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ORIGINAL ARTICLE

Adult vitamin D deficiency disrupts hippocampal-dependent learning and structural brain connectivity in BALB/c mice

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Abstract

Converging evidence from human and animal studies support an association between vitamin D deficiency and cognitive impairment. Previous studies have shown that hippocampal volume is reduced in adults with vitamin D deficiency as well as in a range of disorders, such as schizophrenia. The aim of the current study was to examine the effect of adult vitamin D (AVD) deficiency on hippocampal-dependent spatial learning, and hippocampal volume and connectivity in healthy adult mice. Ten-week-old male BALB/c mice were fed a control (vitamin D 1500 IU/kg) or vitamin D-depleted (vitamin D 0 IU/kg) diet for a minimum of 10 weeks. The mice were then tested for hippocampal-dependent spatial learning using active place avoidance (APA) and on tests of muscle and motor coordination (rotarod and grip strength). The mice were perfused and brains collected to acquire ex vivo structural and diffusion-weighted images using a 16.4 T MRI scanner. We also performed immunohistochemistry to quantify perineuronal nets (PNNs) and parvalbumin (PV) interneurons in various brain regions. AVD-deficient mice had a lower latency to enter the shock zone on APA, compared to control mice, suggesting impaired hippocampal-dependent spatial learning. There were no differences in rotarod or grip strength, indicating that AVD deficiency did not have an impact on muscle or motor coordination. AVD deficiency did not have an impact on hippocampal volume. However, AVD-deficient mice displayed a disrupted network centred on the right hippocampus with abnormal connectomes among 29 nodes. We found a reduction in PNN positive cells, but no change in PV, centred on the hippocampus. Our results provide compelling evidence to show that AVD deficiency in otherwise healthy adult mice may play a key role in hippocampal-dependent learning and memory formation. We suggest that the spatial learning deficits could be due to the disruption of right hippocampal structural connectivity.

Keywords Vitamin D · Hippocampus · Memory · Connectome · Perineuronal nets (PNNs)

Introduction

Vitamin D plays an important role in the nervous system. For example, vitamin D exerts neuroprotective properties, facilitates neurotrophic function (Naveilhan et al. 1996; Brown et al. 2003) and regulates neuronal calcium homeostasis (Gezen-Ak et al. 2011). Epidemiological studies have shown that vitamin D deficiency is associated with a wide range of neuropsychiatric and neurological disorders, including schizophrenia (Cieslak et al. 2014; Chiang et al. 2016), autism (Fernell et al. 2015; Saad et al. 2016), depression (Spedding 2014; Brouwer-Brolsma et al. 2016), multiple sclerosis (Goral et al. 2015; Eskandari et al. 2015), dementia (Afzal et al. 2014) and Alzheimer's disease (Balion et al. 2012). The association between lower serum vitamin D and cognitive impairment has also been reported in meta-analytic studies (Etgen et al. 2012; van Schoor et al. 2016), and

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is associated with various aspects of memory impairment, including visual, (Kuzma et al. 2016) and verbal memory (Vicente et al. 2015). However, a causal role of vitamin D deficiency on adult brain function has not been established.

Several neuroimaging studies have examined the association between cognitive impairment, altered brain morphometry and vitamin D deficiency. However, the results have been inconsistent. For example, Annweiler et al. studied elderly individuals (> 65 years) and reported that the level of serum vitamin D may be associated with the size of the lateral cerebral ventricles (Annweiler et al. 2013), periventricular white matter abnormalities (Annweiler et al. 2014), intracranial volume (Annweiler et al. 2015) and brain volume (Hooshmand et al. 2014). In addition, the level of serum vitamin D was shown to be associated with impaired neuronal integrity, as measured by lower fractional anisotropy (FA) values in several brain regions of cognitively impaired elderly people (Moon et al. 2015). By contrast, no association was shown between serum vitamin D and neuroimaging abnormalities (Littlejohns et al. 2016). With respect to brain morphology, a lower hippocampal volume was observed in subjects with low serum 25-OHD (25-hydroxy vitamin D) (Karakis et al. 2016; Al-Amin et al. 2018), and a reduction of hippocampal grey matter volume in vitamin D-deficient patients with schizophrenia (Shivakumar et al. 2015). Because the hippocampus is an important brain region involved in memory processing, including the conversion of short-term to long-term memory formation (Virley et al. 1999; Cho et al. 2015), encoding and retrieval (Greicius et al. 2003; Lepage et al. 1998), these neuroimaging studies showed that hippocampal atrophy could be a basis of memory impairment associated with vitamin D deficiency. However, most of these studies were conducted on elderly people and the results may have been affected by many factors, such as changes in diet or outdoor activity that may confound the results and these studies cannot establish causality. Therefore, animal models of adult vitamin D (AVD) deficiency may be a feasible way to control for unknown confounding factors and examine the impact of vitamin D deficiency on hippocampal function and hippocampal microstructure.

Animal studies have shown that vitamin D deficiency has an impact on learning and memory performance. However, the results have been inconsistent. For example, there was no effect of AVD deficiency in Sprague–Dawley rats on two-way active avoidance (Byrne et al. 2013), whereas impaired spatial learning was shown in the Morris water maze (Taghizadeh et al. 2013) and 8-Arm Radial Maze (Altemus et al. 1987). By contrast, AVD-deficient mice showed a reduced escape latency during acquisition and a decreased response score on a two-way active avoidance task, indicating improved performance compared to control mice (Groves et al. 2013). These findings suggest impaired hippocampal function, because hippocampal lesions typically lead to the seemingly paradoxical findings of reduced freezing and faster acquisition on active avoidance learning and impaired learning on place avoidance tasks (Black et al. 1977).

The behavioural deficits in AVD deficiency could also be linked with altered brain neurochemistry. For example, AVD-deficient BALB/c, but not C57BL6/J, mice had increased levels of γ-aminobutyric acid (GABA) and reduced glutamine, glutamate (Groves et al. 2013). An increased level of GABA may indicate a possible impact of vitamin D on GABAergic inhibitory interneurons in the brain, e.g., those that express parvalbumin (PV). PV interneurons regulate the activity (such as synchronous firing) of principal neurons through their extensive connections (Willems et al. 2018). For example, PV in the CA2 oriens layer extend horizontally into pyramidal layers of the CA1 and CA3 subregions with their spiny dendrites (Mercer et al. 2012) to regulate the activity of the neighbouring pyramidal cells (Botcher et al. 2014). A previous study showed that the expression of PV was associated with cognitive performance (Volman et al. 2011). However, little is known about the hippocampal expression of PV in AVD deficiency.

Mature GABAergic interneurons are encased by perineuronal nets (PNNs). The PNNs are a lattice like extracellular matrix (ECM) that encloses cell bodies and proximal neurites of the neuron and are found in the various hippocampal subfields (Lensjo et al. 2017). In the mouse hippocampus, PNNs are found predominantly around the PV interneurons (Yamada and Jinno 2017) and PNNs have been shown to play a role in plasticity and memory formation (Morikawa et al. 2017; Kochlamazashvili et al. 2010). However, the effect of AVD deficiency on PNNs within mouse brain has not been examined.

