Horticulture Innovation Australia

# Final Report

## Evaluation of Efficacy of Vitamin D-Rich button mushrooms in a Mouse Model of Alzheimer's Disease

Louise Bennett
CSIRO Preventative Health Flagship

Project Number: MU10019

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# Evaluation Of Efficacy Of Vitamin D-Rich Button Mushrooms in a Mouse Model Of Alzheimer's Disease

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# Contents

# 1 Media Summary

Alzheimer's disease (AD) is a progressive neurodegenerative disorder that manifests with aging as memory loss. Progression of AD is associated with deposition of plaques in the brain comprised of amyloid-β peptide. At present there is no cure for AD, although retrospective studies have linked vitamin D deficiency with cognitive deficit, including risk of AD. Vitamin D3 is produced in the skin upon exposure to sunlight and in limited dietary sources such as oily fish, while low levels of Vitamin D2 are present in some plants and fungi, including mushrooms. The prevalence of Vitamin D deficiency, its links with cognition, and the recent development of methods for stimulating production of Vitamin D2 in mushrooms have converged within this research project. In this project, we aimed to determine whether or not vitamin D2-enriched White Button mushroom (VDM) could exert a beneficial effect on learning and memory in mice that were genetically modified to develop pathologies and symptoms associated with AD. The transgenic 'AD' mice express two human genes linked with familial AD, and consequently display progressive memory loss as a result of the brain deposits of plaque, thus modelling key pathologies of AD. Wild type or AD mice were fed either a control diet or a diet supplemented with White Button mushrooms enriched with Vitamin D2 (VDM) for 6 months post-weaning. Key outcomes of the research were as follows:

When compared to the control diet, the VDM-supplemented diet:

- improved memory in both AD *and* wild type mice and,
- reduced amyloid-β plaque deposits in the brains of AD mice;

These results suggest that VDM may protect against AD-related memory loss by reducing amyloid-β plaque accumulation in the brain. The effects can be attributed to Vitamin D2 or other components of the mushroom, which have been shown to interact with the amyloid-β protein. In addition, the improvement in memory in wild type mice means Vitamin D-enriched mushrooms may have broader health implications for the general community, presumably affecting additional pathways important in cognitive health. For industry, these research outcomes may support growth in consumer demand for this unique, bioactive food, offering potential population-wide opportunity for promoting brain health.

It is recommended that future research aims to substantiate the findings of the mouse study in humans, either healthy aged or at-risk AD subjects including those with family history of AD or early stages of memory impairment, in order to test the preventative efficacy of Vitamin D mushrooms. Human clinical studies should determine the comparative efficacy of Vitamin D2 and D3 for cognitive function and the specific contribution to effects, from Vitamin D2 versus other components of mushroom. Practical usefulness of the outcomes of the mouse study and future human studies to the industry will be achieved by the publication of the research and its subsequent promotion through mainstream media channels.

If human studies prove successful, Vitamin D2-enriched mushrooms may be confirmed to represent a unique, natural, functional food offering significant benefits for cognitive health. Based on the assumption that Vitamin D is responsible for observed cognitive improvements effects in mice, these results might infer that Vitamin D2 exerts differential/superior effects to Vitamin D3 on cognition. Furthermore, the lower bioavailability profile of Vitamin D2 may in fact, render it the safer form of oral supplementation of Vitamin D.

# 2 Technical Summary

The worldwide prevalence of dementia, which includes Alzheimer's Disease (AD) as the predominant pathology, is predicted to rise from 35.6 million people in 2010 to 115.4 million in 2050, with substantial projected social and economic burden. Vitamin D insufficiency and deficiency is also highly prevalent affecting 50-70% of adults, and is particularly elevated in the aged, partly due to diminished exposure to sunlight, and also because of the low natural abundance of dietary sources of Vitamin D. Vitamin D status is strongly correlated with cognitive performance but intervention research demonstrating that elevating Vitamin D status leads to enhanced cognitive functional capacity is lacking. By extension, the potentially protective activity of Vitamin D in onset and progression of dementia in general and AD in particular, is also lacking. This research project sought to conduct a dietary intervention study in a transgenic mouse model of AD using Vitamin D-enriched mushrooms (VDM), to determine if the intervention could produce beneficial effects on memory and biomarkers of AD pathology, specifically, circulating levels of amyloid beta peptide and plaque load in the brain.

The project examined the effect of VDM on memory and amyloid peptide production and as brain deposits of plaque, in both transgenic and control mice. Mice were maintained on standard chow (~0.25 µg Vit D3/day, 10 IU/day) from weaning until 2 months of age, whereupon feeding intervention commenced. During the 6 following months of dietary intervention, mice were fed either Vitamin D-deficient chow (0 µg/g added Vitamin D3, that contained trace levels of Vitamins D2 and D3) or Vitamin D-deficient chow supplemented with 5% VDM solids, delivering a daily dose of ~1 µg (40 IU) Vitamin D2 (in addition to trace levels of Vitamin D3). Mice were killed at 7.5-8 months of age and blood and brain samples were taken for examination.

The results provided strong cumulative evidence from behavioural and biochemical measures, that dietary supplementation with VDM improved memory and cognitive performance in both AD *and* wild type (WT) mice, and furthermore, that elevated ratios of Vitamin D2 to D3 metabolites, 25-OH-D2/25-OH-D3, were associated with memory improvement (WT and AD mice) and lowering amyloid peptide production and plaque deposition (AD mice only). A range of additional measurements were made to verify the findings and interpret biological processes underpinning observed effects. Other measurements included: Vitamin D and mineral analysis of mushroom solids; amyloid fibril inhibition by Vitamin D2, Vitamin D3 and mushroom solids, mouse body weight; liver toxicity biomarkers, Vitamin D metabolite and Ca levels in sera, Ab42 levels in sera and plaque loads in brains, cytokine levels in sera. An unexpected finding was that the positive effects for primary endpoints (memory and amyloid brain load) were associated with the VDM groups (WT and AD) and with elevated ratios of Vitamin D2 to D3 metabolites, and not with the reverse metabolite ratio, as was present in Control groups. This finding demonstrated that mice were not Vitamin D-deficient and that either enhanced specific activity of Vitamin D2 and/or other mushroom-derived components were responsible for the effect. These results may implicate superior specific activity of Vitamin D2 and/or other mushroom-derived components, compared with Vitamin D3 for impacting cognition and memory in both wild type and AD transgenic mice.

It is recommended that this research is suitably prepared for peer review and publication and thereafter may be used for other forms of communication to scientific and non-scientist audiences.

