Article

Vitamin D Levels Are Not Associated with Hippocampal-Dependent Learning in Young Adult Male C57BL/6J Mice: A Negative Report

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ABSTRACT

It is well established that vitamin D is essential in calcium homeostasis and bone metabolism. Recent evidence has exposed further roles of vitamin D in adult brain function, specifically indicating that low vitamin D levels during adulthood may be related to cognitive impairment. We have recently shown that adult vitamin D (AVD) deficiency disrupts hippocampal-dependent learning and structural brain connectivity in BALB/c mice. The BALB/c mouse strain is more vulnerable to social stress compared with other resilient mouse strains, such as C57BL/6J mice. Therefore, the primary aim of this research was to examine C57BL/6J mice exposed to varying levels of vitamin D (0, 1500 and 15,000 IU/vitamin D_3/kg referred to as deficient, control and elevated, respectively) for 10 weeks. The mice were assessed for hippocampal-dependent learning using the active place avoidance (APA) task. Mice were tested for behaviours that could alter performance on the APA task, and hippocampal tissue was analysed for catecholamine and protein expression. Vitamin D status did not affect spatial learning and memory, general behavioural domains, or catecholamine or protein expression in C57BL/6J mice. Overall, these results indicate that, in contrast to BALB/c mice, vitamin D status does not impact on hippocampal-dependent behaviour in young and healthy, adult male C57BL/6J mice.

Keywords: vitamin D; brain function; hippocampus; animal model; adult deficiency; dopamine

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INTRODUCTION

Vitamin D_3 (cholecalciferol) can be acquired through the diet or synthesised in the skin in response to ultraviolet-B (UVB) radiation, where 7-dehydrocholesterol is converted into vitamin D_3 [1]. Vitamin D_3

is biologically inactive, and undergoes two hydroxylation steps before the biologically active 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) is formed [2]. Vitamin D_3 is converted to the major circulating form, 25-hydroxyvitamin D_3 ($25\text{-}OHD_3$), in the liver by 25-hydroxylase and predominantly converted to $1,25\text{-}(OH)_2D_3$ in the kidney by 1α-hydroxylase [3]. However, 1α-hydroxylase can also act in the brain [4]. $1,25(OH)_2D_3$ regulates calcium and phosphorous homeostasis [3], and elicits physiological regulation of gene transcription by binding to the vitamin D receptor (VDR) [5]. VDR [6] and the enzymes involved in vitamin D_3 metabolism [4,7,8] are also found in the brain, including the hippocampus of rats [9] and mice [10], implying a role for vitamin D_3 in brain function. 24-hydoxylase inactivates all forms of circulating vitamin D_3 into 1,24,25-trihydroxy vitamin D_3 ($1,24,25\text{-}(OH)_3D_3$) for excretion [11].

Populations are at risk of vitamin D deficiency when UVB exposure is limited [2] and when foods fortified with vitamin D are not sufficient to counteract decreased vitamin D synthesis [12]. Vitamin D deficiency is a global problem, where 30% of the Australian adult population are deficient [13]. Although there is no clear consensus on the classification of vitamin D deficiency, recent guidelines suggest that vitamin D deficiency is a concentration below 50 nmol/L of $25(OH)D_3$, insufficiency is 50–75 nmol/L and sufficiency is above 75 nmol/L [14].

Vitamin D plays a role in ensuring immune homeostasis [15], controlling inflammatory responses [16] and promoting the production of neuroprotective factors [17]. Recent research has shown that vitamin D has important roles in the developing brain [18] and that adult vitamin D (AVD) deficiency may be associated with adverse brain-related outcomes [19]. Vitamin D is an inexpensive and publically accessible treatment. Therefore, if supplementation is effective in alleviating cognitive decline, this could have important public health implications.

Animal studies have specifically tested the link between vitamin D and cognition, and have shown that vitamin D can alter cognitive function in aged animals [20]. These studies indicate that supplementation with vitamin D improves learning and memory in aged healthy rodents [20,21], whereas only a few studies have confirmed AVD-deficiency impairing spatial performance or significant neurochemical outcomes in younger healthy rodents [22]. We recently examined the effect of AVD deficiency on spatial learning in male BALB/c mice and revealed a specific impairment on the active place avoidance task [23], and this hippocampal-dependent spatial learning deficit was replicated in a larger independent cohort of AVD-deficient mice [23].

However, several studies suggest that the effects of AVD deficiency are dependent on the background strain. For example, BALB/c mice displayed greater effects of AVD deficiency on behaviour when compared directly with C57BL/6J mice [22]. In addition, BALB/c and C57BL/6J mice show a different susceptibility to social defeat stress, with only the BALB/c mice exhibiting long-term social withdrawal [24]. With

respect to AVD deficiency, social stress affected behaviour to a greater extent in BALB/c mice compared to C57BL/6 mice [25].