In the current study, we hypothesized that AVD deficiency in BALB/c mice would be associated with a spatial learning deficit and reductions in hippocampal subfield volumes. In the current study, we examined the performance of AVD-deficient mice on the active place avoidance task as a spatial navigation and memory task and then acquired ex vivo structural brain images. We conducted whole-brain morphometry analysis using voxel-based morphometry (VBM) and quantified the volume of hippocampal subfields. We also measured diffusion tensor imaging (DTI) parametric maps, including axial diffusivity (AD), radial diffusivity (RD), mean diffusivity (MD) and FA on the white matter tracts to measure AVD deficiency associated changes. We recently showed that vitamin D deficiency disrupted right hippocampal central structural connectivity in vitamin D-deficient individuals with mild cognitive impairment (Al-Amin et al. 2018). In this context, we also hypothesized that AVD deficiency would disrupt structural connectivity in mice. Therefore, we used network-based statistics (NBS) (Zalesky et al. 2010) to examine whole-brain connectomes to identify any network differences between the control and

AVD-deficient mice. Finally, we performed immunohistochemistry on ex vivo brain slices to quantify PNNs and PV interneurons in the hippocampal subfields to examine the impact of AVD deficiency on the structural network. We hypothesized that the expression of PV interneurons and PNN density could be altered in AVD deficiency.

Materials and methods

Animals

Ten-week-old male BALB/c mice ($n = 64$) were purchased from Animal Resources Centre (Canning Vale, WA, Australia) and four animals were housed per cage in individually ventilated cages (Optimice, Animal Care Systems, CO, USA), with corn cob bedding (Shepherd Specialty Papers, Inc., TN, USA) at the Queensland Brain Institute Animal Facility (The University of Queensland, Australia). The mice were divided into two groups; control and AVD-deficient mice. Control mice were fed standard AIN93G Rodent diet with 1500 IU vitamin D3/kg (Specialty Feeds, WA, Australia), whereas AVD-deficient mice were fed vitamin-D3 deficient AIN93G Rodent diet (Specialty Feeds, WA, Australia) for a minimum duration of 10 weeks and during the behavioural testing period. The animals were housed in a facility where a 12-h light–dark cycle (lights on at 07:00 h) was maintained. The animals had ad libitum access to food and water.

Active place avoidance test

We tested active place avoidance (APA) using methods previously described (Lobellova et al. 2015; Stuchlik et al. 2013; Wesierska et al. 2005). This behavioural technique was used to assess hippocampal-dependent spatial learning (Wesierska et al. 2005; Cimadevilla et al. 2000). The apparatus was made by Bio-Signal Group, which consists of an elevated arena with a grid floor. A transparent circular boundary (77 cm diameter, 32 cm high, made of plexiglass) is used as a fence and placed on the elevated arena. The stage rotated clockwise (1 rpm) and delivered an electric shock through the grid floor. On this task, mice have to avoid a shock zone consisting of 60° angle on the platform using four external cues hanging on the nearby walls.

The shock zone was defined within a 60° region of the stationary room and kept constant in relation to the room coordinates. The position of the mouse was tracked by PC-based software that analysed images from a camera installed on the top of the apparatus and delivered shocks as required (Tracker, Bio-Signal Group Corp., NY). A mouse was placed opposite to the shock zone facing the wall and trained to avoid an unmarked invisible shock zone using the four external cues. Entrance into the shock zone directed the delivery of a brief constant mild electrical foot shock (0.5 mA, 60 Hz, 500 ms). If the mouse remained in the shock zone, it received additional shocks of the same intensity at 1.5 s intervals until the animal moved out of the area. The experiment was conducted over six days. Initially, the mice were habituated for a 5 min session without shock. Then, the mice were tested for a 10 min testing session with shock for five consecutive days. We have analysed all recorded data including; "number of shocks received", "latency to enter the shock zone" and "distance travelled throughout the experimental sessions" using Track Analysis software (Bio-Signal Group).

The APA test was conducted on two mouse cohorts. The first cohort ($n = 8$/group) was fed the diet for 20 weeks, while the second cohort ($n = 24$/group) was fed the diet for 10 weeks. To confirm that the deficits in spatial cognition were purely dependent on hippocampal function and not a result of impaired motor function or poor muscle strength, we performed rotarod and grip strength tests. The mice were placed on a rotating drum (30 mm diameter, Ugo Basile, VA, Italy) which increases in speed from 8 rpm to 40 rpm over 5 min. The latency to fall off the drum was recorded. Grip strength was measured using a rodent grip strength meter (MK-380CM/F, Muromachi, Tokyo, Japan) over three 1 min trials for both fore and hind paws.

Tissue collection

Following the behavioural experiment, mice in the MRI cohort ($n = 8$/group) were anaesthetized by i.p. injection of Lethabarb (100 mg/kg body weight) and perfused transcardially with 0.1 M phosphate buffered solution (PBS) and 4% paraformaldehyde (PFA) in PBS. The excess skin was removed from the skull, and the brains were removed from the skull and post-immersion fixed in 4% PFA at 4 °C for 24 h. The brains were carefully dissected and preserved at 4 °C in 0.1 M PBS solution containing 0.02% sodium azide.

Acquisition of structural MRI data

MRI data were acquired using a previously established protocol (Sepehrband et al. 2015). The brains were transferred to a solution containing 0.2% Magnevist (Bayer) MRI contrast agent in 0.1 M PBS for 4 days, and subsequently immersed in Fomblin Y06/6 solution (Solvay Solexis, Italy) for imaging. MRI scans were acquired using a 16.4T (89 mm) vertical bore small animal MRI (Bruker Biospin, Rheinstetten, Germany; ParaVision v5.1), equipped with Micro2.5 imaging gradient and a 15 mm linear surface acoustic wave coil (M2M, Brisbane, Australia) at 22 °C. High-resolution ex vivo structural images were acquired using T_1-weighted three-dimensional (3D) gradient echo FLASH (fast low

angle shot) with TR (repetition time) = 50 ms, flip angle = 30 degrees, TE (echo time) = 12 ms, 30 micrometer 3D isotropic resolution, with the acquisition time of 1.5 h.

Voxel-based analysis

Morphological alterations between the two groups were determined using whole-brain voxel-based analysis (VBA). First, a study-based T_1 structural image template was created using a series of affine and diffeomorphic registration protocols as implemented in the "*buildtemplateparallel*" script of the program Advanced Normalization Tools (ANTs). The resulting diffeomorphic transformation matrices were used to calculate each sample Jacobian determinant map, which contain the information of local volumetric changes (shrinkage and expansion) with respect to the template. Group *t* test voxel-wise analyses to detect local volumetric brain changes were performed using FMRIB Software Library (FSL) Randomise, with a design matrix of (8, 8), 5000 permutations and a false discovery rate (FDR) correction for multiple comparison (Bennett et al. 2009). FDR is a program of the FSL neuroimaging data analysis package (https://fsl.fmrib.ox.ac.uk/fsl/fslwiki/FDR). FDR takes in a *p* value image and uses false discovery rate theory to carry out the multiple comparison correction.

Acquisition of diffusion MRI data

High angular resolution diffusion imaging (HARDI) data were acquired using 3D diffusion-weighted spin echo sequence (Kurniawan et al. 2014). The imaging parameters were TE = 20 ms, TR = 400 ms, diffusion encoding times $\delta/\Delta = 2.5/12$ ms, 30 directions with *b* value of 5000s/mm^2 with 2 b = 0 images at 0.1 mm isotropic resolution. Total acquisition time was 15 h.

