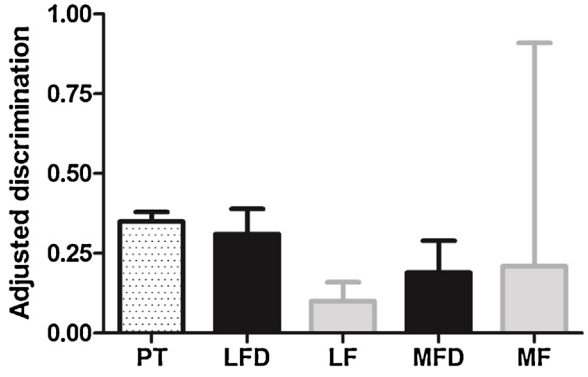

Fig. 6. Object Recognition Test (ORT) performance at 6 (PT) and 22 (LFD, LF, MFD, MF) months old, displayed by the mean with SEM of the adjusted discrimination index. Discrimination was defined as the discrimination between the new and familiar object, specifically time spent in exploring the new object minus the time spent in exploring the old object. Subsequently, this discrimination index was adjusted for total exploration time, namely discrimination divided by the total exploration time. PT: pre-treatment. LF: low fat diet without vitamin D. MFD: moderate fat diet with vitamin D. MF: moderate fat diet without vitamin D.

Table 1
Behavioral test results at 6–8 months and 22–23 months of age. Values displayed are absolute means with standard errors of the mean (SEM).

	Pre-treatment	22–23 months	22–23 months	Time[a]	Vitamin D*fat[b]	Vitamin D[b]	Fat[b]
Open Field Test							
Total distance traveled (cm)				$T = 4.5$, $P < 0.0001$	$F = 0.08$, $P = 0.78$	$F = 0.03$, $P = 0.88$	$F = 0.33$, $P = 0.57$
LFD	3737 ± 110	3052 ± 129	3026 ± 216				
LF			2926 ± 170				
MFD			3018 ± 210				
MF			3328 ± 525				
Center time (s)				$T = -2.7$, $P = 0.01$	$F = 0.12$, $P = 0.74$	$F = 0.02$, $P = 0.89$	$F = 0.20$, $P = 0.66$
LFD	9 ± 1	13 ± 1	14 ± 1.6				
LF			14 ± 2.1				
MFD			12 ± 3.3				
MF			11 ± 2.6				
Elevated Plus Maze							
Open arm time (s)				$T = 3.7$, $P = 0.001$	$F = 1.67$, $P = 0.21$	F = 0.88, $P = 0.36$	$F = 3.48$, $P = 0.07$
LFD	61 ± 6	34 ± 4	25 ± 8				
LF			38 ± 5				
MFD			32 ± 8				
MF			45 ± 13				
Open arm entries (%)				$T = 6.0$, $P < 0.0001$	$F = 0.20$, $P = 0.66$	$F = 3.21$, $P = 0.08$	$F = 9.72$, $P = 0.004$
LFD	35 ± 2	19 ± 2	13 ± 2				
LF			21 ± 2				
MFD			19 ± 4				
MF			26 ± 6				
Open arm entries (frequency)				$T = 6.4$, $P < 0.0001$	$F = 0.13$, $P = 0.72$	$F = 0.17$, $P = 0.68$	$F = 15.8$, $P < 0.0001$
LFD	12 ± 0.8	5 ± 0.6	3.3 ± 0.6				
LF			5.1 ± 0.6				
MFD			5.3 ± 1.3				
MF			7.5 ± 2.3				
Total arm entries (frequency)				$T = 5.7$, $P < 0.0001$	$F = 0.05$, $P = 0.83$	$F = 1.82$, $P = 0.19$	$F = 5.31$, $P = 0.03$
LFD	34 ± 1.3	26 ± 0.9	26 ± 2				
LF			25 ± 1				
MFD			26 ± 2				
MF			26 ± 3				
Object Recognition Test							
Adjusted discrimination index				$T = 3.5$, $P = 0.001$	$F = 3.83$, $P = 0.06$	$F = 0.21$, $P = 0.65$	$F = 0.17$, $P = 0.68$
LFD	0.35 ± 0.03	0.21 ± 0.04	0.31 ± 0.08				
LF			0.11 ± 0.06				
MFD			0.19 ± 0.10				
MF			0.21 ± 0.07				
Morris Water Maze							
Area under the curve for escape latency				$T = -0.31$, $P = 0.76$	$F = 0.69$, $P = 0.41$	$F = 2.96$, $P = 0.10$	$F = 0.08$, $P = 0.79$
LFD	87 ± 4	88 ± 3	83 ± 6				
LF			85 ± 8				
MFD			95 ± 6				
MF			94 ± 8				
Area under the curve for distance traveled				$T = -2.1$, $P = 0.05$	$F = 0.62$, $P = 0.44$	$F = 3.55$, $P = 0.07$	$F = 0.06$, $P = 0.80$
LFD	1264 ± 57	1448 ± 64	1362 ± 106				
LF			1418 ± 158				
MFD			1484 ± 96				
MF			1585 ± 166				
% in target quadrant during probe trial				$T = -6.1$, $P < 0.0001$	$F = 0.56$, $P = 0.46$	$F = 2.01$, $P = 0.17$	$F = 0.53$, $P = 0.47$
LFD	41 ± 2	56 ± 2	60 ± 5				
LF			57 ± 5				
MFD			55 ± 2				
MF			49 ± 5				