The research provides a significant contribution to understanding the protective nature of Vitamin D in general and Vitamin D2 in particular (and/or in combination mushroom bioactives) for improving memory in both AD and normal mice, presumably as a result of lowering production of Ab42 in the transgenic mouse model. The positive effect observed for wild type mice also suggests benefits induced by non-AD pathways that regulate cognition. These research findings justify progression into a human clinical study where comparative effects of Vitamin D2, Vitamin D3 and non-Vitamin D mushroom bioactives should be systematically explored. The results of clinical studies are expected

to inform recommendations for public health policy on dietary supplementation of Vitamin D, with Vitamin D-enriched mushrooms offering a convenient source of Vitamin D2 and possible synergistic effects over Vitamin D alone. In addition, these clinical studies can inform the controversial debate regarding the comparative safety of Vitamin D2 versus D3 supplementation and their relative efficacy for protection against cognitive decline and possibly, against progression of AD pathology.

# 3 Introduction

In general, consumption of edible mushrooms is considered good for health with particular types having a long history of medicinal use in Eastern cultures. For example, treatment of 50-80 year old Japanese men and women with mild cognitive impairment, using a dried form of Yamabushitake mushroom (*Hericium erinaceus*) was found to improve cognitive function, that reverted after cessation of treatment [1]. In addition, Vitamin D has been proposed to play roles in multiple aspects of central nervous system development and this is supported by the association between Vitamin D deficiency and incidence of neurological and psychiatric disorders [2]. Furthermore, Vitamin D intake and circulating levels of metabolites have each been positively correlated with cognitive performance [3] [4]. A number of cross-sectional studies investigating relationships between Vitamin D status and cognitive function have been published since 2009 with several supporting a linear relationship between Vitamin D status and cognitive functioning including a large population study [5]. Linear responses suggest that an effect of restoring Vitamin D status could be expected to benefit those of both insufficient and deficient status, representing a substantial proportion of the adult population [6]. However, an optimal range of Vitamin D intake appears to be necessary to avoid mortality risk associated with high levels of Vitamin D metabolites in serum [7].

Linkages between Vitamin D status and treatment of Alzheimer's Disease (AD) have also been considered. An observational study involving 225 aged individuals with probable Alzheimer's Disease exhibited Mini Mental State Examination scores that were positively correlated with serum 25-hydroxyvitamin D3 but not vitamins B1, B6 or B12 [8]. This study, as for other cross-sectional studies, have supported a relationship between Vitamin D status, whether from diet, sun exposure or drug supplements, with promoting healthy cognition, but studies of this type are not able to demonstrate causality or mechanism. Nevertheless, the first reported randomized trial in mild to moderate AD patients found that neither physiological nor supra-physiological doses of Vitamin D significantly improved cognition [9].

Vitamin D2 is typically present at 0.2 to 29.8 ng/g fresh weight or 2 to 298 ng/g dry weight (for average total solids of 10%), averaged across several types of mushrooms [10]. At 100 ng/g or 10 µg/100 g serve, this represents around the adult Adequate Intake (AI) level per 100 g serve. However, without fortification in foods, collective dietary sources of vitamin D are not considered likely to deliver the daily Adequate Intake for adults [6]. While, oral supplementation of Vitamin D is preferred to extended UV exposure from sunlight [11] the relative merits of supplementation with Vitamin D2 versus D3 is currently unresolved [12]. A recent study showed that dietary supplementation of Vitamin D3 in AD transgenic mice (mutant chimerical mouse/human AβPP695-PS1-dE9) improved memory and lowered Ab42 load in the brain by diverting towards the α-secretase processing pathway of APP [13]. Mushrooms represent an important dietary source of Vitamin D, specifically Vitamin D2 [14] that is bioavailable [15]; [16], however the possible protection of vitamin D2 and potentially other mushroom-related bioactives, against amyloid beta toxicity or other aspects of AD pathology in humans, has not been previously investigated.

The aim of this study was to investigate the effects of dietary supplementation with Vitamin D2-enriched mushroom solids, thereby providing a source of both Vitamin D2 and other

putative bioactives implicated to be present in previous studies of AD and cognition, involving mushrooms without Vitamin D enrichment. A dietary intervention study was conducted using both wild type and AD transgenic mice. After weaning at 3 weeks of age, all mice were maintained on a diet of standard mouse chow for5 weeks. Subsequently, mice were randomised into 4 groups (2 groups within each genotype) and were maintained on the experimental feeds for a further 6 months. The groups were as follows wild type–control feed; transgenic–control feed; wild type–VDM-supplemented feed; transgenic VDM-supplemented feed. The Control feed contained trace amounts of trace Vitamin D, mostly as Vitamin D3 (~0.15 µg per day). VDM diet was the Control feed with 5% mushroom solids added, delivering approximately 1.0 µg of Vitamin D2 per day. Primary endpoints of the study were related to effects on memory and amyloid loads in brain and sera, in addition to serum levels of Vitamin D and Ca. The results showed that Vitamin D2-rich mushroom solids promoted improved memory in both wild type and transgenic mice and probably reflected differential effects of Vitamin D2 (in combination with mushroom bioactives) versus Vitamin D3 on memory in these mice. While providing interesting insights into comparative effects of Vitamin D2 and Vitamin D3, the results from this study do not allow the beneficial effects of Vitamin D2 to be resolved from the other components of mushroom.

# 4 Materials and methods

## Elemental analysis

The dried mushroom product was analysed for total carbon, hydrogen and nitrogen by flash combustion method using a Carlo Erba Elemental Analyser (model 1108, Carla-Erba, Milan, Italy). The coefficient of variation for replicates was <2%.

## Mineral analysis

The dried mushroom product was digested by microwave digestion in a mixture of concentrated nitric and hydrochloric acids (80:20, v/v) according to the US EPA Method 3051 (1994). Analysis of digestates was conducted using a Varian Vista Pro ICP-AES (Varian Australia, Melbourne, Australia) under optimised settings, in at least duplicate, with appropriate reagent blanks and reference samples. The average coefficient of variation between replicates was 10%.