Diffusion MRI data pre-processing

HARDI data were converted from DICOM (Digital Imaging and Communications in Medicine) format to NIFTI, and image inhomogeneity was corrected using N4 correction with the program MIPAV (Medical Image Processing, Analysis and Visualization, version 7.4.0) (McAuliffe et al. 2001). Fibre orientation distribution was calculated using constraint spherical deconvolution (CSD) (Tournier et al. 2007) in MRTRix 3. Diffusion imaging parameters fractional anisotropy (FA), axial diffusivity (AD), radial diffusivity (RD) and median diffusivity (MD) were calculated using the tensor2metric script in MRTRix (Jenkinson et al. 2012). Data were corrected for family-wise error rate (FWE).

Segmentation and determination of DTI parameters

Seeding region of interests (ROIs) of three of the major white matter tracts (anterior commissure, hippocampal commissure and corpus callosum) were manually drawn on the colour FA maps at the midline orthogonal to the white matter tracks. CSD (Constrained spherical deconvolution) probabilistic tractography was generated from the ROIs at 50 seeds per voxel. The resulting tractograms were converted into track-density imaging (TDI) maps and thresholded at 10% to remove spurious background from the probabilistic tractography. These maps were used to measure DTI parameters from each of the white matter structures.

Pipeline of structural connectome construction

The structural connectome was generated using a whole-brain CSD probabilistic tractography with ten seeds per voxel. A combined AMBMC (Australian Mouse Brain Mapping Consortium) and John Hopkins C57BL/6J mouse brain atlas (Liu et al. 2016) was registered into the $b = 0$ image of the individual brain (Fig. 1). The MRTRix command tck-2connectome was used to generate the connectivity matric from 76 brain regions.

Connectivity analysis

We used network-based statistics (NBS) (Zalesky et al. 2010) to determine the global differences in connectivity between control and AVD-deficient mouse brains. NBS controls the family-wise error rate when analysing the difference between groups. The extent (size of the module) and the intensity of the connections could be measured with NBS. "Extent" comprises a total number of connections; whereas "intensity" consists of total test statistics value of all connections of a component. A series of *t* test statistical analysis were performed with a threshold range from 2.5 to 3.5 and 5000 permutations. Two contrasts were used to determine network changes (control > vitamin D deficiency; vitamin D deficiency < control).

Immunohistochemistry

The brain was removed and stored in PBS (phosphate buffered saline) overnight following the MRI experiment, and processed in a Leica TP1020 tissue processor, followed by paraffin embedding. The brains were sectioned (slice thickness 10 µm) coronaly using a rotary microtome. A one-in-ten series of the coronal sections were stained to identify PNNs and PV interneurons. The sections were treated with Antigen Recovery Solution (citrate buffer solution consisting of 10 mM Sodium Citrate, 0.05% Tween 20, pH 6.0) at 60 °C for 30 min followed by three washes in PBS. A

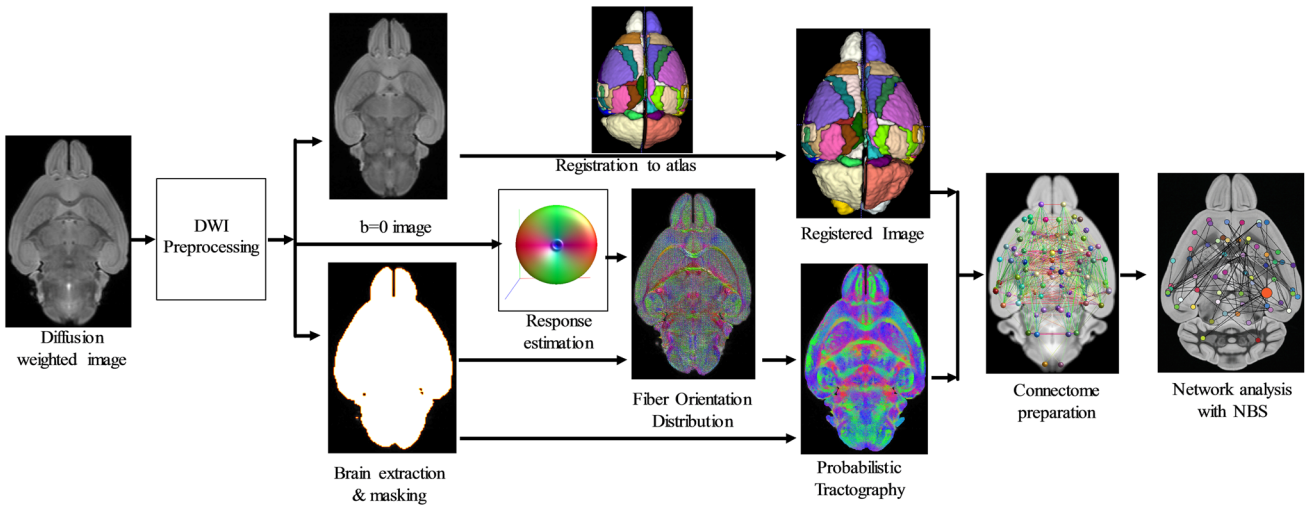

Fig. 1 Processing pipeline of tractography and connectome preparation. The fractional anisotropy (FA) map was constructed from the preprocessed diffusion-weighted image (DWI) file and registered with the combined atlas. The mask image was prepared from DWI. The response function was estimated. Fibre orientation distribution was calculated and subsequently used for probabilistic tractography. The registered atlas was used to extract the connectomes from the tractrography. The network analysis was performed with network-based statistics (NBS). *DWI* diffusion-weighted image

blocking solution consisting of 3% normal goat serum, 0.05% saponin, 0.1% Triton X-100 and 10% sodium azide in PBS were used to block the sections at room temperature for 1 h. The sections were then incubated with primary antibodies; mouse anti-PV (1:2500) (P3088, Sigma-Aldrich), and biotinylated WFA (*Wisteria floribunda* agglutinin) (B-1355, Vector Laboratories, Funakoshi Co., Tokyo, Japan) (1:2500) at room temperature for 48 h. We used secondary antibodies that were conjugated with fluorescent markers such as Alexa fluor-555 anti-mouse (1:1000) (Invitrogen) and Alexa fluor-647 anti-WFA (1:1000) (Invitrogen) at room temperature for 24 h. We then washed the sections with DAPI (1:5000) in 0.9% sodium chloride solution for 15 min. The sections were then mounted with Vectashield (Vector laboratories, USA) mounting medium.

Microscopy and image analysis

We captured the fluorescence images on a Zeiss Axio Imager Z1 microscope using a 20x objective. Cells that immunolabelled with WFA and DAPI (4′,6-diamidino-2-phenylindole), or PV and DAPI were included in the imaging, and multiple tiled images of each hippocampus were taken and stitched together. The number of WFA-positive cells, PV-positive cells and colocalized PV cells with WFA were counted from the dorsal and ventral hippocampal subregions (CA1, CA2, CA3, DG), retrosplenial cortex (Rs), ectorhinal cortex (Ect), amygdala (Am), parietal association cortex (Pta), perirhinal cortex (Prh), secondary motor cortex (S2) and magnocellular nucleus of the lateral hypothalamus (Mclh). An experimenter blind to the treatment conditions performed image analysis using a custom pipeline developed in cellprofiler. The hippocampal subfield areas were measured in Fiji (ImageJ). For the hippocampus, we analysed four coronal sections matched for anatomical location between mice, three dorsal sections (starting 1.22 mm posterior to bregma) and one ventral section (3.28–3.40 mm posterior to bregma).

