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Vitamin D deficiency impairs neurobehavioral development in male mice

Lin Fu[a,c,1], Yuan-Hua Chen[b,c,*,1], Xue Chen[d], Shen Xu[e], Zhen Yu[c], De-Xiang Xu[a,c,**]

[a] Department of Toxicology, Anhui Medical University, Hefei, China
[b] Department of Histology and Embryology, Anhui Medical University, Hefei, China
[c] Anhui Provincial Key Laboratory of Population Health & Aristogenics, Hefei, China
[d] Department of Preventive Medicine, BengBu Medical College, Bengbu, China
[e] The First Affiliated Hospital, Anhui Medical University, Hefei, China

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ABSTRACT

Vitamin D deficiency is prevalent especially in pregnant women and children. Several studies found that vitamin D status was negatively correlated with risk of senile neurobehavioral abnormality. The aim of this study was to investigate the effects of vitamin D deficiency on neurobehavioral development in mice. In vitamin D deficiency group, dams and their male pups were fed with vitamin D deficient (VDD) diet, in which vitamin D was depleted. Anxiety-related behavior, depressive-like behavior, spatial learning and memory were measured. As expected, serum 25(OH)D level was reduced in VDD diet-fed mice. An anhedonia state, a key depressive-like behavior, was observed in VDD diet-fed mice. In open-field test, peripheral time was decreased and internal time was increased in VDD diet-fed mice. In elevated plus maze, the latency of the first entry into open arms was increased and the number of crossing in open arms was elevated in VDD diet-fed mice. Morris Water Maze showed that VDD-fed mice showed longer escape latency in the first six days. On the seventh day, escape latency was increased in VDD diet-fed mice. These results provide evidence that vitamin D deficiency impairs neurobehavioral development.

1. Introduction

Vitamin D, a secosteroid hormone, is mainly synthesized in the skin from cholesterol precursor 7-dehydrocholesterol and is converted to vitamin D3 upon exposure to sunlight. Vitamin D is known for its classical functions in calcium uptake and bone metabolism [1]. Vitamin D deficiency, defined as lower than 20 ng/ml (50 nmol/l) of serum 25(OH)D, is prevalent especially in pregnant women and children and is increasingly recognized as a global public health problem [2–4]. Indeed, vitamin D deficiency among children not only resulted in the occurrence of rickets but also prevented children from reaching their genetically programmed height and peak bone mass [5,6]. On the other hand, several studies showed that maternal vitamin D deficiency during gestational period was associated with adverse pregnant outcomes [7,8]. A recent study found that maternal vitamin D deficiency during pregnancy elevated risks of small for gestational age and low birth weight infants [9]. Moreover, maternal vitamin D deficiency during gestational period was associated with the reduced bone-mineral content, the impaired muscle development, the increased asthma and multiple sclerosis in offspring [3,10–12].

Increasing evidence demonstrated that vitamin D status in old people was negatively associated with risk of neurodegenerative and neuropsychiatric disorders, such as Alzheimer's disease and dementia [13], mood disorders [14], and schizophrenia [15]. Moreover, numerous epidemiological studies observed that there was an association between vitamin D deficiency and an increased risk for cognitive disorders in elderly people [16–18]. Recently, several animal experiments showed that vitamin D deficiency in adulthood or old age resulted in anxiety-related behaviors and cognitive declines in mice [19–21]. A recent study found that supplementation with vitamin D3 improved performance on cognitive function in diabetic rats [22]. Another study showed that vitamin D3 supplementation prevented age-related cognitive decline in aging rats [23]. Nevertheless, it remains unclear whether vitamin D deficiency impairs neurobehavioral development.

The aim of the present study was to investigate the effects of vitamin D deficiency on neurobehavioral development in male mice. Our results showed that vitamin D deficiency resulted in anhedonia state, a key depressive-like behavior. Moreover, vitamin D deficiency increased anxiety-like activities. In addition, vitamin D deficiency impaired ability of spatial learning and memory in adulthood. The present study provides evidence that vitamin D deficiency impairs neurobehavioral development.

* Correspondence to: Yuan-Hua Chen, Department of Histology and Embryology, Anhui Medical University, Hefei, China.
** Correspondence to: De-Xiang Xu, Department of Toxicology, Anhui Medical University, Hefei, China.
E-mail addresses: yuanhuach@126.com (Y.-H. Chen), xudex@126.com (D.-X. Xu).
[1] These authors contributed equally to this work.