## Vitamin D2 analysis

Vitamin D2 analysis in mushrooms was conducted by the method described in [17]. Dried mushroom sample (2-3 g) was accurately weighed into a 250 l flask and saponified with 30 ml of alcoholic KOH in the presence of the anti-oxidant stabiliser sodium ascorbate (0.5 g), overnight at 22°C. A sample aliquot (10 ml) was then loaded onto a solid phase extraction column (Chromabond XTR, 8.3 g adsorbent cartridge size, Machery-Nagel GmbH and Co. Kg, Duren, Germany) and eluted with petroleum ether (75 ml). The eluant was dried by rotary evaporation and nitrogen purging before redissolving in heptane. The extract was then separated by normal phase HPLC using a silica column (Phenomenex, CA, USA) and the fraction at the standard retention time for Vitamin D2 recovered. This fraction was dried under nitrogen and redissolved in acetonitrile. Vitamin D2 was determined by Reverse Phase HPLC using a C18 Synergy 4 µm Hydro column (Phenomenex) and with UV detection at 265 nm. The calculation of vitamin D concentration was made by comparison against standards of Vitamin D2 (Sigma-Aldrich, MO, USA). Mushroom samples were analysed in triplicate and the results reported as µg of Vitamin D2 per g of sample.

## Preparation Of Vitamin D-Rich Mushrooms

Light treatment of mushrooms was conducted using a light-proof box assembled with an array of 6 UV-C tubes (Philips TUV 36W.G36 T8 Longlife UV-C lamps) in a parallel configuration secured to the inside roof of the box. The dimensions of the box were 1300 mm width × 1300 mm depth × 500 mm height, as described in Liu et al, 2009 [18]. The light tubes were spaced at intervals of 215 mm, with the first tube positioned 170 mm from the side door. The distance from the base of the box to the tube edge was 375 mm. Calibration of the irradiance from the UV-C light box was conducted using a research radiometer with a solar blind photodiode SED 240 sensor and cosine-correction diffuser (Model IL1700, International Light, MA, USA), set to detect at 253.7 nm, at each of nine points across a symmetrical 3 x 3 grid. The height of the light intensity measurement plane was 55 mm above the base of the box compared with 25 mm for the treatment plane height for mushrooms so the light intensity values would be expected to be lower at the mushroom treatment level and cannot be directly related to Vitamin D production levels. The output from 6 tubes was monitored at each grid position until the irradiance was stable so as to identify the time required for the light output to stabilise and the stabilised UV-C light intensity. The lights were then switched off for 2 min before switching on and monitoring the stabilisation time and irradiance level at the central sensor position. This permitted assessment of the expected stabilisation time during sample changeover.

The calibration of UV-C treatment time versus Vitamin D production was determined by exposing fresh, quartered mushrooms positioned at each point of the grid, to increasing times of UV-C

irradiation. The treatment plane of the mushrooms was approximately 25 mm above the base of the light box. The mushrooms from triplicates of each treatment time were pooled and dried before analysis of Vitamin D content. A calibration curve was fitted to the relationships between UV-C exposure time and Vitamin D content per dry or fresh weight of mushrooms as follows, and the value of chi-squared for the fit was 0.98:

$$\text{Vitamin D (ug/g dry)} = 28.62\ (1 - e^{-0.0158.\ \text{exposure time}})$$

$$\text{Vitamin D (ug/g fresh)} = 2.325\ (1 - e^{-0.0158.\ \text{exposure time}})$$

The calibration relationship was used to estimate the required UV-C irradiation time and the Vitamin D content of dried mushrooms was subsequently verified by chemical analysis.

## Aβ42 ThT Binding Assay

Aβ42 (GL-Biochem Ltd, Shanghai, China) was dispersed at 1.0 mg/ml in 1,1,1,3,3,3 – Hexafluoro-2-propanol (HFIP, Sigma, St. Louis, MO, USA) with gentle vortexing (1 min) followed by incubation at 22°C for 30 min. Vials containing 0.5 ml aliquots (0.5 mg of peptide) were dried under nitrogen (high purity, BOC Gases, Australia) leaving a 'film' of Aβ42, and stored at -18°C until required. Aggregate-free solutions of Aβ42 were prepared according to the method of Broerson *et al* (2011) [19]. Vials containing 0.5 mg Aβ-HFIP films were thawed (22°C for 10 min) before redispersing in 0.5 ml HFIP and redrying under nitrogen as described above. The Aβ-HFIP film was redissolved in 0.5 ml DMSO (Sigma, St. Louis, MO, USA), by the same method as that for HFIP. This solution was immediately loaded onto a desalting column (HiTrap™ Desalting column, GE Healthcare, Upsala, Sweden) washed and pre-equilibrated with chilled buffer solution (50 mM phosphate (Merck, Damstadt, Germany), 100 mM NaCl, pH 7.2). The peptide solution in DMSO (500 µl) was loaded onto the column and washed with buffer (1 ml) which was discarded. Aggregate-free Aβ42 was then eluted in 2 × 0.5 ml aliquots of chilled buffer, into pre-cooled micro-centrifuge tubes which were maintained on ice during adjustment of concentration and pending use in assay. Using the Extinction Coefficient of 0.33 $mg.ml^{-1}.cm^{-1}$ at 280 nm [20], the concentration of aggregate-free Aβ42 was adjusted to 0.1 mg/ml with chilled buffer and used in the ThT assay within 30 min.

Amyloid fibril inhibition by Aβ42 was adapted from [21]. Aggregate-free Aβ42 (0.1 mg/ml in buffer) was incubated with test samples and controls using black 96-microwell plates (Ooti-Plate-96F, Perkin Elmer, Shelton, USA) at 37°C for 24 hr, that were sealed to prevent evaporation. Vitamin D-rich mushroom sample (dissolved in 10% ethanol and filtered through 0.2 µm filter) was tested over the concentration range from 0.001 to 1 mg/ml. After the incubation period, plates were cooled to 22°C and Thioflavin T (ThT, Sigma-Aldrich, St. Louis, MO, USA) solution added to a final concentration of 2 µM. Fluorescence was measured using a fluorescence plate reader (VarioSkan, Thermo Scientific, Flash, USA) at excitation and emission wavelengths of 442 and 482 nm respectively. Positive and negative controls were Eosin (3.4 µM in 10% ethanol, BDH Laboratory, Poole, England), and buffer containing 10% ethanol, respectively. Percentage fibril inhibition (Fi) was calculated as follows: Fi (%) = 100 – [100 × ((S-C)/(K-B))), where S and C are fluorescence intensities of samples in the presence and absence (control) of Aβ, respectively. K is the fluorescence intensity of the uninhibited control of Aβ42 and B is the reagent blank. Analysis was conducted in triplicate for all samples.

## Dietary intervention study

Transgenic (Tg) mice expressing human genes for the Swedish variant of amyloid precursor protein and exon-9 deleted presenilin-1 (APPswe/PS1d9) were obtained from the Jackson Laboratory (Stock 004462; Bar Harbour, ME, USA). All male experimental subjects were selected from a breeding colony of hemi-zygous male crossed with wild type (WT) female mice and genotype identification was performed prior to experiments by the PCR protocol recommended by the Jackson Laboratory.