[a] Degrees of freedom: 32.
[b] Degrees of freedom: 29.

3.5.2. Recognition memory, spatial learning and reference memory

We did not observe significant effects of vitamin D, fat or the interaction between vitamin D*fat on the adjusted discrimination index in the ORT, but there was a tendency toward an vitamin D by fat interaction ($F_{1,29} = 3.83$, $P = 0.06$) (Fig. 6 and Table 1). Our treatments did also not significantly affect spatial learning (Fig. 7A–D and Table 1) or reference memory, assessed in the probe trial (Fig. 8 and Table 1) as assessed in the MWM. Though, the figures of the MWM do suggest a weak tendency for a beneficial effect of vitamin D. Specifically, the absolute increase in time needed to locate the platform tended to be larger in vitamin D deficient mice than in vitamin D adequate mice (absolute change ± SEM in vitamin D adequate vs. vitamin D deficient: -7 ± 7 vs. 10 ± 7, $F_{1,29} = 2.96$, $P = 0.10$). Accordingly, a similar pattern was observed when studying the distance traveled before locating the platform (absolute change ± SEM in vitamin D adequate vs. vitamin D deficient: 31 ± 119 vs. 345 ± 123, $F_{1,29} = 3.55$, $P = 0.07$) and reference

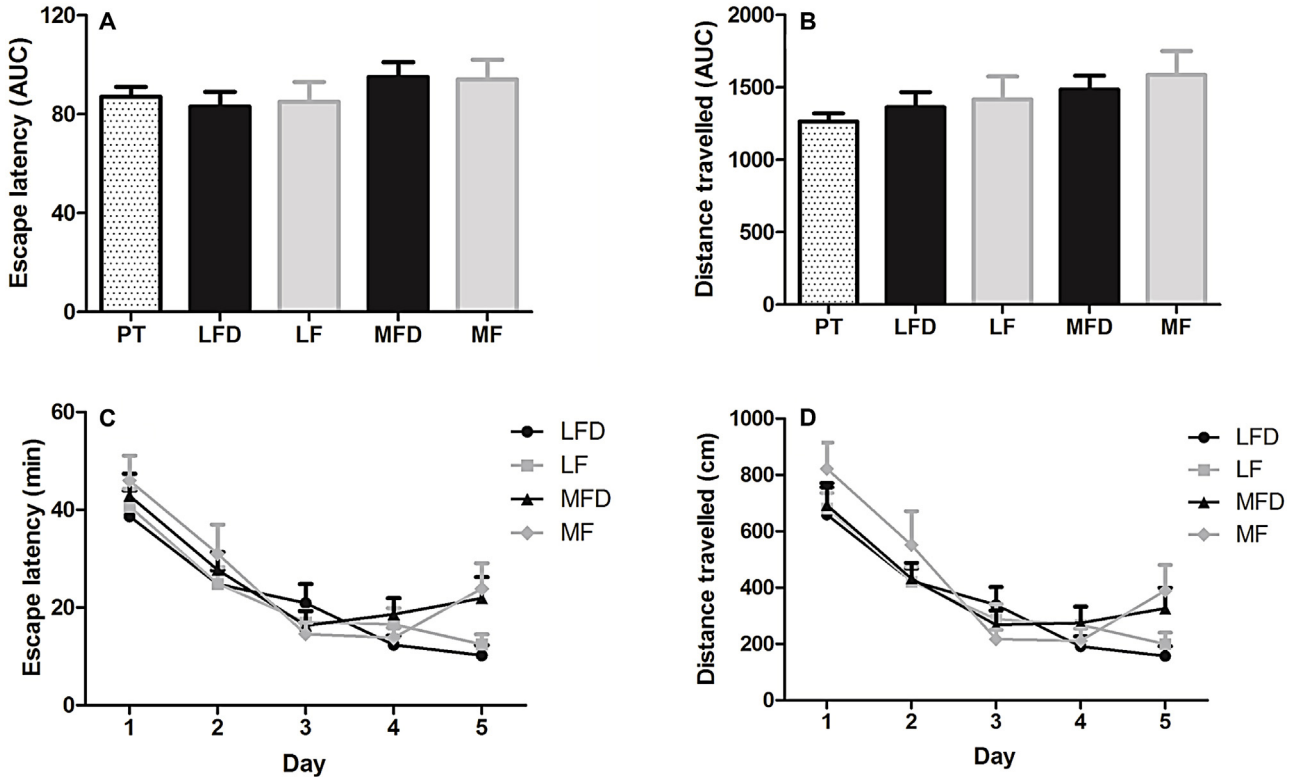

Fig. 7. Morris Water Maze (MWM) performance at 7 (PT) and 23 (LFD, LF, MFD, MF) months old. Performance was assessed by means of 4 trails during 5 daily acquisition sessions; the mean with SEM of each daily session is displayed in the figure. (A) Area under the curve (AUC) for escape latency. (B) AUC for distance traveled to platform. (C) Learning curve for escape latency at 23 months old. (D) Learning curve for distance traveled to platform at 23 months old. PT: pre-treatment. LF: low fat diet without vitamin D. MFD: moderate fat diet with vitamin D. MF: moderate fat diet without vitamin D.

memory in the probe trial of the MWM (absolute change ± SEM in vitamin D adequate vs. vitamin D deficient: 18 ± 4 vs. 12 ± 3, $F_{1,29} = 2.01$, $P = 0.17$).

4. Discussion

4.1. Main findings