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2. Materials and methods

2.1. Animals and treatments

The ICR mice (8–10 week-old), one of most commonly outbred strain (ICR strain), were purchased from Beijing Vital River whose foundation colonies were all introduced from Charles River Laboratories, Inc. The animals were allowed free access to food and water at all times and were maintained on a 12-h light/dark cycle in a controlled temperature (20–25 °C) and humidity (50 ± 5%) environment for a period of 1 week before use. To investigate the effects of vitamin D deficiency on neurobehavioral development, ten dams were randomly divided into vitamin D deficiency and control groups. In vitamin D deficiency group, dams were fed with vitamin D deficient (VDD) diet (lower than 25 IU vitamin D3/kg), beginning 4 week before mating throughout gestational period and suckling period. After weaning, 3 male offspring were randomly selected from each litter. Total 15 male offspring were fed with VDD diet until the end of the experiment. In control group, dams and their male offspring were fed with standard feed (1000 IU vitamin D3/kg). After weaning, 3 male offspring were randomly selected from each litter. Total 15 male offspring were fed with standard feed until the end of the experiment. All animals were anesthetized with phenobarbital sodium (50 mg/kg) and sacrificed at postnatal 18 week. Blood samples from eye socket were collected for 25(OH)D, calcium and phosphorus levels. This study was approved by the Association of Laboratory Animal Sciences and the Center for Laboratory Animal Sciences at Anhui Medical University (Permit Number: 12-0005). All procedures on animals followed the guidelines for humane treatment set by the Association of Laboratory Animal Sciences and the Center for Laboratory Animal Sciences at Anhui Medical University. In this study, all mice were monitored at least twice per day. In addition, the rules of humane endpoints were strictly performed to determine when mice should be euthanized. All efforts were taken to minimize suffering when mice met our euthanasia criteria.

2.2. Measurement of 25(OH)D

Non-fasting blood samples of male offspring at postnatal 18 week were collected from eye socket and stored at − 80 °C, with no further freeze-thaw cycles, until 25(OH)D measurement. Serum 25-(OH)D was measured by Radioimmunoassay (RIA) with ^{125}I labelled 25(OH)D as a tracer, using a kit from DiaSorin (DiaSorin Inc., Stillwater, MN, USA) following manufacturer's instructions (9). Serum 25(OH)D is expressed as ng/ml.

2.3. Sucrose preference test

Anhedonia state in male offspring, a key depressive-like behavior, was analyzed by sucrose preference test (SPT) at postnatal 14 week [24]. Prior to SPT, all mice were housed individually and habituated to 48 h of forced 1% sucrose solution consumption in two bottles on each side. Then after 16 h water deprivation, we randomly placed two re-weighed bottles, one containing 1% sucrose solution and another containing tap water to each mouse. The bottles were weighted again after 1 h. The weight difference was calculated as amount of liquid intake from each bottle. Sucrose preference was calculated as a percentage of the consumed 1% sucrose solution relative to the total amount of liquid intake.

2.4. Anxiety-related activities

2.4.1. Black-white alley

Black-white alley was conducted to test anxiety-related activities of male offspring at postnatal 15 week. The apparatus for black-white alley is a narrow galvanized iron box (120 cm × 30 cm × 9 cm, half was black and the other half was white). Each mouse started in the less aversive black alley and was observed for 90 s. For each mouse, latency to enter into the white alley and total time spent in the black alley were recorded. The number of crossings was counted.

Table 1
Serum 25-(OH)D, calcium and phosphorus concentration.

	Control	VDD	F	*P* value
Serum 25-(OH)D (ng/ml)	27.06 ± 4.99	2.25 ± 0.48	25.519	0.003
Serum calcium (mmol/l)	3.182 ± 0.066	2.867 ± 0.099	5.356	0.033
Serum phosphorus (mmol/l)	3.152 ± 0.141	2.761 ± 0.092	6.985	0.017

2.4.2. Open field test

Open field test was conducted to test anxiety-related activities of male offspring at postnatal 15 week. The apparatus was a black wooden box (28 cm high). The box floor was painted with white lines to form 16 equal squares (20 cm × 20 cm) with a colored box (8 cm × 5 cm × 3 cm) in the center of the area. The mice were placed in a corner square, facing the walls and was permitted to explore the environment for 5 min ad lib. The following parameters were recorded: the number of rearing, the number of grooming, the number of manure, latency to the first grid crossing, total number of squares crossed, peripheral and internal distance, peripheral and internal times.