The overall aim of this research was to investigate the effect of vitamin D_3 on cognition and brain function, in healthy adult C57BL/6J mice. Specifically, this study aimed to (a) examine the role of vitamin D_3 status on spatial learning and memory and to (b) provide evidence for an impact of vitamin D_3 status on neurochemistry and protein expression in the hippocampus. We hypothesised that vitamin D supplementation in healthy, young adult C57BL/6J mice would improve deficits in spatial learning and memory and hippocampal neurochemistry and protein expression induced by AVD deficiency.

METHODS

Animal and Housing

Ninety-three naïve adult male C57BL/6J mice (10 weeks old) (Animal Resources Centre, WA) were housed in groups of four in individually ventilated OptiMICE cages (Animal Care Systems, USA). Each cage of C57BL/6J mice was assigned one of three diets that varied in vitamin D_3 (Standard AIN93G Rodent Diet, Specialty Feeds, WA, Australia) (Appendix 1); either deficient (0 IU of vitamin D_3/kg, n = 30), control (1500 IU/kg, n = 32) or elevated (15,000 IU/kg, n = 31). These values were before irradiation with 25 kGy. These diets were selected to maintain serum 25-hydroxyvitamin D_3 ($25(OH)D_3$) levels of approximately <5 nmol/L, 35 nmol/L and >75 nmol/L respectively [26], while not depleting calcium stores.

Mice were housed under standard conditions (water and food available *ad libitum*, 21 ± 2 °C, 50 ± 10% humidity, 12 h light-dark cycle, lights on at 07:00 h), using incandescent lighting free from UVB radiation. Mice were maintained on diets for a minimum of 8 weeks and for the entire duration of experimental procedures. Testing began on C57BL/6J mice at 20 weeks old. All work was undertaken with approval of The University of Queensland Animal Ethics Committee (QBI/202/13/NHMRC/QCMHR) under the guidelines of the National Health and Medical Research Council of Australia.

Behavioural Apparatus and Procedures

In behavioural experiments, the apparatus was cleaned between animals using 70% ethanol. Mice were habituated in the test room for at least 30 min prior to testing and all experiments were performed from 08:00 h to 18:00 h. Experiments were conducted to minimise bias such that different diets were tested in an alternating fashion. One cohort of C57BL/6J was examined for a number of behavioural domains and another cohort of C57BL/6J was tested on active place avoidance (APA).

Active Place Avoidance

Behaviourally naïve C57BL/6J mice were tested on APA in order to assess hippocampal-dependent memory [27]. The apparatus (Bio-Signal

Group, USA) consisted of an elevated arena with a grid flood, surrounded by enclosed transparent circular boundary (35 × 77 cm). This was located in an enclosed room with a different visual cue on each wall. The arena rotated counter-clockwise (1 rpm). Mice were habituated (Day 0) to the apparatus and the room 24 h before the experiment, where they were placed in the rotating arena for 5min with the shock turned off.

During the trials, the mice were placed in the arena and trained to avoid a frame-defined sector (60° shock zone), based on external cues. The start position of the mice was opposite the shock zone, near the barrier of the arena. Entrances to the shock zone elicited a brief foot shock (500 ms, 60 Hz, 0.5 mA). If the animal remained in the shock zone, shocks were delivered at 1500 ms intervals until the animal exited the zone. Mice were tested over 5 consecutive days and each training session lasted 10 min. Activity was monitored by an overhead camera linked to Tracker software and analysed using Track Analysis software (Bio-Signal Group, USA, version 2.36). This software measured two parameters that related to memory retention (number of entries and latency to first entry) and the distance travelled. Light was maintained at 300lx using white light-emitting diode (LED) lighting.

Behavioural Test Battery

Behaviourally naïve C57BL/6J mice were subjected to a behavioural test battery, beginning with the most sensitive and least aversive tests: elevated plus maze (EPM), activity monitors, hot plate test (HPT) and forced swim test (FST). Each test was performed on a separate day.

Elevated plus maze

The EPM was used to test for anxiety-like behaviours. The EPM was a plus-shaped opaque Perspex platform consisting of two enclosed arms (30 × 5 × 30 cm) and two open arms (30 × 5 cm) radiating from a centre platform (5 × 5 cm), elevated 50 cm above the ground. Mice were placed on the centre platform, facing towards the front open arm. Activity was monitored for 10 min using an overhead camera linked to video capturing software (PowerDirector, Cyberlink Corp, Taiwan). Image analysis software (Ethovision XT 9, Noldus, The Netherlands) was used to calculate the distance travelled, time spent in the open arms, the closed arms and the centre. The time spent in the open arms relative to the closed and open arms was used as the primary measure of anxiety-related behaviour [28]. Light was maintained at 520lx using white LED lighting.