Cognitive performance and emotional reactivity of mice at the age of 22–23 months – as assessed with the ORT, MWM, OFT and the EPM – significantly differed from the performance of the same mice at the age of 6–8 months. Furthermore, a significant effect of fat was observed on emotional reactivity in the EPM, suggesting that a moderate fat diet may prevent or delay the age-related increase in emotional reactivity. This study did not provide evidence for an effect of adult vitamin D deficiency on the behavioral indices in 22–23 months old C57BL/6 mice.

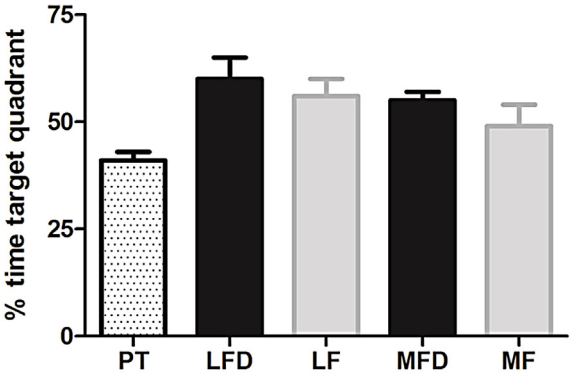

Fig. 8. Probe trial performance at 7 (PT) and 23 (LFD, LF, MFD, MF) months old. Bar graph shows the percentage of time spent in the target quadrant during a 1-min trial at the end of the fifth test day. The dotted line indicates the chance level of swimming in the target quadrant. PT: pre-treatment. LF: low fat diet without vitamin D. MFD: moderate fat diet with vitamin D. MF: moderate fat diet without vitamin D.

4.2. Our study in a broader perspective

Laboratory studies have suggested a number of pathways via which vitamin D may beneficially affect brain function [3,28]. When looking into the current literature on studies in rodents it can be concluded that even though the results are mixed [38–42], the majority does point toward a favorable effect of vitamin D on behavior [39–42]. When comparing these studies, however, it is clear that there are large differences between the applied study designs. Three out of five rodent studies were conducted in rats [38,39,41] and two in mice [40,42]. Whereas four studies intervened by means of a modified dietary regimen [38,40–42], one study intervened with a subcutaneous injection of 42 IU 1,α25(OH)D3 per kg [39]. There was also a large variety in the behavioral tests applied. Also physiologically there were major differences between the models studied. The age of the behavioral assessment varied from 3 to 20 months, and the duration of the intervention varied from 2 to 20 weeks. Moreover, in two of the studies that observed a vitamin D effect, a very particular neuropathology was induced, specifically amyloid plaques deposition [41,42]. Thus, the mechanisms underlying the behavioral deficiencies or changes may differ substantially between the used models and therefore the potential molecular "targets" for vitamin D are model-dependent. As already mentioned previously, in this study the aim was to examine the effect of long-term vitamin D deficiency on emotional reactivity and cognitive performance in