2.4.3. Elevated plus maze

Elevated plus maze was conducted to test anxiety-related activities of male offspring at postnatal 16 week. The apparatus was made up of two opposite enclosed arms (30 cm long, 5 cm wide, 15 cm high), two opposite open arms (30 cm long, 5 cm wide, without edges) and a central arena (5 cm × 5 cm). The whole apparatus was elevated 80 cm above the floor. The mouse was placed in the central arena of the apparatus facing an open arm. The mouse was observed for 5 min. Following parameters will be recorded and evaluated: the number of manure and climbing, the latency of the first entry into the open arms, the number of crossing, time in open arms and the enclosed arms.

2.5. Morris water maze (MWM)

MWM was conducted to test learning and memory performances of male offspring at postnatal 17 week. The instrument used was a circular black tank (150 cm diameter, 30 cm high) inside fills with water (temperature 24–26 °C, depth 25 cm). A black escape platform (diameter 10 cm, height 24 cm) was placed in one of the four quadrants of the tank. It included two parts: spatial training trials and spatial probe test. In the spatial training trials, the mice were tested with 4 trials per day in place navigation task for 6 consecutive days. On the seventh day, the spatial probe test was conducted to test spatial memory. A camera linked to a computer was fixed above the tank. Image analysis software was used to record the swim tracks, the latency to find the platform, swim distance, swim velocity, the number of crossing platform quadrant and time proportion in platform quadrant.

2.6. Statistical analysis

Normally distributed data were expressed as mean ± S.E.M. ANOVA and the Student-Newmann-Keuls post hoc test were used to determine differences among different groups. Data that were not normally distributed were assessed for significance using non-parametric tests techniques (Kruskal-Wallis test and Mann–Whitney *U* test). Data for the MWM were analyzed by repeated-measures two-way ANOVA. Data for aggressive performance were analyzed by one-way ANOVA. Other data were analyzed by two-way ANOVA. $P < 0.05$ was considered statistically significant.

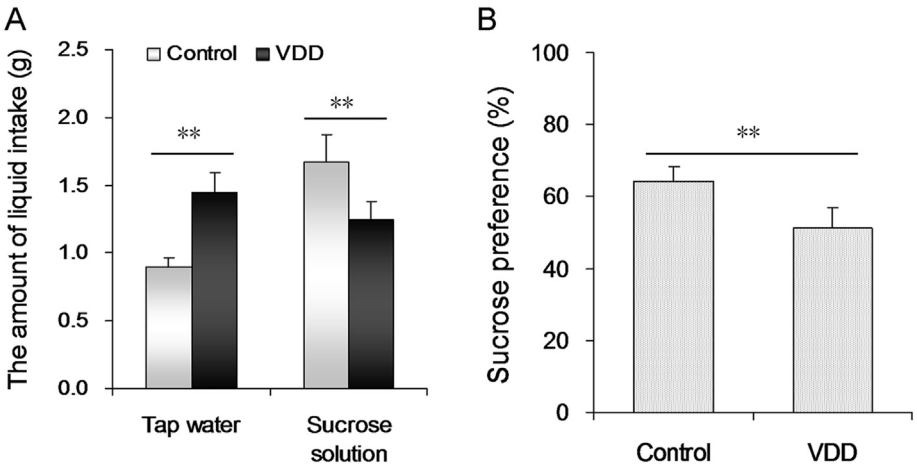

Fig. 1. Depressive-like behavior. In vitamin D deficiency group, dams and their male pups were fed with vitamin D deficient (VDD) diet, in which vitamin D was depleted. Anhedonia state in male offspring, a key depressive-like behavior, was analyzed by sucrose preference test at postnatal 14 week. (A) The amount of tap water and 1% sucrose solution intake. (B) Sucrose preference. All data were expressed as means ± S.E.M (n = 15). $^{**}P < 0.01$.