Statistical analyses

We conducted statistical analyses using SPSS (Version 24.0). We considered the effect of Diet (control and AVD-deficient groups) as an independent variable, whereas the hippocampal subfield and total hippocampal volumes were estimated as a dependent variable. We conducted either repeated measure ANOVA, one-way ANOVA or *t* test, as required. A p value less than 0.05 was considered significant. Data are presented as mean ± SEM.

Results

Assessment of behavioural tests

We assessed the effect of Diet on spatial learning and memory formation using the active place avoidance (APA) task (Fig. 2a). With respect to the MRI cohort, we found a significant main effect of Diet ($F_{1,7} = 9.96$; $p < 0.01$, $\eta^2 = 0.58$), Day ($F_{4,28} = 24.48$; $p < 0.001$, $\eta^2 = 0.77$), and Diet x Day interaction ($F_{4,28} = 4.75$; $p < 0.005$, $\eta^2 = 0.40$) on the latency to avoid the shock zone (Fig. 2d–f; Supplementary

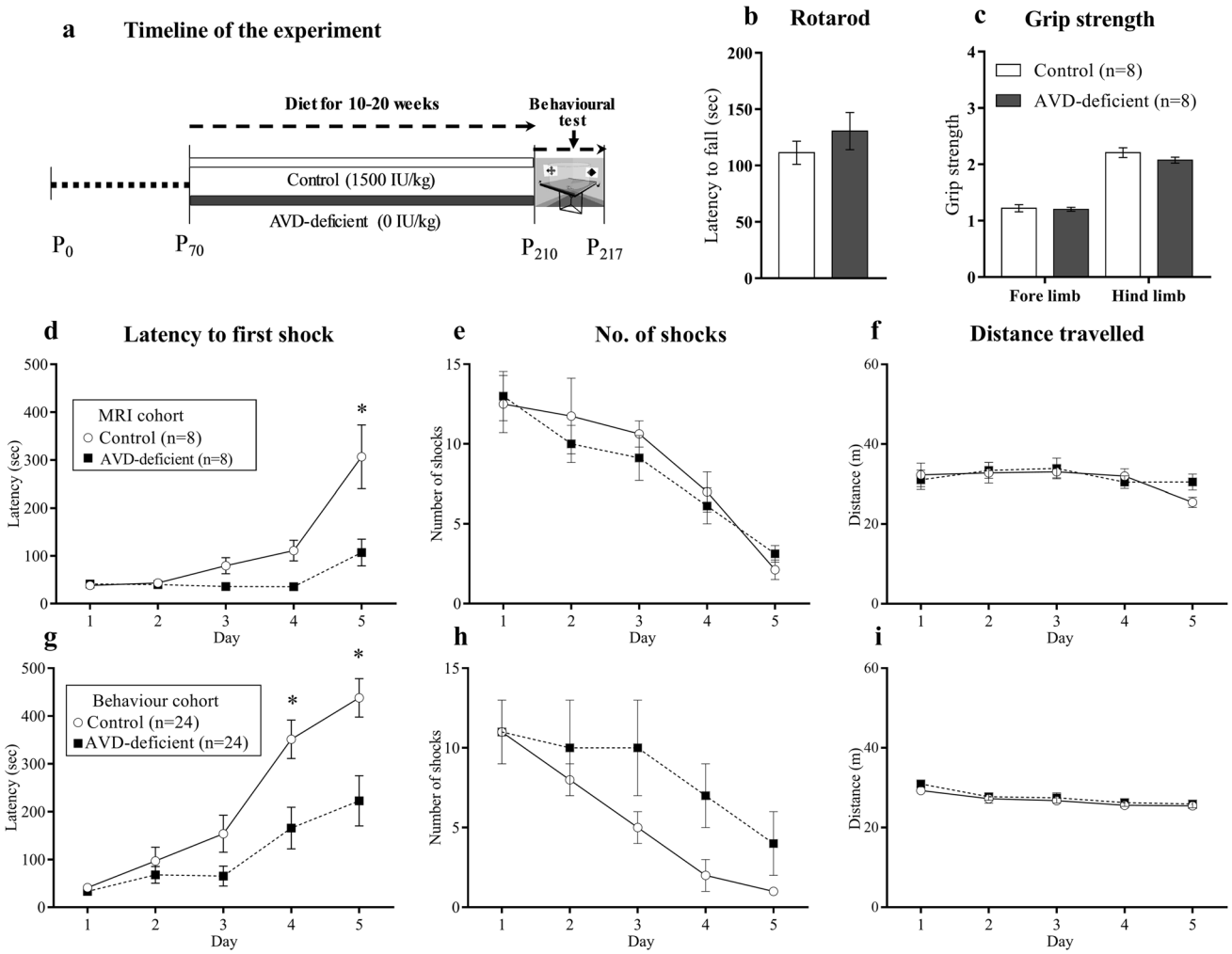

Fig. 2 Effect of Diet on tests of motor coordination, muscle strength, and spatial learning and memory in control and AVD-deficient BALB/c mice. Timeline of the experiment (**a**), latency to fall (second) in rotarod test (**b**), grip strength (**c**). AVD-deficient mice had a shorter latency to first shock (**d**), received the same number of shocks (**e**) and travelled a similar distance (**f**) throughout the sessions of an active place avoidance test. We replicated the findings for the active place avoidance in a larger independent cohort ($n = 24$/group) (**g**–**i**). We conducted repeated measures ANOVA to measure the main effect of Diet and Day on the spatial learning test parameters. Values are means ± SEM, $^*p < 0.05$ compared with the control diet group

Fig. 1). Moreover, AVD-deficient mice had a shorter latency ($M = 107.0$, SD = 78.7) than control mice ($M = 306.7$, SD = 188.6) on Day 5 to first enter into the shock zone. There was a significant main effect of Day ($F_{5,35} = 21.07$; $p < 0.001$) but not Diet ($F_{1,7} = 0.43$; $p > 0.05$) on the distance travelled on the APA task. Control and AVD-deficient mice travelled a similar distance on the APA.

We examined a separate behavioural cohort of control ($n = 24$) or vitamin D-deficient ($n = 24$) mice on the active place avoidance test and we found a significant main effect of Diet ($F_{1,46} = 18.34$; $p < 0.001$), Day ($F_{4,184} = 31.33$; $p < 0.001$), and Diet × Day interaction ($F_{4,184} = 4.31$; $p < 0.005$) on the latency to avoid the shock zone (Fig. 2g–i). There was a significant main effect of Day ($F_{4,184} = 16.23$; $p < 0.001$) but not Diet ($F_{1,46} = 2.12$; $p > 0.05$) on the number of shocks received. There was also a significant main effect of Day ($F_{4,184} = 10.50$; $p < 0.001$) but not Diet ($F_{1,46} = 0.96$; $p > 0.05$) on the distance travelled in the active place avoidance task. The AVD-deficient mice showed spatial learning deficits consistent with the cohort of mice used for MRI, indicating an impaired ability of spatial memory encoding.

To confirm that the deficits of spatial learning were dependent on hippocampal function and not caused by impairment of motor function or poor muscle strength, we performed rotarod and grip strength tests to assess whether AVD-deficient mice had poor muscle strength, which could affect the performance in APA. We found no significant difference between control and AVD-deficient mice in rotarod and grip strength test (Fig. 2b, c). These results indicate altered spatial learning of AVD-deficient mice was not

associated with poor muscle strength or impaired motor coordination.