Activity monitor

The activity monitor was used to assess locomotion [29]. The apparatus was a clear Perspex chamber (45 × 45 × 45 cm) equipped with three 16-beam infrared arrays (Med Associates Inc., USA) within a sound attenuated box with a ventilation fan. Mice were placed in the bottom left hand corner of the box and locomotion was measured for 30 min. Up

to eight mice were tested in separate boxes at one time. Light was maintained at 80lx during the trial using white LED lighting. Locomotion was measured in 5 min intervals.

Hot plate test

The HPT was used to assess response to noxious stimuli [30]. Mice were placed on an automatic hot plate (Harvard Apparatus Ltd., UK) set to 53 °C and contained in a clear Perspex cylinder (20 × 50 cm). The latency of the animal to show a hind limb response (licking or jumping) was recorded as a measure of nociception. The test consisted of three trials (maximum length, 30 s) with an inter-trial period of 60 s.

Forced swim test

The FST was used to measure behavioural despair [31]. Mice were placed in clear, cylindrical plastic containers (13 × 20 cm) filled 11 cm deep with 25 ± 1 °C clean water. Activity was monitored for 10 min using an overhead camera linked to video capturing software (PowerDirector, Cyberlink Corp, Taiwan). Image analysis software (Ethovision XT 9, Noldus, The Netherlands) was used to determine distance moved and swimming ability. Activity between 0% and 5% was defined as immobility and immobility time between 3–6 min was interpreted as a measure of learned helplessness.

Blood Sera and Tissue Collection

Blood sera and brain tissue were collected from a representative sample of mice. Mice were anaesthetised with 2% isoflurane (Attane Isoflurane, Bayer, Australia Ltd.) and then rapidly decapitated. A terminal blood sample was decanted after decapitation. The brain was extracted and placed in a 0.9% saline solution on ice before dissection. The brain was sectioned into left and right frontal pole (olfactory bulbs removed), cerebellum and left and right hippocampus by free hand dissection, using 'Paxinos and Franklin's the Mouse Brain in Stereotaxic Coordinates' as a reference [32]. Samples were frozen immediately in liquid nitrogen, before storage at −80 °C until analysis. Tissue collection was conducted by alternating between genotypes and diets to minimise bias.

Blood samples were kept at room temperature for 1–3 h before centrifugation (5000 rpm, 10 min). Isolated serum was stored at −80 °C until analysis. 25(OH)D_3 levels from mice were measured and quantified using Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS) on a 4000 QTrap API AB mass spectrometer (Sciex Instruments, Canada), as previously described [18].

Western Blot

Western Blot was used to analyse glutamate decarboxylase (GAD) 65/67 expression, normalised to glyceraldehyde 3-phosphate dehydrogenase (GAPDH), in hippocampal tissue as previously described [22].

High Performance Liquid Chromatography

High performance liquid chromatography (HPLC) with electrochemical detection was used to measure catecholamines, indoleamines and their associated metabolites in the hippocampus as previously described [22,33].

Statistical Analysis

Data were analysed using SPSS software (version 23.0) and graphs were generated using GraphPad Prism (GraphPad Software, Version 7.0a). Data were analysed for the main effects of Diet. Data were analysed using one-way or two-way analysis of variance (ANOVA) or repeated measures (RM)-ANOVA. Pearson correlations were performed between behavioural variables and individual 25(OH)D_3 levels. Values were expressed as mean ± standard error of the mean (SEM) with significance determined at $p < 0.05$. Significant differences were followed up with a Bonferroni *post hoc* test.

RESULTS

Blood Serum

Blood serum 25(OH)D_3 levels were measured in mice fed each of the three diets to determine if the diets had effectively altered vitamin D status. There was a main effect of Diet on serum 25(OH)D_3 levels ($F_{2, 90}$ = 211.8, $p < 0.001$) (Figure 1). *Post hoc* tests indicated that all diets were significantly different ($p < 0.001$) compared to controls.