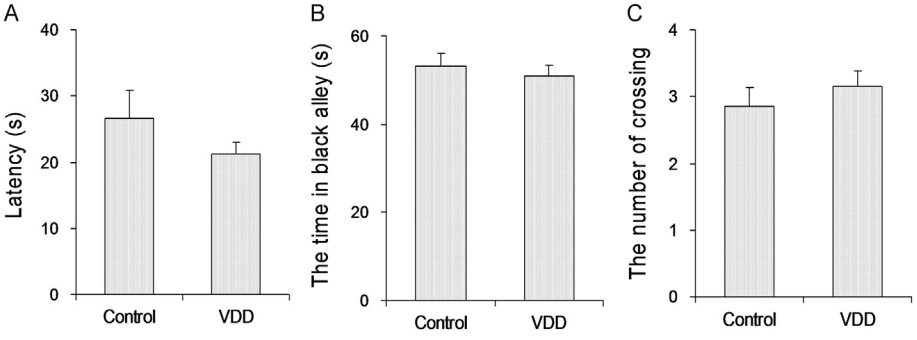

Fig. 2. Anxiety-related activities in black-white alley test. In vitamin D deficiency group, dams and their male pups were fed with vitamin D deficient (VDD) diet, in which vitamin D was depleted. Black-white alley was conducted to test anxiety-related activities at postnatal 15 week. (A) Latency to enter into the white alley. (B) The time in black alley. (C) The number of crossing. All data were expressed as means ± S.E.M (n = 15).

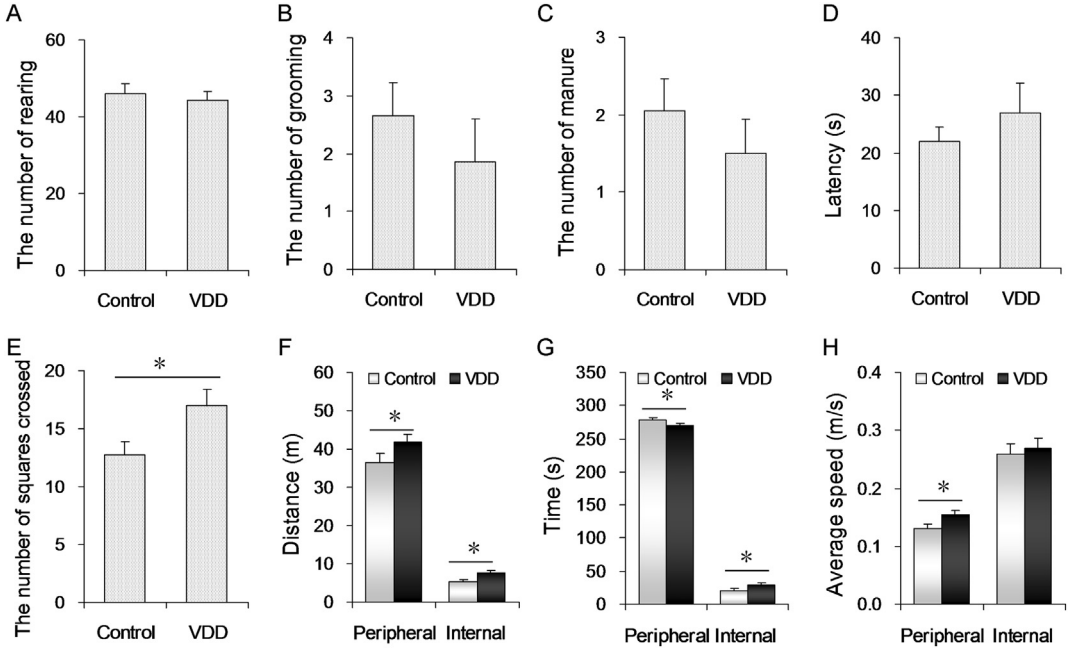

Fig. 3. Anxiety-related activities in open-field test. In vitamin D deficiency group, dams and their male pups were fed with vitamin D deficient (VDD) diet, in which vitamin D was depleted. Open-field was conducted to test anxiety-related activities at postnatal 15 week. (A) The number of rearing. (B) The number of grooming. (C) The number of manure. (D) Latency to the first grid crossing. (E) The number of squares crossed. (F) Distance. (G) Time. (H) Average speed. All data were expressed as means ± S.E.M (n = 15). $^{*}P < 0.05$.