Voxel-based analysis on structural MRI data

Voxel-based morphometry (VBM) was conducted to estimate the volume changes between the control and AVD-deficient mice. Initial VBM analysis showed a reduced volume throughout the hippocampus in AVD-deficient mice (Supplementary Fig. 2). However, FDR correction showed no significant changes in the AVD-deficient brain.

The results of the hippocampal segmentation in FSL have also shown no significant main effect of Diet on total hippocampal volume ($F_{1,14} = 3.69$; $p = 0.075$) (Supplementary Fig. 3). With regard to hippocampal subfields, there was no significant main effect of Diet on any of the hippocampal subfields.

Analysis of the DTI (diffusion tensor imaging) data

We also performed voxel-based analysis on the FA (fractional anisotropy) map. Voxel-based analysis revealed a number of voxels in the FWE uncorrected image showing a reduced FA value on the dorsal hippocampus. However, following FWE correction ($p < 0.05$), the voxels disappeared suggesting that vitamin D deficiency had no impact on FA throughout the brain (Supplementary Fig. 4).

We then extracted the DTI parameters such as FA, AD, RD and MD from the three major brain white matters tracts, namely anterior commissure (AC), hippocampal commissure (HC) and corpus callosum. There was no significant main effect of Diet on the FA, AD, RD and MD values of AC (Fig. 3). There was also no significant main effect of Diet on the FA, AD, RD and MD values of neither HC nor CC.

Analysis of the whole-brain structural connectivity

The interpretation of NBS analysis was completed using a threshold $t = 3.1$, in which the lowest p value in both network extent ($p = 0.025$) and network intensity ($p = 0.021$) were observed (Supplementary Fig. 5). NBS revealed brain network changes in the brain AVD deficiency compared to control mice (Fig. 4).

Connection deficits were observed in 29 brain regions in AVD-deficient mice, 12 nodes in the left and 17 in the right hemisphere. The affected nodes were, right hippocampus, left thalamus, anterior cingulate (right and left), subiculum (right and left), amygdala (right), septum (right), entorhinal

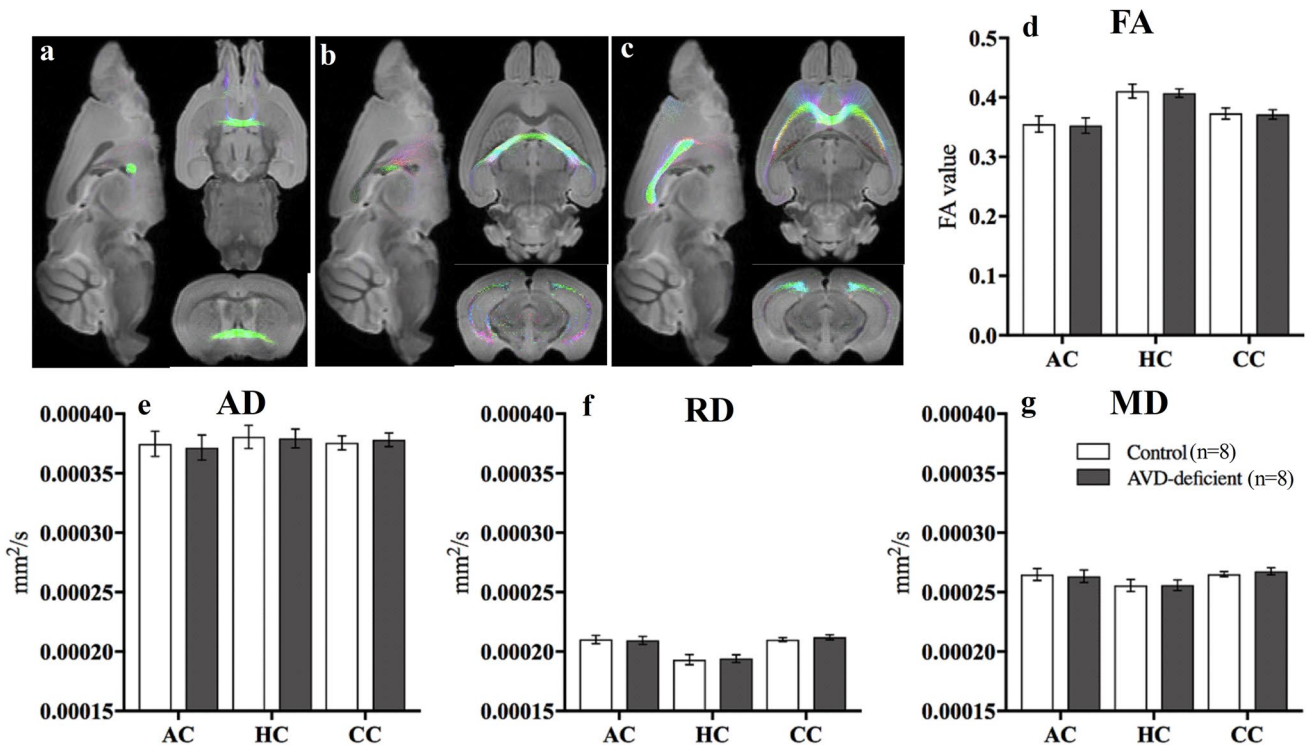

Fig. 3 Effect of Diet on the DTI parameters of the BALB/c mouse brain. Horizontal axis represents the white matter tracts, **a** anterior commissure (AC), **b** hippocampal commissure (HC) and **c** corpus callosum (CC). We conducted *t* test to measure the difference between control and AVD-deficient mice. Vertical axis represents the diffusivity in (mm^2/s). *FA* fractional anisotrophy, *AD* axial diffusivity, *RD* radial diffusivity, *MD* median diffusivity