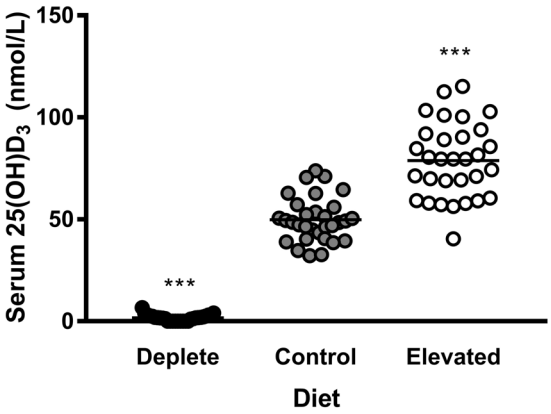

Figure 1. Dietary vitamin D status affected serum 25(OH)D3 levels in healthy male C57BL/6J mice. Serum 25(OH)D3 concentrations of mice on each vitamin D-controlled diet with mean ± SEM; deficient (1.7 ± 1.6), control (49.8 ± 10.8) and elevated (78.9 ± 18.4). n = 29–31 per diet. One-way ANOVA. ***$p < 0.001$ denotes significance compared to control mice.

Active Place Avoidance

The APA task was used to investigate if vitamin D affected spatial learning and memory in healthy adult male C57BL/6J mice (N = 48). Overall, vitamin D status in healthy adult C57BL/6J mice did not have an effect on spatial learning and memory in APA. There were no significant

interactions of Day*Diet on latency to first entry, number of entries or distance travelled ($F_{9, 132} < 1$, $p > 0.1$) (Figure 2A–C). There were no significant main effects of Diet on latency to first entry, number of entries or distance travelled ($F_{3,45} < 1$, $p > 0.1$) (Figure 2A–C).

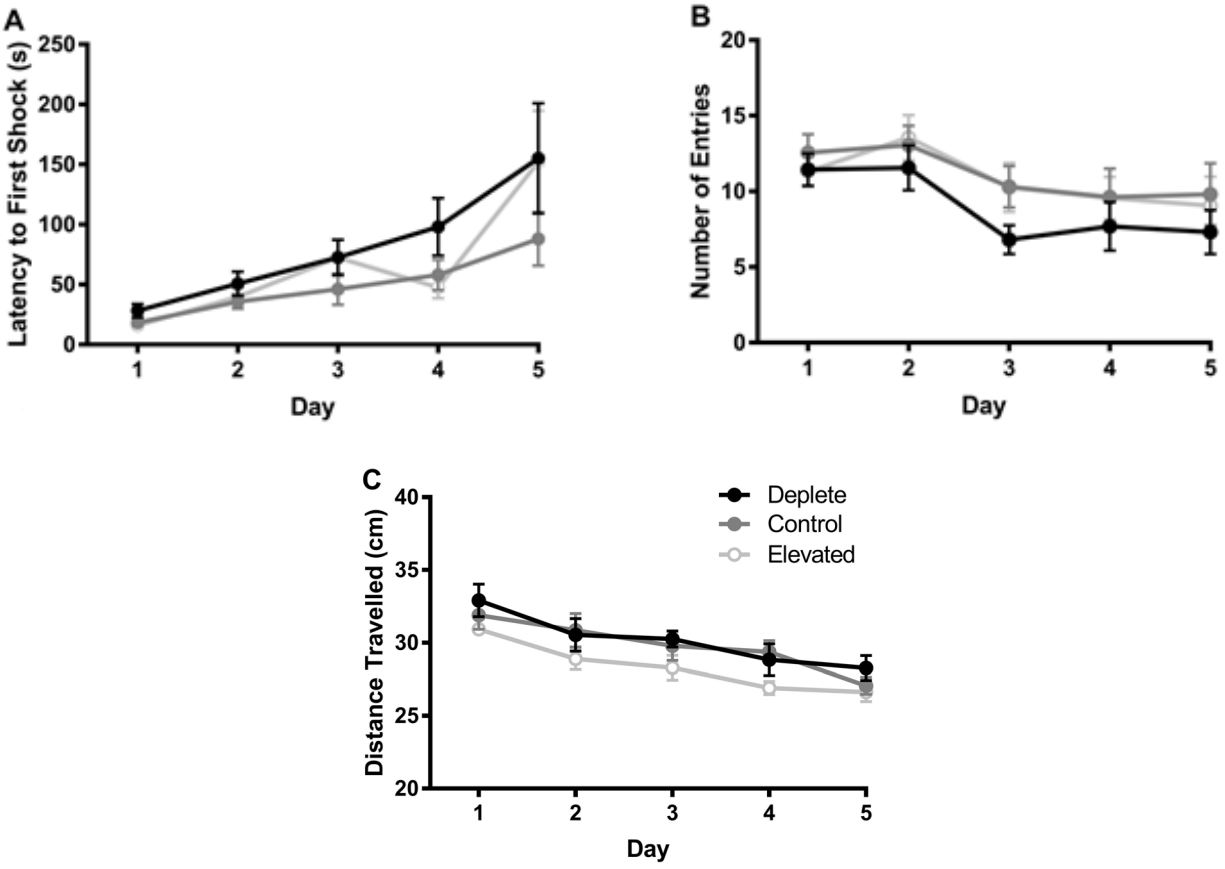

Figure 2. Dietary vitamin D did not affect spatial learning and memory performance on APA in healthy adult male C57BL/6J mice. C57BL/6J mice, exposed to three different vitamin D-controlled diets, were trained on APA. Over the 5 days of training, all mice increased (**A**) the latency to first entry and decreased (**B**) the number of entries and (**C**) distance travelled. There was no significant effect of Diet on any of the parameters (A–C). Data expressed as mean ± SEM, n = 16 per diet. Abbreviations: APA, active place avoidance.