All experiments were performed in accordance with the Prevention of Cruelty to Animals Act, 1986 under the guidelines of the National Health and Medical Research Council (NHMRC) Code of Practice for the Care and Use of Animals for Experimental Purposes in Australia, and approved by the Animal Ethics Committee (AEC) of the Howard Florey Institute, University of Melbourne (AEC No. 11-010).

A total of 21 WT and 25 Tg mice completed the study, which were randomised between four groups based on genotype and feed type: *n* = 10 wild type, control diet (WT-con); *n* = 11 wild type, Vit D mushroom diet (WT-VDM); *n* = 13 transgenic, control diet (Tg-con) and *n* = 12 transgenic, Vit D mushroom diet (Tg-VDM). Two mice from the WT-con group died for unknown reason. One mouse died during the cheek bleeding procedure. The second began losing weight mid-way through the feeding study and was sacrificed according to ethical guidelines. It can be concluded that this death was not attributable to the feed intervention for the following reasons: the remaining mice were not adversely affected, and loss of health occurred mid-way through the study rather than upon first exposure and finally, the subject did not recover after switching its diet to normal mouse chow and mash.

Standard mouse chow (Barastoc mice cubes, Ridley Agriproducts Pty Ltd, Victoria, Australia, Table 2) was fed to all mice up to completion of the baseline behavioural assessment by Morris water maze at 2.0 months of age, after which the standard chow was replaced by 'Control' chow, which was deficient in Vitamin D (nominally <0.05 µg/g, reported as limit of detection of analytical method). The Control chow was administered either as supplied (WT-con and Tg-con groups) or after blending with mushroom solids at 5% w/w (WT-VDM and Tg-vdm groups). Mice were maintained *ad libitum* on experimental solid feeds and water. Monitoring for weight and health was performed twice per week. Mice were housed in treatment groups (3-5 per cage) in an artificially-lit environment with 12 h light/dark cycles and no exposure to natural light that might affect Vitamin D synthesis. Behavioural testing was conducted at the same point in the photo-period.

## Preparation of mouse feeds

Mouse feed pellets formulated from Control chow were crushed using a custom built hammer mill to yield particles of <2 mm. Crushed mouse feed (1-3 kg per batch) and Vitamin D-rich mushroom powder (blend of UV-treated mushroom Batches 1 and 2, Table 1) were dry blended at 5% (w/w) so that the final Vitamin D concentration was 0.20 $\pm$ 0.01 µg/g, before dispersing in de-ionised water (~950 ml) so as to make a moist dough. Control feed was prepared in the same manner but without addition of mushroom solids (and was prepared first to avoid chance cross-contamination). After thorough mixing, the dough was manually fed through a mincer with a sausage attachment (Weston No. 8 Manual Meat Grinder, Pragotrade USA) to make pellets of approximately 4 cm long and 1.5 cm diameter. The pellets were oven-dried overnight at 60°C before vacuum sealing and storing at 4°C until use. Feed batches were prepared as required with 7 batches prepared over the complete study (Table 1). Assuming an average daily consumption of 3 to 5 g per mouse, the daily dose of Vitamin D2 was 0.6 to 1.0 µg.

## Blood sampling

Blood samples were taken from mice prior to the commencement of feeding at 2 months (T-early), during the feed intervention at 6 months (T-mid), and at the conclusion of the study at 9 months of age (T-end). At the two former time points, samples were collected via sub-mandibular bleeds using Goldenrod animal lancets (MEDIpoint Inc., Mineola, NY) in K3 EDTA tubes (Greiner bio-one, item no. 450475). Final bleeding was performed under anaesthesia via cardiac puncture with a syringe. Blood was then divided between either EDTA tubes (Greiner Catalogue No. 450475, for Aβ42 analysis), or lithium-heparin tubes (Greiner Catalogue No. 450477, for Vitamin D analysis). The tubes were mixed by inversion 5-10 times and then centrifuged at 2000 × g for 10 min. The upper plasma layer was carefully removed by pipette and several aliquots prepared in 1.5 ml tubes before being snap-frozen in liquid nitrogen and stored at -80°C until use.

## Analysis of Vitamin D in plasma

Re-analysis is in progress using LC-MS-based analysis, after obtaining inconsistent results from 2 independent competitive immunoassays. Preliminary data from a limited set of replicates are presented and final results are yet to be confirmed.

## Analysis of Ca in plasma

A random sub-set of 4 plasma samples per treatment group taken at the final timepoint (7.5 months) were analysed for calcium levels, and corrected for total albumin levels. Samples were analysed by Melbourne Pathology (Collingwood, Vic, Australia) using Cobas methods for calcium and albumin (Roche Diagnostics, IN, USA) according to recommended protocols.

## Analysis of Aβ42 in plasma

Plasma analysis for Ab42 was conducted by commercial ELISA kit (Wako Human β-Amyloid (1-42) kit, product No. 298-62401). The required number of rows of antibody-coated microplate cells were removed from the refrigerator and allowed to equilibrate at 22°C. A calibration curve was obtained using supplied standard Aβ42 and diluents from 1 to 100 pM. Transgenic mouse plasma samples were diluted 1:16 with standard diluent (or 1:32 if plasma volume was insufficient). Samples or standards (100 µl) were loaded in duplicates before sealing and incubating overnight at 4°C. Wells were washed five times with supplied wash solution before loading 100 µl of HRP-conjugated antibody solution, sealing and incubating at 4°C for 1 hour. After washing, colour development was conducted using 3,3',5,5'-tetramethylbenzidine and stop solutions as per manufacturer instructions before reading absorbance at 450 nm. The concentration of test samples was calculated from the standard curve and reported as the average of duplicates.

## Analysis of cytokines in sera

Mouse sera taken at the mid-point of the study, after 3 months of experimental feeds, were analysed for a selection of cytokines including: interferon gamma, interleukins 2, 4, 10 and 17 and tumor necrosis factor alpha. Analysis was conducted using antibody-immobilized beads and the Luminex 100™ IS, 200™, High Throughput Screening system (Luminex, Austin, TX, USA). Reagents specific for detection of mouse cytokines were obtained from Merck Millipore (Milliplex Map Mouse Cytokine-Chemokine Panel, Cat. No. MPXMCYTO-70K, Merck KGaA, Darmstadt, Germany) and analysis conducted according to the recommended protocol. A calibration curve was prepared for each analyte over the range 3.2 to 2,000 pg/mL and serum samples were diluted 2-fold into assay buffer before analysis. The median fluorescent intensity (MFI)response data was analysed by interpolating values from fitting standards to a 5-parameter logistic curve. Results are reported as the mean of duplicates which were then averaged across respective treatment groups.