'normal' aging C57BL/6 mice. In view of the often relatively slowly decreasing vitamin D status while aging in humans, we believe that our model more closely resembles the clinical condition of vitamin D deficiency and natural aging in the elderly than the models that have been described previously. However, in future studies the design could be optimized further, for instance by reducing the vitamin D content of the food more gradually. Such a gradual decrease may even more closely mimic the development of vitamin D deficiency as observed in human aging. Groves and colleagues examined the effect of a 10-week vitamin D-deficient diet in 20-week-old C57BL/6 mice [40], which we consider physiologically most comparable to our design. The findings in these 20-week-old C57BL/6 mice were in line with the current results, specifically no effect of vitamin D was found with regard to behavior in the EPM, a familiar OFT, the hole-board test, the light/dark test, the forced swim test, or the social interaction test. Vitamin D deficient C57BL/6 mice did show signs of hyperlocomotion in an unfamiliar OFT when compared to their vitamin D adequate counterparts. More robust vitamin D effects were observed in the BALB/c strain, showing that deficient mice spent more time on the open arms of the EPM and responded faster to heat, sound and shock [40]. However, as 20-week-old mice cannot yet be considered aged and the period of an absolute vitamin D deficiency in these mice was relatively short, Groves and colleagues did not specifically target the aging mechanisms related to brain function like we did. This study, however, does suggest that BALB/c mice may be more susceptible to vitamin D deficiency than C57BL/6 mice, which might advocate the use of BALB/c mice instead of C57BL/6 in future behavioral studies on the effect of vitamin D deficiency. When extending our comparison with the literature by looking at the behavioral tests used, we saw that three of the five previous studies assessed spatial learning using the MWM [39,41,42]. All suggested an effect of vitamin D on spatial learning. We also assessed spatial learning in the MWM. Our data only suggest a weak tendency for a vitamin D effect in the MWM.

4.3. Methodological considerations

4.3.1. Aging

Our longitudinal study showed that age was an important determinant of behavior. Two of these findings warrant clarification. First of all, the finding that old mice spent more time in the center of the OFT. This may be explained by the fact that – in view of health monitoring throughout the trial – mice had already been exposed to the OFT for five times before the final measurements. Thus, whereas the open field at 6 month of age was a novel open field, at old age the open field had become familiar to the mice, resulting in habituation and hence less signs of emotional reactivity. Secondly, although of borderline significance, our results showed that the distance traveled toward the platform in the MWM was longer in old mice than in young mice, and that probe trial performance improved with age. Stronger age effects might have been expected. As shown by previous longitudinal studies, carry-over effects may occur when administering the MWM at multiple time points [43,44]. This may explain the modest age effect observed in the MWM in this study. Guidi and colleagues also showed that once the reference memory task is learned, animals remain capable to successfully complete the task, irrespective of age [43]. Since the MWM with varying platform locations did appear to be sensitive to detect subtle cognitive changes [43], using this modified version may refine future studies.

4.3.2. Vitamin D deficiency

This study did not provide evidence for a significant effect of adult vitamin D deficiency on measures of emotional behavior (EPM and OFT) or cognitive performance (ORT and MWM) in aged mice. Neither did we observe an effect of the type of fat diet in combination with vitamin D on emotional reactivity, learning and memory. In order to substantiate our conclusion that vitamin D does not seem to play a major role in learning, memory and emotional behavior in aging C57BL/6 mice at the age of 22–23 months, it should be evident that the vitamin D status differed sufficiently between the treatment groups. Serum 25(OH)D measurements in pooled samples collected at seven months after the initiation of the vitamin D deficient diet indicated that the levels in the deficient group were below the detection limit of the assay. Serum 25(OH)D levels in the adequate groups were around 55 nmol/L, which is considered to be an adequate level in humans [45]. Measurements in individual blood samples collected at sacrifice showed that serum 25(OH)D levels were still very low or non-detectable in the deficient mice. Thus, these results provide evidence that mice had an absolute vitamin D deficiency for at least 6 months preceding the final behavioral tests, which to our knowledge has never been done before. Based upon these data we argue that the difference in vitamin D intake between the adequate and deficient group was large enough to show a potential effect of vitamin D on brain function and behavior. Since we did observe weak tendencies for a vitamin D effect in the MWM, it might also be postulated that we did not observe an effect of the treatments on cognitive decline because brain functions were still too well preserved at 22–23 months of age – mice as old as 18–24 months are considered to be comparable to humans at the age of 56–69 years [46] – resulting in behavioral differences between treatment groups that were too small to detect.