Behavioural Test Battery

To investigate if vitamin D affected behaviours that could alter performance on the APA task, healthy adult male C57BL/6J mice (N = 48) were tested on EPM, activity monitor, HPT and FST. In the EPM, there were no significant main effects of Diet on total distance travelled or percentage of time spent in the open arms relative to the closed arms ($F_{2,32} < 4$, $p > 0.5$) (Figure 3A). In the activity monitor, there was no significant interaction of Time*Diet ($F_{9,93} < 1$, $p > 0.5$) or main effect of Diet ($F_{3,43} < 1$, $p > 0.5$) on locomotion over 5min blocks (Figure 3B). On the HPT, there was no significant interaction of Trial*Diet ($F_{4,64} < 2$, $p > 0.1$) or main effect of Diet ($F_{2,32} < 1$, $p > 0.5$) on the latency to react over 3 trials (Figure 3C). In the FST, there were no significant interactions of Time*Diet on

time spent immobile or distance moved between 3–6 min ($F_{4,90} < 1$, $p > 0.5$) (Figure 3D). In the FST, there was no significant main effects of Diet on time spent immobile or distance moved between 3–6 min ($F_{2,45} < 1$, $p > 0.5$) (Figure 3D). Overall, these results indicate that AVD status did not affect any behavioural domains that could also affect performance on APA. There were no significant correlations between individual 25(OH)D_3 levels and any behavioural measure (data not shown).

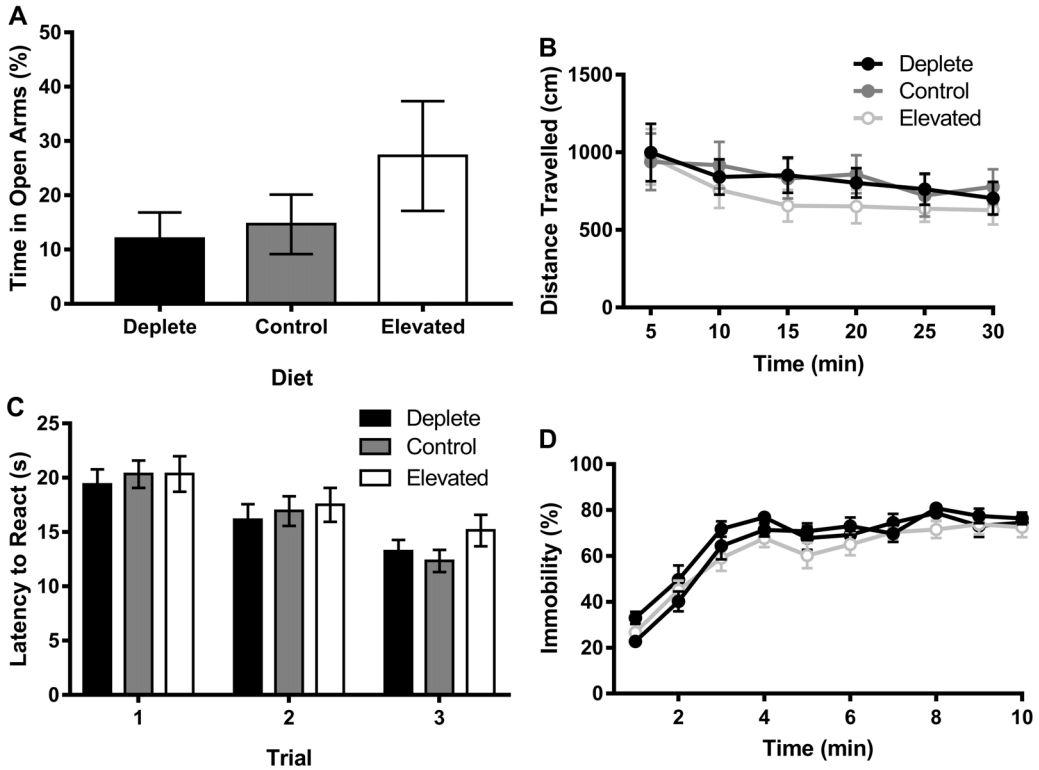

Figure 3. Dietary vitamin D status did not affect general behaviours in healthy adult male C57BL/6J mice. (**A**) In the EPM, there was no main effect of Diet on percentage of time spent in the open arms. (**B**) In the activity monitor, there was no main effect of Diet on distance travelled over 5min blocks. (**C**) On the HPT, there was no main effect of Diet on latency to react over the three trials. (**D**) In the FST, there was no main effect of Diet on time spent immobile between 3–6 min. Data expressed as mean ± SEM, n = 11–16 per diet. One-way ANOVA and RM-ANOVA. Abbreviations: EPM, elevated plus maze; HPT, hot plate test; FST, forced swim test.