3. Results

3.1. Serum 25(OH)D, calcium and phosphorus levels

In the present study, no significant difference on conception rate, gestation length, litter size, sex ratio and body weight was observed between VDD-fed mice and controls. Serum 25(OH)D level was measured among male offspring. As shown in Table 1, serum 25(OH)D level was significantly reduced in VDD diet-fed mice as compared with controls. Moreover, serum calcium and phosphorus levels were also reduced in VDD diet-fed mice (Table 1).

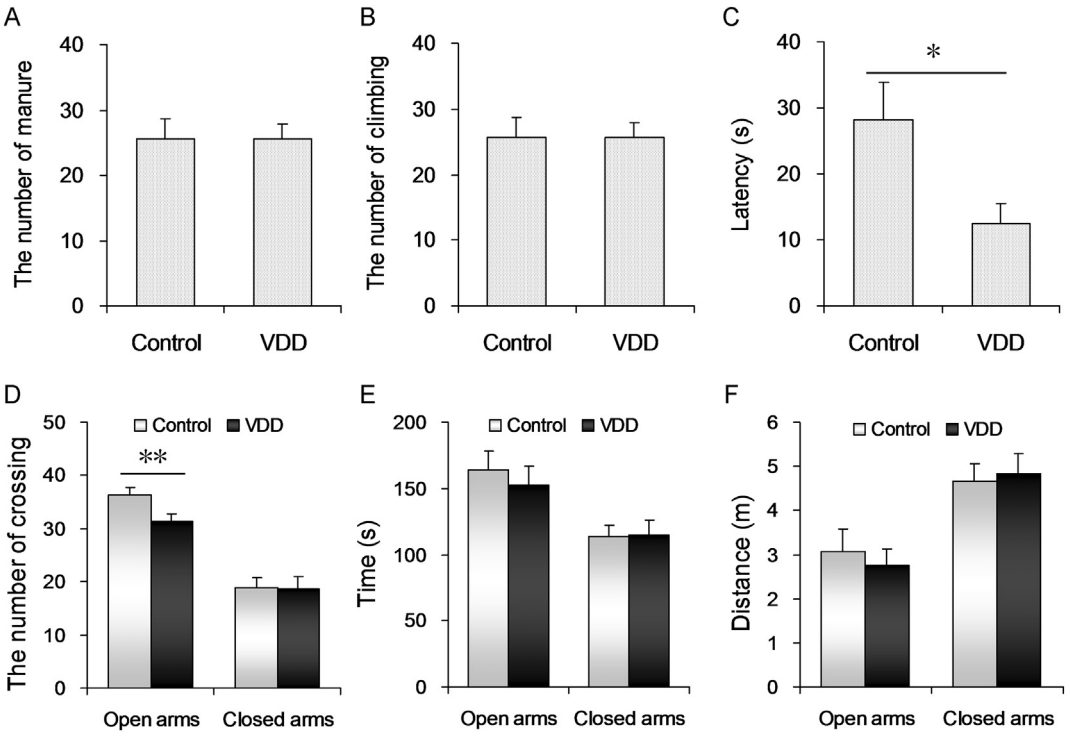

Fig. 4. Anxiety-related activities in elevated plus maze test. In vitamin D deficiency group, dams and their male pups were fed with vitamin D deficient (VDD) diet, in which vitamin D was depleted. Elevated plus maze was conducted to test anxiety-related activities at postnatal 16 week. (A) The number of manure. (B) The number of climbing. (C) The latency of the first entry into the open arms. (D) The number of crossing. (E) Time in open arms and closed arms. (F) Distance in open arms and closed arms. All data were expressed as means ± S.E.M (n = 15). *P < 0.05, **P < 0.01.

3.2. Depressive-like behavior

The effects of vitamin D deficiency on anhedonia state, a depressive-like behavior, were analyzed. As shown in Fig. 1A, mice drank more tap water in VDD diet-fed mice as compared with controls (F = 8.910, P = 0.005). By contrast, mice drank more sucrose solution in control group than in vitamin D deficiency group (F = 10.331, P = 0.002). Compared with controls, mice in VDD diet-fed mice showed a significant reduction of sucrose preference (Fig. 1B, F = 8.224, P = 0.007).