4.3.3. Impaired glucose homeostasis

Another aim of this study was to examine the role of an impaired glucose homeostasis in the potential relationship between vitamin D and behavior. Byrne and colleagues postulated that the effect of a vitamin D deficiency may be stronger in the presence of a second neurobiological stressor as Parkinson's Disease or brain ischemia [47]. However, we have to conclude that in our study the effect of vitamin D deficiency on behavioral outcomes was not stronger in mice with an impaired glucose homeostasis compared to mice with a normal glucose tolerance. Our data did suggest that mice with an impaired glucose homeostasis did not have an age-related increase in emotional reactivity, whereas mice with a normal glucose homeostasis did. We do not have an explanation for this unexpected finding. To induce an impaired glucose homeostasis a subsample of mice in this study were exposed to a moderate fat load – a diet where 20% of energy was provided by fat – to mimic the relatively slow development of glucose intolerance as often observed at older age in humans. Thus, to reflect a more relevant physiological state, the fat load fed in this study was deliberately lower than the high fat loads used in previous short-term studies that provoked diet-induced diabetes [32]. The moderate fat load appeared to be potent enough to induce a higher weight gain and modest differences in fasting glucose and insulin concentrations, which indicates a state of impaired glucose homeostasis in MF(D) mice. Since there was no significant difference in glucose response in the OGTT it cannot be concluded that the moderate fat diet resulted in an impaired glucose clearance. This profile of metabolic changes combined with elevated body weight in MF(D) mice mimics a human state which can be described as pre-diabetic. However, we feel that the achieved pre-diabetic state was not as strong as we aimed at. To increase the impact of a moderate fat diet, future studies might consider the use a diet with a higher fat content, specifically a diet containing 25 En% from fat. However, the rapid increase in body weight following the switch to the moderate fat diet as observed in our study, does imply that caution is warranted. Besides the fact that a too extensive weight gain may result in early dropouts, it may also become a limiting factor in the behavioral assessment.

4.3.4. Other factors that may have interfered with the relations studied

Since the cognitive and emotional tests involve a clear physical component, it might be argued that the results of the behavioral tests are influenced by sensorimotor impairments and as such influenced the internal validity of this study. Another factor that may have been involved in the relationships studied is hypocalcaemia. Specifically, vitamin D deficiency may lead to hypocalcaemia, which has been linked with neuromuscular as well as neuropsychiatric symptoms [48]. Unfortunately, serum calcium levels have not been measured in this study. However, tests of sensorimotor performance and muscle strength – specifically the Horizontal Wire Test, the Balance Rod, the Open Field Test as well as ex vivo muscle measurements [49] – did not point toward differences between the treatment groups (data not shown). Nor did we observe large differences in anxiety related behavior between vitamin D deficient and vitamin D adequate mice.

4.3.5. Novelty

During the past decade interest in vitamin D has grown enormously [50]. Since vitamin D receptors are located on many tissues throughout the body including the brain, vitamin D is hypothesized to target a variety of bodily tissues. Likewise, a large number of epidemiological observational studies have shown associations linking vitamin D not only to brain health but also to cardiovascular disease, fat metabolism, muscle function, and glucose homeostasis [50,51]. A major issue in many of these human studies, however, is the possibility of reverse causation and residual confounding. In randomized clinical trials (RCTs) these factors are less likely to disturb the potential relationships studied, and from these studies the effects of vitamin D seem to be less clear-cut than they appear from observational studies. A very recent review summarized the data of RCTs that studied the effect of vitamin D supplementation on a variety of non-skeletal health outcomes in humans, showing no clear vitamin D effect [50]. Based on their findings the authors speculate that the discrepancy between observational and intervention studies in humans may indicate that low 25(OH)D is rather a marker of ill health than a risk factor of disease. In view of these previous RCTs, our mouse study can be regarded as a proof of principle experiment, albeit with a negative result. Thus, despite the fact that there are aspects of our study that can be debated, it should be realized that this study is unique in its kind. It is the first study that investigated the effect of a 1-year vitamin D deficient diet – and an absolute verified vitamin D deficiency for at least 6 months – on various behavioral parameters related to emotional reactivity and memory in natural aging mice.

5. Conclusion

This study showed a clear aging effect on various measures of emotional behavior and cognition in male C57BL/6 mice at 22–23 months of age. No large differences in behavioral phenotype, as assessed with the OFT, EPM, ORT and MWM, between vitamin D adequate mice and vitamin D deficient mice was observed.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbr.2014.03.038.

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