Western Blot

To investigate if vitamin D status affected enzymes involved in ɣ-aminobutyric acid (GABA) synthesis, hippocampal tissue from C57BL/6J mice (N = 27) was analysed for protein content using a Western Blot. There were no significant main effects of Diet on levels of GAD65 or GAD67 ($F_{2,78} < 0.05$, $p > 0.5$) (Figure 4A,B).

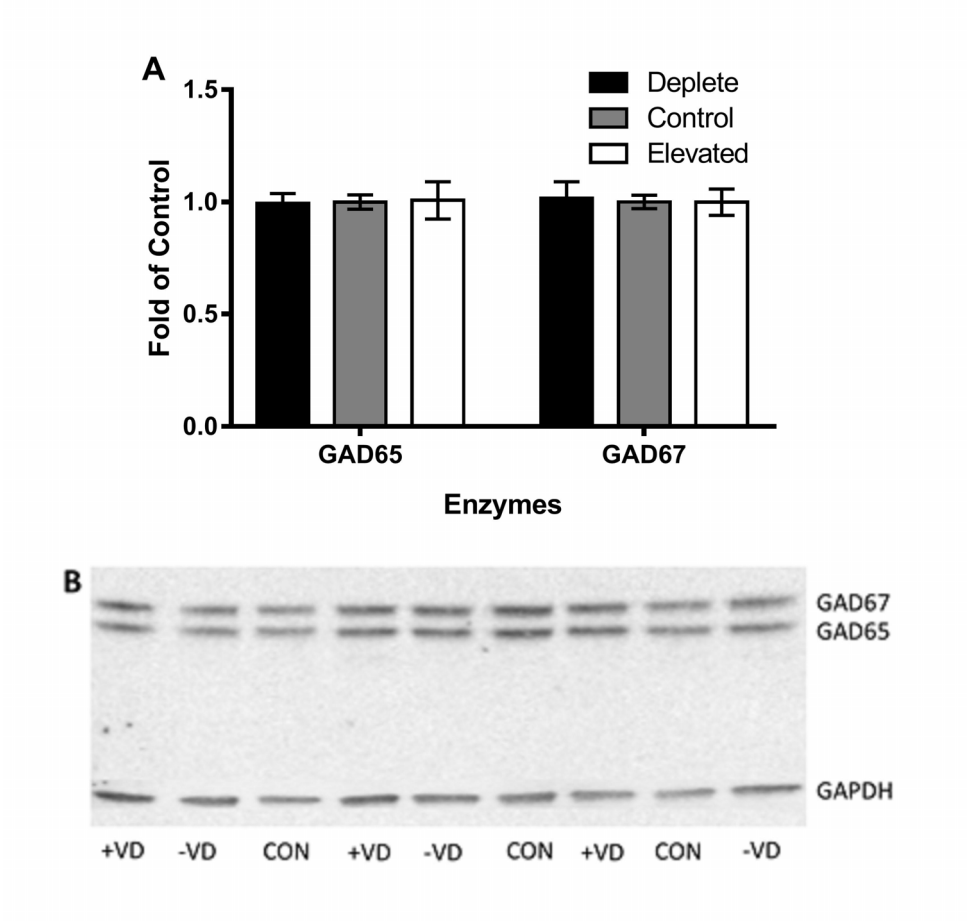

Figure 4. Dietary vitamin D did not affect expression of GAD65/67 in healthy adult male C57BL/6J mice. (**A**) There was no main effect of Diet on normalised levels of GAD65 or GAD67. (**B**) A representative Western Blot gel is shown, which includes AVD-deficient (-VD), AVD-control (CON) and AVD-elevated (+VD) samples. Data are expressed as mean ± SEM, n = 9 per diet, repeated in triplicate. One-way ANOVA.

HPLC Analysis

To investigate if vitamin D status affected neurotransmitter expression, hippocampal tissue from C57BL/6J mice (N = 18) was analysed using HPLC. There were no significant main effects of Diet on neurotransmitter levels in hippocampal tissue ($F_{2,14} < 2$, $p > 0.1$) (Table 1).