3.3. Anxiety-related activities

The effects of vitamin D deficiency on anxiety-related activities were detected by three tests: black-white alley, open-field and elevated plus-maze. In black-white alley, there were no significant differences in latency to enter into the white alley, the time in black alley and the number of crossing (Fig. 2A–C). In the open-field test, there were no significant differences in the number of rearing, grooming and manure (Fig. 3A–C). In addition, no significant difference was observed in latency to the first grid crossing (Fig. 3D). Of interest, the number of squares crossed was significantly increased in VDD diet-fed mice (Fig. 3E, F = 3.846, P = 0.023). Moreover, both peripheral distance (F = 3.231, P = 0.040) and internal distance (F = 5.647, P = 0.023) were significantly increased in VDD diet-fed mice (Fig. 3F). As shown in Fig. 3G, peripheral time was decreased in VDD diet-fed mice (F = 5.358, P = 0.026). By contrast, internal time was increased in mice fed with VDD diet (Fig. 3G, F = 5.368, P = 0.026). The average speed in Peripheral Square was increased in mice with VDD diet (Fig. 3H, F = 4.791, P = 0.035). In the elevated plus maze, there were no differences in the numbers of manure and climbing between two groups (Fig. 4A, B). Of interest, the latency of the first entry into the open arms was significantly reduced in VDD diet-fed mice (Fig. 4C, F = 5.677, P = 0.024). Further analysis showed that the number of crossing only in open arms (F = 6.335, P = 0.009) but not in closed arms (F = 0.134, P = 0.717) was reduced in VDD diet-fed mice (Fig. 4D). There were no significant differences in time and distance in open arms and closed arms between two groups (Fig. 4E, F).

3.4. Learning and memory performances

The effects of vitamin D deficiency on learning and memory performance were detected by Morris Water Maze. First, the learning performances in the first six days are analyzed. Representative MWM reversal probe path tracings were shown in Fig. 5A (F = 4.913, P = 0.023). Of interest, the latency to find the platform on days 3 (F = 3.267, P = 0.041) and 4 (F = 4.850, P = 0.034) was longer in VDD diet-fed mice than controls. In addition, There were no significant differences in swim distance and swim velocity in Morris Water Maze between two groups (Fig. 5C, D). Spatial memory was then assessed on the seventh day. Representative MWM reversal probe path tracings were shown in Fig. 5E. As shown in Fig. 5F, the latency (F = 7.545, P = 0.011) to find the platform is significantly longer in VDD diet-fed mice than in controls. No significant difference in the number of crossing platform quadrant was observed between two groups (Fig. 5G). Moreover, there was no significant difference on swim distance and time proportion in platform quadrant between two groups (Fig. 5H, I).

4. Discussion

In the present study, we investigated the effects of vitamin D deficiency on neurobehavioral development in male mice. Our results showed that an anhedonia state, a key depression-like behavior, was observed in VDD diet-fed mice. Moreover, anxiety-related activities, as determined by black-white alley, open-field and elevated plus-maze, were significantly elevated in VDD diet-fed mice. In addition, the ability of spatial learning and memory, as determined by Morris Water Maze, was impaired in VDD diet-fed mice. These results suggest that vitamin D