## Brain tissue sectioning and final bleed

At the conclusion of behavioural assessments, blood samples were taken prior to perfusion with 4% paraformaldehyde. Mice were anaesthetised (sodium pentobarbitone 80 mg/kg, i.p.) and blood was collected via cardiac puncture as described above. Finally, mice were transcardially perfused with 10 ml phosphate-buffered saline at 37°C (PBS; containing 50 mM $Na_2HPO_4$, 50 mM $NaH_2PO_4$, 154 mM NaCl; pH 7.4) followed by 50 ml of ice-cold PBS containing 4% paraformaldehyde (PFA; Merck, Hohenbrunn, Germany). Brains were then removed, hemisected and stored in 10% neutral buffered formalin prior to paraffin embedding.

Immuno-histochemical processing was performed by Histology Core Service Laboratory at the Melbourne Brain Centre (University of Melbourne). Brains were sectioned on a cryostat (5 µm thickness) and were assessed for plaque load using IE8 antibody (mouse monoclonal specific to Aβ17-24, University of Melbourne) with secondary antibody and streptavidin-biotin-

diaminobenzidine image development. Image capture was conducted using a Leica DM LB2 microscope (Leica Microsystems Inc. Bannockburn, IL, USA) under 20-fold magnification, in transmittance mode. Amyloid beta peptide plaque loadings were determined by image segmentation methodology, enabling subtraction of background and interferences such as nuclei, from amyoid plaque-specific signals. Image registration, quantitation and statistical analysis were conducted using customised scripts developed by CSIRO in MatLab (Natick, MA, USA).

## Behaviour testing by Morris Water Maze

Prior to introducing test feeds (~2 months of age) and at the conclusion of the feeding study (~9 months of age), hippocampal-dependent, long term spatial learning and memory retention were assessed in wild type and APPswe/PS1d9 mice using a Morris water maze [22, 23]. Distinct visual cues were placed at each of the four points of the compass of a circular tank (diameter = 144 cm), and Ethovision XT tracking software (Noldus, Leesburg, VA, USA) was programmed to divide the tank into four theoretical quadrants by drawing straight lines from east to west and north to south. The tank was then filled with opaque water (25°C) and a platform was situated 1 cm below the surface in the centre of one of the quadrants. Subjects were required to utilise the spatial cues in order to locate the platform. During acquisition of the memory task, mice underwent four trials per day with approximately 15 mins between each trial, and were introduced to the maze at each point of the compass. This ensured that the visual cues were required to locate the platform and prevented the possible influence of kinetic memory if subjects were provided with a consistent starting point. Mice that were unable to complete the task within 2 min were placed on the platform for 20 s before removal from the maze. After each trial, mice were dried and placed under a heat lamp to prevent hypothermia. Acquisition of spatial memory was assessed by examining latency and distance travelled by the mouse to locate the platform. The day after completion of the training phase, a "probe" trial was performed to test memory retention and retrieval, where the platform was removed from the maze and the amount of time the subject spent in the quadrant that previously contained the platform ("home") was recorded. Measures obtained from Morris water maze testing are described in Table 5.

## Behaviour testing by Barnes maze

Long-term, hippocampal-dependent, spatial reference memory was also assessed using the Barnes maze at the conclusion of the feeding studies [24]. The maze comprised a large circular platform (diameter = 120 cm) which was elevated 150 cm above floor level. Equally spaced circular holes (36 in total) were located around the perimeter. An escape box was situated underneath one of these holes, but was not visible until it was inspected by the subject. The escape box was kept in the same location for each mouse. Spatial cues were located outside the maze to provide a means for the mouse to orient itself. In order to increase motivation, mice were exposed to a loud buzzer and aversive bright lights during each trial, both of which were switched off upon entering the target hole. Mice were introduced into the maze from underneath a small box which was remotely raised by the experimenter in the adjoining room via a pulley system. After a pre-trial in which mice were led by the experimenter to the target hole, mice were exposed to the maze twice per day for four days. Parameters examined were primary latency (time until first visit of target hole), latency to enter target hole, primary error score, number of errors and total error score (for the purposes of error scoring, each hole was designated a number between 0 [target hole] and 18 [opposite to target] according to distance from the target). Trials were a maximum of 2 min long, and were terminated prior if the mouse was successful in entering the escape hole. On the fifth day, a probe trial was performed, where the escape box was removed and the latency and number of visits to the target hole recorded. Measures obtained from Barnes maze testing are described in Table 5.

## Behaviour testing by Y-maze

A standard Y-maze was utilised in order to assess short-term spatial memory [25, 26]. The maze comprised three enclosed arms (length = 30 cm, width = 10 cm, height = 16.5 cm), each with a distinct visual cue located at the end, as well as a centre zone located at the point at which the arms met. Protocol was performed as previously described [27, 28]. Briefly, mice were initially exposed to two of the three arms for 10 min, and were then removed to home cages for 2 h. Mice were subsequently re-exposed to all three arms of the Y-maze (including the novel arm) for 5 min. Spatial memory was assessed using Ethovision XT tracking software to record number of entries into the novel arm as well as total duration in the novel arm. Total distance moved was also recorded to control for any influence of altered activity. Measures obtained from Y maze testing are described in Table 5.

# 5 Results

## Preparation and Characterisation Of Vitamin D Button Mushrooms

The UV-C light box was used to treat Button mushrooms so as to prepare a calibration curve describing the relationship between exposure time and mushroom Vitamin D level. The calibration profile and fitted curve (Figure 1a) was used to calculate the exposure time required to produce 4-5 µg/g Vitamin D in dried mushrooms. However, the treatment of 30 kg of mushrooms in batches of 4 kg yielded a lower Vitamin D level than predicted (Table 1). This was attributed to the difference in layer density and proportion of mushrooms distributed away from the middle, compared with the calibration sample set. It was therefore necessary to treat a second batch of mushrooms (5 kg treated for 30 s, Table 1), and blend the 2 dried batches to the target level of 4 µg/g dry weight. The mixed batch of dried mushrooms was bagged and stored at 4°C in portions required for mouse feed preparation. The Vitamin D content of each stored batch was determined at the time of use, to assess storage stability of the Vitamin D in the dried mushroom product.