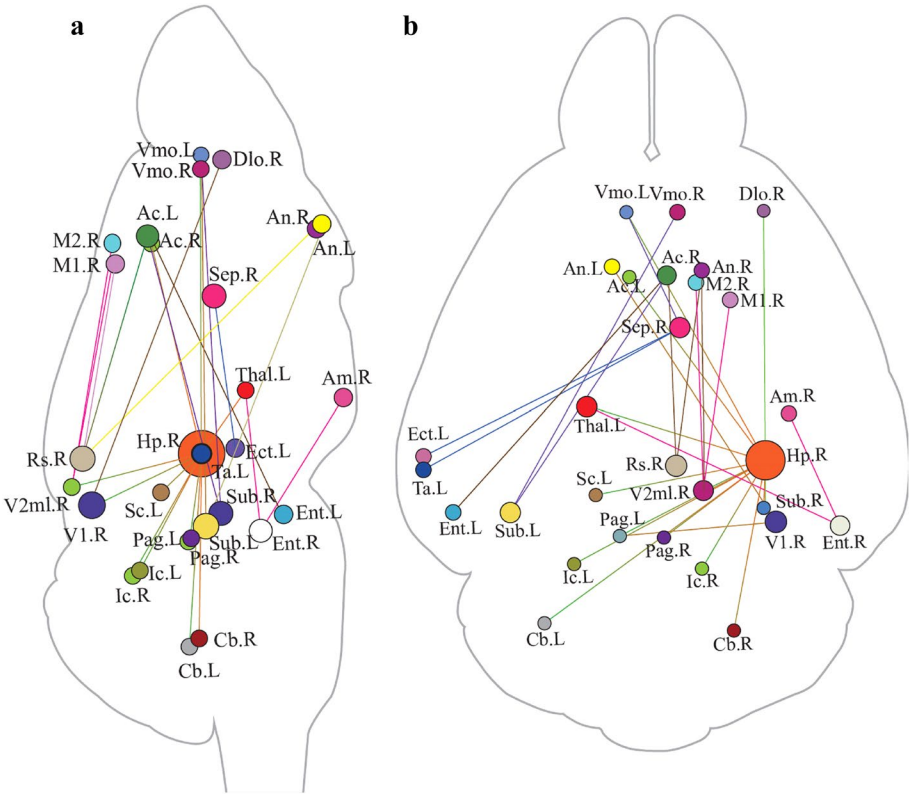

Fig. 4 Disrupted inter-hemispheric connectivity in AV D-deficient BALB/c mice. This network was observed at a statistical threshold of $t=3.1$. This network was selected based on the highest statistical significance in terms of extent and intensity. The affected nodes are, *Hp.R* right hippocampus, *Thal.L* left thalamus, *Ac.L and Ac.R* anterior cingulate (right and left), *Sub.R and Sub.L* subiculum (right and left), *Am.R* amygdala (right), *Sep.R* septum (right), *Ent.R and Ent.L* entorhinal cortex (right and left), *V1.R* primary visual cortex (right), *M1.R* primary motor cortex (right), *M2.R* secondary motor cortex (right), *Pag.R* periaqueductal grey (right), *Ta.L* temporal association area (left), *Ect.L* ectorhinal cortex (left), *Vmo.L* ventromedial orbital cortex (left), *Dlo.R* dorsolateral orbital cortex (right), *Rs.R* retrosplenial area (right), *Sc.L* superior colliculus (left), *Ic.L* inferior colliculus (right) and *Cb.R and Cb.L* cerebellum (right and left). NBS results are shown in sagittal (**a**) and axial view (**b**)

cortex (right), primary visual cortex (right), primary motor cortex (right), secondary motor cortex (right), periaqueductal grey (right), temporal association area (left), ectorhinal cortex (left), ventromedial orbital cortex (left), dorsolateral orbital cortex (right), retrosplenial area (right), superior colliculus (left), inferior colliculus (right) and cerebellum (right and left) (Fig. 4).

A number of modules ($n=9$) appeared in the disrupted network. Based on the number of edges, the biggest disrupted module was the right hippocampus (8 edges). There were 16 inter-hemispheric connections and 13 intra-hemispheric connections in the right cerebrum disrupted in vitamin D deficiency.

WFA and PV labelling in the hippocampus

We conducted two-way repeated measure ANOVA (Diet × Subfield) to analyse the average PNN and PV density in the hippocampal subfields. We showed a significant main effect of Diet on the PNN expression ($F_{1,14} = 25.9$; $p<0.001$), but no significant interaction with Subfield. The PNN expression was significantly reduced in each subregion from the dorsal and ventral hippocampus in AVD-deficient mice (Fig. 5d). There was no significant main effect of Diet on PV expression in whole hippocampus. To determine if the changes in WFA-labelled PNNs were restricted to the hippocampus or seen in other brain regions, we quantified WFA-labelled PNNs in three additional nodes under the disrupted network (Rs, Ect, Am), and four regions (Pta, Prh, S2, Mclh) that were outside of the disrupted network. Additional analysis showed no changes in the expression of PNNs except for Ect ($t_7=3.78$; $p=0.007$, Supplementary Fig. 6). Therefore, it seems that the hippocampal PNNs are selectively susceptible to vitamin D deficiency.

Discussion

Our aim was to examine the effect of adult vitamin D (AVD) deficiency on spatial learning, hippocampal subfield volumes and structural connectivity in male BALB/c mice.

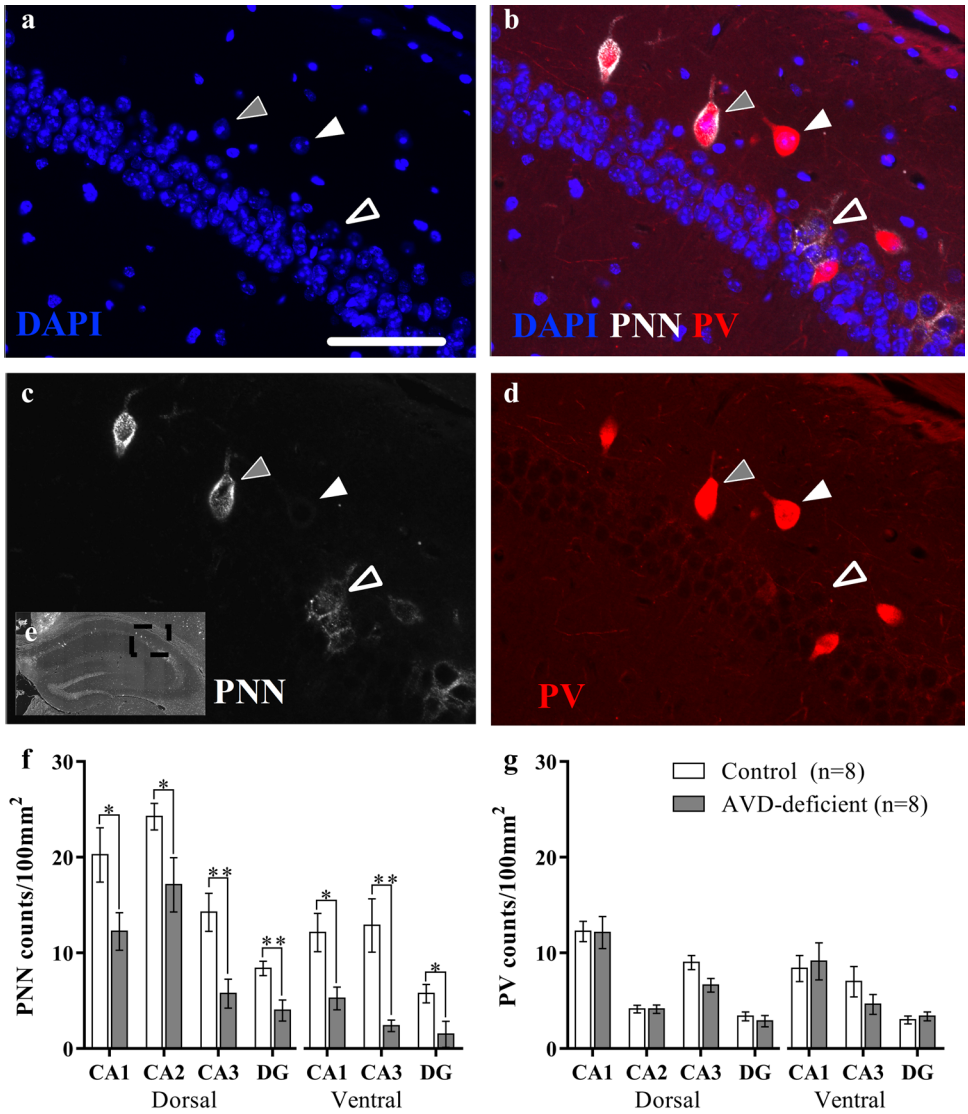

Fig. 5 AVD deficiency alters PNN expression in the hippocampus. DAPI-labelled nuclei (**a**), colocalization of WFA, PV and DAPI (**b**), WFA-labelled perineuronal nets (PNN) (**c**), parvalbumin (PV+) labelled cells (**d**), representative image (black outline) of hippocampus (**e**), PNN counts (**f**) and PV counts (**g**) for hippocampal subregions. Solid white arrowhead indicates PV + PNN cell; grey arrowhead indicates PV + PNN+; black arrowhead shows PNN + PV cell. Scale bar = 50 µm; values are means ± SEM, $n = 8$/group. *$p < 0.05$ compared with the controls