Table 1. Mean (± SEM) levels for catecholamines in hippocampal tissue of C57BL/6J mice fed either a vitamin D deficient, control or elevated vitamin D diet (n = 6/group).

ng/g Wet Tissue	Deficient	Control	Elevated
Noradrenaline	155.9 ± 8.6	168.2 ± 12.8	179.1 ± 9.6
Dihydroxyphenylacetic acid	101.6 ± 31.1	143.9 ± 14.3	116.3 ± 32.1
Dopamine	18.9 ± 9.2	10.6 ± 1.5	7.7 ± 1.8
5-Hydroxy-indoleacetic acid	288.2 ± 35.6	285.1 ± 30.3	234.2 ± 21.6
Homovanillic acid	32.8 ± 4.6	31.5 ± 3.7	32.1 ± 10.1
3-Methoxytyramine		not detectable	
5-Hydroxytryptamine	208.3 ± 25.4	224.4 ± 26.6	214.7 ± 23.3

DISCUSSION

There is growing evidence to support AVD deficiency as a risk factor for cognitive decline [34]. This study examined the effects of AVD status in healthy, adult male C57BL/6J mice. After 10 weeks on diets that varied in vitamin D_3 (deficient, control, elevated), serum levels of 25(OH)D_3 were altered, but this was not associated with alterations in learning and memory or in neurochemical processes in the hippocampus. Overall, we showed that vitamin D status did not affect behaviour or brain function in healthy, adult C57BL/6J male mice.

Numerous studies have shown that there is a more pronounced effect of vitamin D supplementation on cognitive function in aged mice [20,21], and only a subtle effect in young, healthy adult mice [35]. Furthermore, there has been limited evidence to suggest that AVD-deficiency significantly affects spatial performance and neurochemistry [36,37] in young, healthy rodents. Thus, the results we obtained are consistent with the literature and confirm our hypothesis. By contrast, we have shown that AVD deficiency impairs spatial learning and memory performance in male BALB/c mice [23], confirming that the effects of AVD deficiency are dependent on the background strain.

In previous studies, different methodologies have led to inconsistencies in results. For example, there is considerable variation in the duration of vitamin D exposure, administration of vitamin D, levels of dietary vitamin D, age of rodents, rodent strain, behavioral tests used and brain regions tested. However, AVD deficiency, for as little as 10 weeks, was shown to regulate neurotransmitter systems in whole brain samples. In previous studies, AVD-deficient C57BL/6J mice showed a small but significant decrease in enzymes involved in GABA signalling (GAD65/67) and dopamine turnover (5-Hydroxy-indoleacetic acid and homovanillic acid) in whole brain tissue [22]. In the same study, AVD-deficient BALB/c mice exhibited an imbalance between excitatory and inhibitory neurotransmitters, showing reductions in glutamate, glutamine and GAD65/67 levels and elevations in GABA. In rats, vitamin D treatment resulted in elevated dopamine in the brain stem and homovanillic acid in the hypothalamus and striatum [38]. Although vitamin D regulates a wide range of genes [39], perhaps the relatively short time of exposure or the lack of secondary challenge may have limited the action of vitamin D in the hippocampus in the present study. Moreover, vitamin D may not exert its actions in the hippocampus, unless rodents are subject to certain vulnerabilities.

There was no effect of vitamin D status on a number of behavioural domains that may affect performance on APA. Although spontaneous locomotion in a novel open field has been observed in some rodent models of developmental and adult vitamin D deficiency [22,40], the effect is not consistent between studies [37,41]. Previous studies did not report an effect of AVD-deficiency on behaviour in the EPM, HPT or the FST [22,41]. Thus, in contrast to BALB/c mice our results provide no

evidence for an effect of vitamin D status on a number of behavioural domains in healthy young C57BL/6J mice.

In our previous work [22] we showed that the non-emotional, high locomotor C57BL/6J strain showed very little effect of AVD deficiency except on spontaneous hyperlocomotion. By contrast, the highly emotional, neophobic BALB/c strain showed significant effects of AVD deficiency, particularly involving limbic system functions. We can only speculate as to the reasons why BALB/c and C57BL/6J mice may show different effects of AVD deficiency. These two strains differ on a range of physiological parameters, behavioural responses, and drug-induced behaviours, as well as in levels of several neurochemicals, and future studies will be required to establish the precise mechanism that accounts for these differences.

Observational studies in humans indicate an association between low vitamin D levels and cognitive impairment [42], but this appears to be dependent on a number of factors, such as age and health. This association is seen in mild cognitive impairment [43], in healthy older adults [44] and seems to be predictive of cognitive outcome in adults older than 65 [45]. However, there is little association between low vitamin D levels and cognitive impairment in younger (< 60 years) adults [46]. There is also no effect of supplementation in younger individuals [47]. Conversely, a select number of studies have suggested a U-shaped, non-linear association between vitamin D status and cognitive function, indicating that both deficient and elevated levels of vitamin D could be unfavourable [48,49]. Collectively, these findings suggest that AVD deficiency accompanies the onset of an aged-related cognitive impairment and suggest a possible age threshold.