## Chemical Composition of Vitamin D-enriched Button mushrooms

Compositional properties of 7 batches of dried mushroom used to prepare mouse feeds throughout the feeding study are summarised in Table 1. Two preparations of UV-treated, dried mushroom were combined initially at a ratio of 3:1 (batches 1 and 2) and then at 2:1 (batches 3 to 7) so as to achieve the target Vitamin D2 level of approximately 4.0±0.2 µg/g. The pre-mixed batches of dried mushroom were stored at -20°C in vacuum-sealed packages suitable for making ~2-3 kg batches of mouse feed. Results for Vitamin D analysis indicate good storage stability over 36 weeks (Table 1). Elemental analysis was conducted on the mushroom product used in feed batch 3 giving C, H, N and ash contents of 40.0; 6.5, 5.7 and 12.4%, respectively. The protein level computed from the N content using the recommended factor of 4.55 for Button mushroom [29] was 25.9%, which was in good agreement with the reported value for fresh Button mushroom (2.09% on fresh weight basis, that computes to 26.1% on a dry weight basis at 92% moisture, [29]. However, 23-40% of nitrogen in mushrooms is known to be present as free amino acids [29].

## Amyloid fibril inhibition by Vitamin D Button mushrooms

Inhibition of assembly of amyloid fibrils by Vitamin D mushroom product was measured for respective batches of feed (Table 1, Figure 2). Previous studies (MU8015) indicated that Vitamin D2 and dried standard Button mushroom inhibited assembly of RCM-κCn fibrils with $IC_{50}$ values of 340 and 20 µg/ml, respectively, suggesting that Vitamin D was not likely to account for the anti-fibril activity in the mushroom product. Inhibition of self-assembly of Aβ42, which is more biologically relevant to AD, indicated that $IC_{50}$ values for inhibition progressively increased from 18 to >500 µg/ml, possibly suggesting that bioactive factors were not stable over the storage period. Alternatively, protease activity that was evident in dried fresh mushrooms (and not present in processed forms of mushrooms reported in project MU08015), might also introduce a concentration-dependent interference with this assay. A comparison of the anti-fibril effects of pure Vitamin D2 and D3 will also be obtained for inclusion in the manuscript.

## Study design

All mice were initially fed standard chow containing 0.05 µg/g Vitamin D3 and trace levels of Vitamin D2 (exact amount of each form to be confirmed). When mice reached 2 months of age, baseline memory testing was performed using the Morris water maze. Both mothers and newborn mice therefore received the standard dosing of Vitamin D3 until commencement of the dietary intervention at 2 months. From 2 months, the WT and Tg groups were each split in half and received either Vitamin D mushroom solids at 5% (w/w) in Control chow (containing trace levels of Vitamin

D2 and D3, to be confirmed) while the remaining half of each group received Control chow alone (containing trace levels of Vitamin D2 and D3, to be confirmed), without any mushrooms solids (Figure 3). This design was intended to test effects of supplementation with Vitamin D2 in mushroom together with unspecified putative mushroom bioactives in WT and AD transgenic mice. The use of a Vitamin D3-depleted base diet from 6 weeks onwards was expected to lead to Vitamin D deficiency, that is known to occur in adult humans. As such, 2 effects were planned to occur: firstly, depletion of Vitamin D3 and unchanged zero levels of mushroom bioactives in the Control diet and secondly, elevation of both Vitamin D2 and mushroom bioactives in the mushroom diet. The study design did not permit the effects of Vitamin D2 from mushroom bioactives to be resolved.

However, actual levels of serum metabolites of Vitamin D2 and D3 (Figure 6a) indicated a different outcome to that planned, in comparative Vitamin D sub-type status. The trace levels of Vitamin D3 in the Control diet combined with the longer residence time of Vitamin D3 metabolites, actually provided for Vitamin D adequacy in the Control groups (high ratio of D3/D2 metabolites) while the VDM groups manifested the opposite ratio of D3/D2 metabolites. As such, the effects observed in the VDM treatment groups reflect the high ratio of D2 (+mushroom solids)/D3 and allow some comparison to be made between specific activity of Vitamin D2 (and mushroom solids) and Vitamin D3.

## Body weight monitoring

Wild type and APPswe/PS1d9 mice exhibited normal and equivalent rates of body weight gain over the trial period of ~230 days without significant differences between diet or genotype on rate of weight gain (2-way ANOVA, Figure 4). While the mean weight gain over 230 days was significant ($P<0.05$), the mean initial and final body weights of each group were not significantly different from each other. It can be concluded that the rate of body weight gain was independent of both genotype and diet and that respective dietary compositions did not have a differential effect on weight gain.

## Liver toxicity biomarkers in mouse blood

Blood samples taken at the mid-timepoint of the feeding study were analysed for liver toxicity biomarkers. Results for 7/8 biomarkers are shown in Figure 5 while results for levels of gamma glutamyl transpeptidase (GGT) were incomplete due to insufficient blood sample volume. Results indicated that levels of GGT were <3 units/L for 4/4 wild type mice and 3/4 transgenic mice receiving Vitamin D mushroom feed with remaining results for control fed mice unavailable. Statistical comparisons for each biomarker between mice groups indicated lack of significant differences for any biomarker. These results demonstrated that neither the Control diet nor the supplementation of Vitamin D mushroom solids into the mouse chow produced a biochemical effect on liver function indicative of toxicity.

## Vitamin D and Ca levels in plasma

Vitamin D analysis was conducted on mouse plasma collected at 7.5 months using LC-MS-based analysis (Figure 6). These results are preliminary and analysis of additional mice per group is underway. Total levels of the metabolite 25-OH-D ranged from 55 to 67 nM across the 4 groups of mice which was in good agreement with the range reported by Yu et al (2011) for their transgenic and wild type mice fed from 2.4 to 12 IU of Vitamin D3, over 5 months. These results indicate firstly, that all animals received feeds with identical levels of Vitamin D3, that is, 0.05 ug/g (2 IU/g) for the first 6 weeks and trace levels of Vitamin D2 and D3 (ie, <0.05 ug/g, to be confirmed) for the following 6 months. The VDM groups received an *additional* 0.2 ug/g (8 IU/g) of Vitamin D2 for 6 months post-weaning. Secondly, the results showed that the additional load of Vitamin D2 from the mushrooms produced 25-OH-D2 as the predominant metabolite in both WT and Tg VDM groups and that the Vitamin D2 intake prevented the same levels of Vitamin D3 metabolites, known to have a

slower turnover [12], to accumulate to the levels measured in the Control groups. Importantly, the feeding study design produced inverse ratios of Vitamin D2 to Vitamin D3 metabolites and allows the following data to be interpreted in terms of comparative effects of Vitamin D2 (and mushroom solids) versus D3, on effect measures. Ca levels in VDM groups (WT and Tg) taken at the same timepoint as for Vitamin D metabolite analysis were significantly lower than for Tg-Control, demonstrating apparent advantages of Vitamin D2 in VDM for Ca absorption (and implicitly bone density, Figure 6b).