Performance on the APA task indicated a specific impairment in hippocampal-dependent spatial learning in the AVD-deficient mice, and this finding was replicated in a larger independent cohort. To understand the impact of AVD deficiency on structural brain alterations, we acquired structural and diffusion-weighted images of the behaviourally tested animals that were fed either control or vitamin D-deficient diet for 20 weeks. Structural image analysis indicated that AVD deficiency had no impact on the global hippocampal volume in BALB/c mice. Diffusion-weighted image data showed that the neuronal integrity was not affected by AVD deficiency. However, the results of the structural connectome analysis suggested a disrupted network, whereby the right hippocampus was the largest module of the disrupted network in AVD-deficient mice. In addition, the reduction of PNN expression in the hippocampal subfields was associated with the hippocampal central connectivity disruption and thereby resulted in a spatial learning deficit in BALB/c mice.

Spatial learning deficits

The APA test consists of five consecutive (10 min/day) testing sessions in which the mice were required to process and encode new spatial information (learning) to avoid a shock zone. Each day, the mice had to retrieve information from the past and store for future recall (memory) in the successive testing sessions. Four conspicuous external spatial cues were available for the mice to remember the location of the invisible shock zone. We obtained a number of parameters from the active place avoidance, including 'latency to first enter the shock zone', 'total number of shocks' and 'distance travelled throughout the session'. Latency to first enter the shock zone data represents learning and memorizing of spatial information, because mice have to avoid a previously visited shock area. We observed that the AVD-deficient mice entered the shock zone with a shorter latency compared to the control mice on Day 5, suggesting that these animals

failed to retrieve the spatial information of the shock zone, which may be due to a failure of encoding location-associated spatial information. Importantly, the hippocampus encodes the spatial information of the environment and is required for spatial navigation (Eichenbaum 2017). The APA test has been used in the past to investigate hippocampal-dependent spatial learning (Vukovic et al. 2013; Wesierska et al. 2005; Arora et al. 2017). The findings observed in the current study mirror those of previous studies that have examined the effect of AVD deficiency on spatial learning deficits (Taghizadeh et al. 2013; Altemus et al. 1987). Previous studies have used two-way active avoidance and shown either improved performance in AVD-deficient mice (Groves et al. 2013) or no change in AVD-deficient rats (Byrne et al. 2013). The role of the hippocampus in these two tasks is different. For example, hippocampal lesions typically facilitate active avoidance learning and impair place avoidance learning in rodents (Black et al. 1977). The active avoidance task requires a mouse to learn to avoid a shock by shuttling between two boxes between trials and impaired hippocampal function leads to reduced freezing and faster acquisition. By contrast, the active place avoidance task requires integration of spatial information to learn the position of the shock zone, and impaired hippocampal function leads to slower acquisition to form the association and avoid the shock by moving to a neutral area. We think that AVD-deficient mice perform well on the active avoidance and poorly on the active place avoidance task because of differential requirement of spatial information and impaired hippocampal function.

We also analysed parameters such as "total number of shocks" received by these animals throughout the experiment. Both control and AVD-deficient mice received a similar number of shocks throughout the experiment indicating that the mild shock had the same effect on each group. Other brain regions, including the amygdala, infralimbic prefrontal cortex and nucleus accumbens, could also be involved in learning the avoidance of the shock zone (Ramirez et al. 2015; Moscarello and LeDoux 2013). In terms of stress response, a recent study showed that the APA test did not alter the serum corticosterone level and suggested that this test is no more stressful than an unreinforced exploration of a known situation (Lesburgueres et al. 2016).

One potential confound from vitamin D deficiency may be reduced muscle strength and motor coordination, which might be associated with a reduced performance in the APA task. To address this issue, we first analysed the parameter "path travelled" throughout the experimental sessions in the APA test. We showed that the AVD-deficient mice travelled the same distance as controls, indicating that AVD deficiency had no impact on locomotion. We then measured motor coordination and grip strength on the rotarod and grip strength tests, and there were no significant differences in performance between control and AVD-deficient mice. Therefore, the impaired performance on hippocampal-dependent spatial learning did not appear to be influenced by changes in motor coordination or muscle strength.

The overall white matter integrity did not change in AVD-deficient mice

To evaluate the neuronal properties of the whole-brain fibre tracts, we conducted voxel-based analysis on FA map. The FA value is dependent on a number of tissue properties, including myelination, organization and density of fibre and diameter of the axon (Scholz et al. 2014). Recent evidence suggests that the myelination is significantly associated with the FA value (Chang et al. 2017). The result of voxel-based analysis on FA map showed no significant changes throughout the brain in AVD-deficient mice following FDR correction. A second analysis involving the value of DTI parameters (FA, AD, RD and MD) of the brain's major white matter tracts (AC, CC, HC) also showed no effect of Diet. By contrast, a lower FA value was shown in the white matter tracts of elderly patients with vitamin D deficiency (Moon et al. 2015). It is possible that vitamin D deficiency is associated with impaired neuronal integrity only in the aged population, and we only examined relatively young mice (~7 months of age) in the current study.

Hippocampal volume and connectivity

Initially, the voxel-based analysis revealed a number of clusters in the hippocampal region, but these failed to reach significance following FDR correction. Therefore, a second analysis involving an image registration technique to quantify the volume of each subfield also showed no effect of Diet. We next aimed to identify whether AVD deficiency was associated with altered connectivity and networks, and so we performed a whole-brain connectome analysis. The result of the network analysis revealed 29 nodes that had deficits in connectivity. Most of the nodes are involved in the regulation of memory, navigation, fear, emotional stimuli processing, and the hippocampus is required for spatial learning and navigation (Eichenbaum 2017). However, the connection between thalamus and hippocampus is also necessary for spatial learning (Aggleton and Nelson 2015; Dumont et al. 2015). The thalamic nucleus known as nucleus reuniens (Re) has GABAergic projecting neurons (Dolleman-Van der Weel and Witter 2000) which connects with the hippocampus (Bokor et al. 2002). It has been shown that the stimulation of the Re causes excitatory effects at the hippocampal CA1 area (Dolleman-Van der Weel et al. 1997). Re regulates the interactions between the hippocampus with the PFC in spatial working memory formation (Griffin 2015). We observed an inter-hemispheric connection deficit between the hippocampal-thalamic circuit. However, a

recent study showed evidence of the cross-hemispheric connection of thalamic nuclei with the hippocampal formation (Mathiasen et al. 2017). Taken together, the deficit of hippocampal–thalamic connectivity could underly the impaired spatial learning and memory formation in AVD-deficient mice.