Despite clear findings, there are a number of limitations that should be considered. Blood serum analysis demonstrated a relatively broad overlap of diets. The diets were formulated to counteract the effects of irradiation (required in the animal facility), by adding an additional 50% of vitamin D, but perhaps this was not sufficient. Although $25(OH)D_3$ is an adequate indicator of vitamin D status [50], it does not confirm that the active metabolite, $1,25(OH)_2D_3$, has been altered. Serum levels do not accurately represent a rodent's capacity to use vitamin D metabolites. Furthermore, elevated levels of vitamin D may affect metabolism within the brain, leading to increased 24-hydroxylation and a reduced concentration of the biologically active $1,25(OH)_2D_3$.

Previous epidemiological, observational and animal studies indicate that AVD deficiency is associated with cognitive decline in healthy and disease states, and that vitamin D treatment can improve such impairment. We have shown that vitamin D status did not affect cognition and brain function in healthy adult male C57BL/6J mice.

AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: KJ, NG, MA, SA, TB. Performed the experiments: KJ. Analyzed the data: KJ, TB. Contributed reagents/materials/analysis tools: DB, NG, MA, SA, RS. Wrote the paper: KJ TB.

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CONFLICTS OF INTEREST

The authors have no conflict of interests to declare.

Appendix 1. Specialty Feeds Diet–SF09-088 AIN93G Rodent Diet

Table A1. Base components of the AVD-controlled mouse feed.

Ingredients	Inclusion Rate
Casein (Acid)	200 g/Kg
Sucrose	100 g/Kg
Canola Oil	70 g/Kg
Cellulose	50 g/Kg
Wheat Starch	404 g/Kg
Dextrinised Starch	132 g/Kg
DL Methionine	3.0 g/Kg
Calcium Carbonate	13.1 g/Kg
Sodium Chloride	2.6 g/Kg
AIN93 Trace Minerals	1.4 g/Kg
Potassium Citrate	2.5 g/Kg
Potassium Dihydrogen Phosphate	6.9 g/Kg
Potassium Sulphate	1.6 g/Kg
Choline Chloride (75%)	4.1 g/Kg
Oxicap E2	0.14 g/Kg
AIN93 Vitamins	15 g/Kg
Vitamin K 0.23%	0.87 g/Kg

Note: Values given are prior to irradiation with 25 kGy.

Table A2. Vitamin composition of AVD-controlled mouse feed.

Calculated Total Vitamins	Inclusion Rate
Vitamin A (Retinol)	6000 IU/Kg
Vitamin D (Cholecalciferol)	0 IU/Kg (Deficient)
	1500 IU/Kg (Control)
	15,000 IU/Kg (Elevated)
Vitamin E (α Tocopherol acetate)	115 mg/Kg
Vitamin K (Menadione)	3.5 mg/Kg
Vitamin C (Ascorbic acid)	None added
Vitamin B1 (Thiamine)	9.1 mg/Kg
Vitamin B2 (Riboflavin)	9.3 mg/Kg
Niacin (Nicotinic acid)	45 mg/Kg
Vitamin B6 (Pyridoxine)	11 mg/Kg
Pantothenic Acid	24.5 mg/Kg
Biotin	300 µg/Kg
Folic Acid	3 mg/Kg
Inositol	None added
Vitamin B12 (Cyancobalamin)	152 µg/Kg
Choline	2380 mg/Kg

Note: Values given are prior to irradiation with 25 kGy.

Table A3. Mineral composition of AVD-controlled mouse feed.

Calculated Total Minerals	Inclusion Rate
Calcium	0.47%
Phosphorous	0.35%
Magnesium	0.08%
Sodium	0.15%
Chloride	0.16%
Potassium	0.40%
Sulphur	0.23%
Iron	68 mg/Kg
Copper	7.0 mg/Kg
Iodine	0.2 mg/Kg
Manganese	19 mg/Kg
Cobalt	No data
Zinc	46 mg/Kg
Molybdenum	0.15 mg/Kg
Selenium	0.3 mg/Kg
Cadmium	No data
Chromium	1.0 mg/Kg
Fluoride	1.0 mg/Kg
Lithium	0.1 mg/Kg
Boron	2.5 mg/Kg
Nickel	0.5 mg/Kg
Vanadium	0.1 mg/Kg

Note: Values given are prior to irradiation with 25 kGy.

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