## Aβ42 levels in mouse sera

The levels of serum Aβ42 reflect comparative effects of mouse feeds on the peptide that is over-expressed in the brain of the transgenic mice and is accumulated as plaque. This peptide is of human origin and is not detected in the wild type mice (data not shown). For the transgenic mouse, the Aβ42 detected in serum would be expected to be balanced by increased retention of Aβ42 in brain as plaque, unless overall lowering of Aβ42 expression is implicated. Serum levels of Ab42 were significantly elevated after 6 months of intervention in both control and Vitamin D mushroom feeds, compared with earlier timepoints (0 and 3 months intervention, Figure 7). However, serum levels of Aβ42 were significantly lowered by feeding with Vitamin D mushrooms compared with the control feed. Elevation of Aβ42 in sera at the 8 month timepoint may suggest a change in biochemistry of Aβ42 expression or increased rate of deposition occurring in the brain in aging mice, but the VDM treatment (high Vitamin D2/D3 metabolite ratios) was more effective in lowering the serum levels of Ab42 compared with the Control diet (high Vitamin D3/D2 metabolite ratios).

## Ab42 plaque loads in mouse brains

Plaque deposition in the brain was measured at the end of the study (~8 month old mice) and indicated significant lowering of both the fractional area plaque per area of brain cortex or hippocampus (Figure 8a) and also significant lowering of mean plaque area in the cortex and hippocampus (Figure 8b). As Aβ42 levels in the periphery (Figure 7) and in brain (Figures 8 and 9) were significantly lowered in the Tg-VDM group, this indicates that the Vitamin D2 or mushroom bioactives were either effective in lowering Aβ42 expression, or that Vitamin D3 or deficiency of mushroom bioactives in the Control group promoted Aβ42 expression. In support of this, *in vitro* data from Project MU8015 showed that mushroom bioactives in Wood Ear mushroom, but not Vitamin D2 per se, inhibited BACE1, the enzyme that releases Aβ42 in the brain of the Tg mice. While not measured here, it is possible that BACE1 inhibitory activity, as for Wood Ear mushroom, could be present in the low molecular mass fraction of the VDM product, and be contributing to lowering of Aβ42 production via BACE1 inhibition. An alternate interpretation of the data is that the vitamin D3-containing Control diet increased Aβ42 deposition in the Tg mice and this was reversed at least partially by the VDM feed containing Vitamin D2 via a cholesterol-dependent pathway. The lowering of blood cholesterol levels by Button mushroom has been demonstrated in rats [30], with the efflux efficiency of Ab42 from brain known to be strongly linked with the genotype of the Apolipoprotein E receptor.

## Effects on cytokines in sera

After 3 months on the experimental feed, serum cytokine levels were measured in order to evaluate any effects of Vitamin D status on biomarkers of immune system activation (Table 4). The results indicated that pro-inflammatory (IGNg, IL2, IL4) regulatory (IL10, IL17) and anti-inflammatory (TNFa) cytokines were not altered by any treatment. Statistical analysis of the averages of each cytokine fail the null hypothesis, ie, none are statistically significantly different to zero, however it may be noted that elevated cytokine levels above the limit of detection were only seen in the Control groups with all mice in VDM groups exhibiting non-detectable levels of cytokines.

## Morris water maze testing of learning and memory

The rationale and sensitivities of respective memory tests are described in Table 5, including a summary of statistical analyses for all training and probe (recall) testing parameters. Baseline evaluation of mice (2 months of age) in the Morris water maze was compared for genotype groups (n=24 mice per group) prior to commencing dietary intervention. There were no significant differences between genotypes in any training (Figure 10) or probe (Figure 11a) testing parameters before commencing the dietary intervention.

Morris water maze testing at completion of the study indicated that rate of learning during training by latency to platform measure was significantly faster for WT-VDM compared with WT-control groups but there was no difference between Tg-control and Tg-VDM groups (Figure 12a). There was no difference between groups for distance travelled during training (Figure 12b), however the average speed of the Tg-VDM group diverged significantly from either Tg-control or WT-VDM groups (Figure 12c) reflecting relatively longer latency times for the Tg-VDM group (Figure 12a). This observation indicated that the Tg-VDM groups became slower as a result of their diet and that this altered capacity should be considered when interpreting other behaviour data. Results for memory recall in probe testing were not significantly different between any groups (Figure 11b).

## Barnes maze testing of learning and memory

Barnes maze testing was conducted only at completion of the study to avoid loss of sensitivity due to familiarisation. No differences due to diet or genotype were detected for any training parameters (Figures 13 and 14) except a diverging trend for distance travelled to find escape hole, suggesting a benefit of the VDM compared with control diet (Figure 13c). However, for Tg's, *all* training parameters diverged (Figures 13 and 14) with the results implicating that the VDM-fed group exhibited longer training primary latency (Figure 13a) and latency (Figure 13b) times and travelled shorter distances (Figure 13c) compared with the Tg-control group to find and enter the escape hole. This correlated with significantly slower overall training speed for the Tg-VDM group (Figure 13d), as was observed during Morris water maze training (Figure 12c). Thus, while there was no evidence of comparative physical impairment of this group either by body mass or liver biomarkers, the VDM feed appeared to produce some sort of motor impairment in the Tg mice only. It is not known if this manifestation reflects a relatively positive cognitive status such as level of relaxation for the Tg-VDM compared with Tg-WT group or other physical capacity factor such as bone development but these possibilities will be investigated.

Training error score measures were considered to be independent of motor capacity and the positive effect of VDM versus control was statistically significant for the training primary error score in Tg's but not WT's (Figure 14a). The training results for Barnes maze testing indicated a stronger benefit of VDM in Tg's compared with WT mice, in contrast with Morris water maze training, where the reverse was observed. It is likely that the Tg mice experienced decreasing physical robustness with age as their brain disease progressed and is possible that the Barnes maze test was less physically demanding and stressful than the Morris water maze test, and permitted expression of elevated cognitive performance in a setting without fear of drowning.