AVD-deficient mice showed connection deficits of the ventromedial orbital cortex (Vmo). The right and left Vmo were disconnected from the left subiculum and right hippocampus, respectively. Based on human studies, the ventromedial cortex is connected with the hippocampus via three reciprocal connections including, fornix, cingulum bundle and uncinate fasciculus (Catani et al. 2013). A recent study suggests that damage to the hippocampus and Vmo affects memory recall (McCormick et al. 2017). Collectively, the disrupted connectivity between Vmo and hippocampus in AVD-deficient mice could affect memory recall in the APA test.

We observed that the largest module of the disrupted network contained eight edges, where the right hippocampus was the core of the hub. Interpreting the basis of this laterality should be explained with caution. The right hippocampal-centred disrupted connectivity could be possible if there was a loss of right hippocampal volume. However, we found no evidence of this change. Prior studies have observed a reduction in lower right hippocampal grey matter volume in AVD-deficient patients with schizophrenia (Shivakumar et al. 2015). Notably, we recently showed that vitamin D deficiency disrupted a right hippocampal central structural connectivity in mild cognitive impairment (Al-Amin et al. 2018). The mouse structural connectivity analysis also showed a right hippocampal central connectivity disruption. Therefore, the right hippocampal structural connectome might be vulnerable to vitamin D deficiency. We have not investigated the effect of vitamin D deficiency on laterality. Interestingly, the right hippocampus was shown to be associated with the memory for locations, whereas the left hippocampus was shown to be associated with the retrieval for episodic or autobiographical memory (Burgess et al. 2002). Since our mice were required to memorize the location of a shock zone in the APA task, we argue that the observed spatial learning deficits are associated with the right hippocampal central structural connectivity disruption.

The result of disrupted connectivity could be associated with many potential factors. There is evidence to support both direct and indirect effects of vitamin D deficiency on the hippocampus. For example, vitamin D deficiency impacts the immune system and is associated with higher levels of interleukin-6 in the hippocampus (Samuelsson et al. 2006). In addition, vitamin D deficiency downregulates neuronal antioxidant properties (Garcion et al. 1999) and a lower level of vitamin D may leave the brain with lower levels of neurotrophic factors (Naveilhan et al. 1996; Feron et al. 2005). Furthermore, a loss of hippocampal synapses (Scheff et al. 2006) and synaptic protein (Latimer et al. 2014) might be associated with the disrupted brain connectivity and loss of extracellular matrix (ECM) such as perineuronal nets (PNNs).

We quantified the expression of PNNs in eight brain regions; four regions (hippocampus, retrosplenial cortex, amygdala and ectorhinal cortex) from within the disrupted network and four (parietal association cortex, secondary motor cortex, perirhinal cortex and medial globus pallidus, magnocellular nucleus of the lateral hypothalamus) were outside of the disrupted network. However, the PNN reduction was specifically observed in the hippocampal region, and to a smaller extent in the ectorhinal cortex. It seems that the hippocampal PNNs are selectively susceptible to vitamin D deficiency. The ECM molecules have been shown to contribute to hippocampal volume (Peixoto-Santos et al. 2015). In addition, the ECM has an important role in retaining the neuronal connectivity in the brain (Bikbaev et al. 2015). The neurobiological basis of lower PNN expression in AVD deficiency has not been studied so far. A possible explanation of PNN reduction is the lack of PNN components such as aggrecan and hyaluronan. Interestingly, these proteins are commonly found in both bone and PNNs. Although vitamin D has a well-known role in bone function, its role on PNN formation in the brain has not been studied yet.

Accumulating evidence from in vitro studies have shown that vitamin D contributes to the expression of proteins making up PNNs. For example, the active metabolite of vitamin D ($1,25(OH)_2D_3$) reduces the synthesis of aggrecan in the immortalized rat chondrocyte (IRC) cell line (Horton et al. 1991), and downregulates proteoglycan synthesis in the osteoblastic cell line (Takeuchi et al. 1989). In addition, there might be an association between vitamin D receptor (VDR) polymorphism and aggrecan gene (Eser et al. 2010). Moreover, vitamin D also contributes to hyaluronan in bone formation (Genever and Dickson 1996). However, because we show very restricted effects of AVD deficiency on PNNs in the hippocampus and ectorhinal cortex, compared to other cerebral regions, this argues against a general downregulation of WFA-positive matrix in the adult brain.

There are many possible reasons (mechanisms) behind the disrupted structural connectivity in AVD deficiency and a reduced expression of PNN in the hippocampus could be one of the many unknown underlying reasons. Research regarding PNN's role in disrupted connectivity and spatial learning deficits has not been sufficiently studied. However, a previous study explained how memories can be stored within the lattice of a PNN (Tsien 2013) and contribute to learning and memory formation (Morikawa et al. 2017; Kochlamazashvili et al. 2010). PNNs have been shown to retain structural connectivity (Bikbaev et al. 2015), regulate synaptic plasticity (Wlodarczyk

et al. 2011; Dityatev et al. 2010), provide protection from oxidative stress (Morawski et al. 2004; Cabungcal et al. 2013) and stabilise the synapses maintaining the balance between excitatory and inhibitory neurons. Hippocampal function also depends on adult neurogenesis and has been shown to increase following learning on the APA task in mice (Vukovic et al. 2013), and differentiating hippocampal stem cells and neural progenitors are surrounded by WFA-positive PNNs (Yamada et al. 2018). We have examined rates of proliferation and neurogenesis in adult hippocampal tissue of AVD-deficient mice, but found no significant effects of Diet on hippocampal neurogenesis in the dentate gyrus, including the number of proliferating cells (Ki67 positive), number of doublecortin (DCX)-positive immature neurons or incorporation of bromodeoxyuridine (BrdU) into new neurons (Groves et al. 2016). Therefore, we argue that the deficits of the PNNs could alter hippocampal synaptic plasticity (Jansen et al. 2017) leading to the impaired spatial learning and memory formation observed in the APA test in AVD-deficient mice. It is also possible that vitamin D deficiency impaired axonal wiring in the hippocampus resulting in disrupted connectivity, and a reduced network activity for memory consolidation (Ognjanovski et al. 2014). Since the hippocampus plays a pivotal role in spatial learning and memory formation, we suggest that the spatial learning deficits are associated with an altered hippocampal connectome.

Conclusion

AVD-deficient mice had a shorter latency to enter the shock zone on APA, compared to control mice, suggesting impaired hippocampal-dependent spatial learning. There were no differences in rotarod or grip strength, indicating that AVD deficiency did not have an impact on muscle or motor coordination. AVD-deficient mice displayed a disrupted network centred on the right hippocampus with abnormal connectomes among 29 nodes. With respect to hippocampal PNN, there was a significant reduction of PNN expression throughout the hippocampal subfields in AVD-deficient mice, despite no changes in PV expression. The reduction of hippocampal PNN and the alteration of right hippocampal structural connectivity provide compelling evidence to show that AVD deficiency in otherwise healthy adult mice may play a key role in hippocampal-dependent learning and memory formation. Future studies should examine the time course of these changes using a longitudinal design and establish whether vitamin D supplementation is able to reverse the behavioural and structural changes associated with AVD deficiency.

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Compliance with ethical standards

Informed consent Not applicable.

Ethical approval All experiments conformed to The University of Queensland's Animal Welfare Unit guidelines for animal use in research and was approved by the University of Queensland Animal Ethics Committee (QBI/376/15).

Conflict of interest The authors have no conflict of interests to declare.

Ethical statement All work was carried out in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes under the guidelines of the National Health and Medical Research Council of Australia.

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