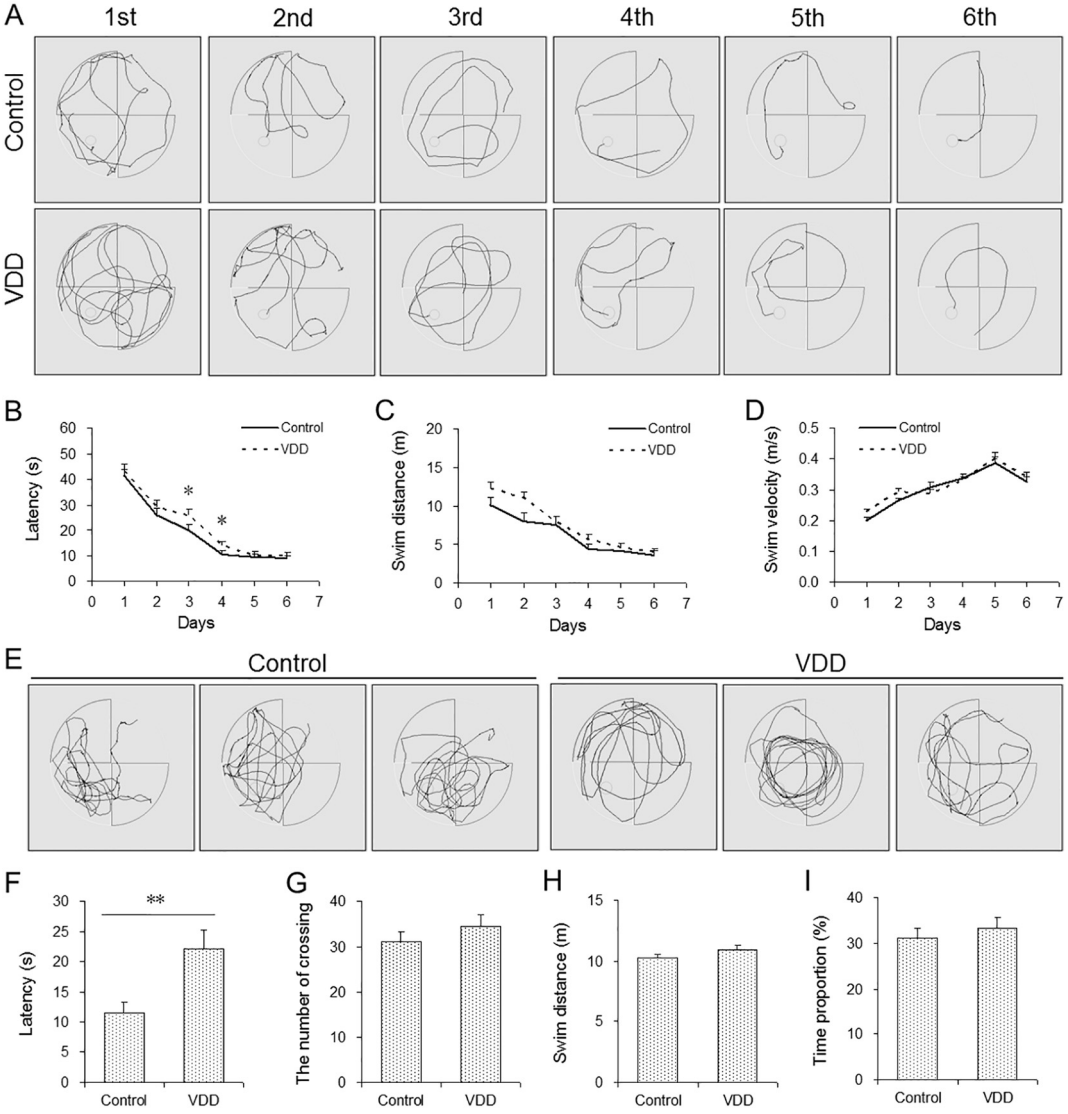

Fig. 5. Learning and memory performance. In vitamin D deficiency group, dams and their male pups were fed with vitamin D deficient (VDD) diet, in which vitamin D was depleted. MWM was conducted to test learning and memory performances at postnatal 17 week. (A-D) Learning performance was examined on the first six days. (A) Representative MWM reversal probe path tracings. (B) The latency to find the platform. (C) Swim distance. (D) Swim velocity. (E–I) Memory performance was assessed on the seventh day. (E) Representative MWM reversal probe path tracings on the seventh day. (F) The latency to find the platform. (G) The number of crossing platform quadrant. (H) Swim distance. (I) The time proportion in platform quadrant. All data were expressed as means ± S.E.M ($n = 15$). *$P < 0.05$, **$P < 0.01$.

deficiency impairs neurobehavioral development.

Several epidemiological investigations explored the association between vitamin D status and risk of depression with contradictory results. Some reports showed that there was a positive association between vitamin D deficiency and an increased risk of depression in middle-aged and elderly population [25]. Other data found that no association between vitamin D status and depression was observed in adult and elderly people [26–28]. Until now, no report analyzed between vitamin D status in early life and risk of depression in adult period. To investigate the effect of vitamin D deficiency on depressive-like behavior in male offspring, pregnant dams and their pups were fed with VDD diet. The depressive-like behavior in male adult offspring was measured using sucrose preference test. Our results showed that there was a significant reduction of sucrose preference in VDD diet-fed mice. These results indicate that vitamin D deficiency induces a depressive-like behavior.

Numerous studies had demonstrated that vitamin D deficiency was associated with anxiety-related activities. According to a case-control study, vitamin D deficiency occurs more frequently in patients with anxiety [29]. Another epidemiological study showed that there was a negative correlation between serum 25(OH)D concentration and anxiety [30]. Recently, an animal experiment found that an anxiety-related behavior, as determined by the elevated plus maze, was observed in mice fed with VDD diet [19]. The present study investigated the effects of vitamin D deficiency on anxiety-related behavior. Anxiety-related activities were determined by black-white alley, open field task or elevated plus maze. In the open-field test, peripheral time was decreased in mice fed with VDD diet. By contrast, internal time was increased in VDD diet-fed mice. In the elevated plus maze, the latency of the first entry into the open arms was decreased in VDD diet-fed mice. The number of crossing in open arms is lower in VDD diet-fed mice than in control. The present study demonstrates for the first time that vitamin D deficiency aggravates anxiety-related activities in adulthood.

Cognitive decline is a predictor of brain function impairment. Indeed, patients with cognitive decline perform worse learning and memory [31]. Several epidemiological reports demonstrated that vitamin D deficiency in elderly people was associated with a reduced capacity of memory [16–18]. On the other hand, an animal study found that vitamin D deficiency in adulthood impaired cognitive function in male mice, as determine by assessing two cognitive tasks, the 5 choice-

serial reaction task and the 5 choice-continuous performance task [32]. Supplementation with active vitamin D3 improved conditioned fear memory in mouse models of Alzheimer's disease [33]. In the present study, the effects of vitamin D deficiency on learning performances and memory ability were evaluated by Morris Water Maze. In the first six days, the escape latency on days 3 and 4 was longer in VDD diet-fed mice than in controls. There were no significant differences in swim distance and swim velocity in Morris Water Maze between two groups. On the seventh day, the escape latency was longer in VDD diet-fed mice than in controls. These results suggest that vitamin D deficiency impairs the ability of learning and memory in male mice.

Accumulating evidence demonstrates that neural stem cell proliferation and newborn neuron differentiation play important roles in mood behavior, learning and memory through contributing to synaptic plasticity of hippocampus [34–38]. On the other hand, several studies indicate that vitamin D3 regulates cell proliferation and differentiation through activating vitamin D receptor (VDR) signaling [39,40]. Moreover, vitamin D3 induced a variety of neurotrophic factors, such as nerve growth factor, Brain-derived neurotrophic factor and Glial-derived neurotrophic factor [41,42]. These neurotrophic factors have been known for their classical functions in neuronal proliferation and differentiation [43]. Indeed, several studies found that VDR was highly expressed in human and rodent brains [44–46]. Mice lacking functional VDR showed a specific anxiety-related behavior [47,48]. Thus, it is reasonable to postulate that vitamin D deficiency impairs neurobehavioral development through down-regulating VDR signaling and subsequent neuronal proliferation and differentiation.

The present study had several flaws. Firstly, the present study found that vitamin D deficiency caused anxiety-related activities and cognitive decline in adult animals. These results need to be demonstrated in a population-based epidemiological investigation. Secondly, the present study had not explored the effects of vitamin D deficiency on neurobehavioral development in female adult offspring. Indeed, a recent study found that vitamin D deficiency in adulthood led to a mild cognitive impairment in male but not female mice [32]. Thus, additional research is required to analyze whether vitamin D deficiency impairs neurobehavioral development in female adult offspring. In addition, several studies showed that supplementation with calcium restored the behavioral phenotype seen in whole of life vitamin D deficiency [49,50]. Actually, the present study found that serum calcium and phosphorus levels were also reduced in VDD diet-fed mice. Thus, the present study cannot exclude the possibility that vitamin D deficiency impairs neurobehavioral development through reducing serum calcium level.

In summary, the present study investigated the effects of vitamin D deficiency on neurobehavioral development. Our results showed that vitamin D deficiency induced anhedonia state, a key depressive-like behavior. Moreover, vitamin D deficiency increased anxiety-related activities. In addition, vitamin D deficiency impaired the ability of spatial learning and memory in adulthood. The present study provides evidence that vitamin D deficiency impairs neurobehavioral development. Thus, supplementation with vitamin D3 may be a potential strategy for preventing anxiety disorders and cognitive declines.

Disclosure

The authors have declared that no competing interests exist.

Competing financial interests

The authors report no conflict of interests.

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