Upon probe testing in the Barnes maze, there were no significant differences between diets for either genotype in latency or distance travelled (Figure 15a). However, as observed previously, the average speed of mice in the Tg-VDM group was significantly slower than that of the Tg-control and WT groups (Figure 15a). In terms of remembering the location of the escape hole, the WT-VDM group retained its training and demonstrated significantly lower primary and total error scores compared with the WT-control group (Figure 15b), whereas for the Tg groups, the error scores were not significantly different (Figure 15b). This may suggest that the delayed memory of Tg's were generally inferior to WT mice, which may not be surprising considering the disease state of the Tg mice at 8 months of age.

## Y-maze testing of cognitive awareness

The Y maze test represented the least physically demanding and stressful cognitive test. Results are reported for the probe phase after a prior familiarisation period in which one arm of the Y maze was blocked. The results indicated that, compared with mice on a control diet, mice on the VDM diet spent significantly more time in the novel arm and this occurred in both WT and Tg groups (Figure 16a). Compared with WT mice on a VDM diet, WT mice on a control diet were faster to enter the novel arm (Figure 16b). In contrast, there was no difference in speed between Tg mice fed a control or VDM diet (Figure 16b).

# 6 Discussion

## 6.1 Characterisation of chemical composition of Vitamin D mushrooms

The convenience and utility of increasing Vitamin D2 levels in Button mushrooms by UV-C treatment has been noted [31]. Vitamin D2 in Button mushrooms was elevated from 0.07 to 10.14 µg/g dry weight after treatment for 2 hr with UV-C at 53 µW/cm2 [32] and from 2.2 to 7.3 µg/g dry weight in Button mushrooms after 2 hours exposure to UV-C light at 200 µW/cm2 [33]. In the current study, treatment times of 12 and 30 seconds produced Vitamin D2 levels of 2 and 8 µg/g dry weight, respectively from a source of approximately 1200 to 1700 µW/cm2 (intensity is expected to be ~20% lower in the treatment plane of the mushrooms compared with that of the UV-C sensor). While the Vitamin D2 production rate is expected to be positively correlated with UV-C intensity and treatment time [34], the surface area of exposed mushroom [35] and spatial configuration (this study) also influenced the conversion efficiency, as was observed in the difference between predicted and measured Vitamin D in calibration versus study batches. In calibration batches, the mushrooms were relatively sparsely distributed compared with the spatial distribution for treatments of batches 1 and 2.

At 5% (w/w) of the mouse base diet, VDM solids contributed a range of potential nutrients to the diet apart from Vitamin D2. Proximate analyses of standard Button mushroom solids indicate 26.8% crude protein; 1.9% fat; 10.3% ash and 61.0% total carbohydrate, by difference [36]. Up to a third of the protein content (26% of dry weight) in Button mushroom is expected to be present as amino acids [29] and minerals accounting for 4.9% of solids were identified (Table 3). The Vitamin D2 levels were elevated using post-harvest UV light treatment [32] to approximately 4.0 ug/g dry weight so as to deliver approximately 1.0 ug per day, based on a daily chow intake of 5 g. The levels of minerals present in the mushroom solids boosted the levels of metals in the base chow by 17.9%, 3.6%, 17.3% and 5.2% for Cu, Fe, Mo and Zn respectively (Table 3). The VDM solids provided a source of primary metabolites including sugars, organic acids, fatty acids and other compounds (data not shown), and also a source of secondary metabolites including hispidins (data not shown). While the study design controlled for effects of Vitamin D2, the effects of these additional components of mushroom were not resolved from the total VDM effect.

## 6.2 Comparative bioavailability of Vitamin D2 and D3

The daily dose of Vitamin D2 given in the solid chow by inclusion of mushroom solids at 5% (w/w) did not alter any liver function biomarkers in comparison with control feeds (Figure 5), suggesting that neither the Vitamin D nor non-Vitamin D solids produced harmful effects.

The Adequate Intake of Vitamin D 400 IU/day for middle-aged adults and the upper safe level for humans over 1 year old is 3200 IU/day [37]. The current upper limit of Vitamin D intake is considered to be a conservative estimate as others have recommended the level be raised to 250 ug/day [38]. For humans, a plasma level of 25(OH)D >750 nm/L has been defined as a toxic range of Vitamin D [39] however, recently, a 'J'-shaped curve for all-cause mortality risk was reported with negative consequences for survival associated with 25-OH-D levels above ~90 nM [40]. It is likely that the previous data is specific for 25-OH-D3, based on the prescription-only availability of Vitamin D2 in Denmark, where the study was conducted (personal communication with author).

In the current study, Vitamin D intake levels were not considered to be in toxic ranges, but nevertheless provide interesting insights into the competitive bioavailability of Vitamin D2 and D3 when administered together, in mice, which appears to be without precedent in the literature.

However, a recent publication has shown that a similar effect occurs in humans [41]. Indeed, Vitamin D metabolite status is commonly reported as total 25-OH-D, which does not take into account either the intake ratio of Vitamin D2 to D3 nor the metabolite ratio. Therefore, because of the low proportion of Vitamin D2 in food and its low use as the source of Vitamin D in supplements, most reported data can be interpreted to reflect intake and effects mainly of Vitamin D3. In spite of the Control and VDM mice receiving only trace levels of Vitamin D3 throughout the intervention, the serum level of 25-OH-D3 in the Control groups was equivalent to that of 25-OH-D2 in the VDM groups, with approximately constant total 25-OH-D levels across all groups. This most probably reflected the relatively higher efficiency of Vitamin D3 metabolism into 25-OH-D3 compared with Vitamin D2 metabolism [12] such that a >4-fold higher ratio of Vitamin D2 was required to produce the same metabolite levels of 25-OH-D2 as 25-OH-D3. Given the potential for Vitamin D3 toxicity at elevated doses, and no reported toxicity associated with Vitamin D2, it might be concluded that Vitamin D2 represents a safer form of Vitamin D, and the preferred form of Vitamin D for oral supplementation. As such, this study can inform the ongoing debate regarding the comparative bioavailability and safety of Vitamin D2 versus D3 [12], and specific recommendations for supplementation.

## 6.3 Vitamin D, mushrooms, memory and motor function

This study represents the first to investigate effects of vitamin D2 administered within a total mushroom solids supplement, on a transgenic mouse model of Alzheimer's disease [42], and as such, is the first study to focus on specific effects of Vitamin D2 on cognitive outcomes in mice. Learning impairments were observed in both wild type and transgenic mice that were maintained on a diet deficient in vitamin D (Control), whereas memory performance was relatively improved for mice fed a diet replete with vitamin D2, supplied by mushroom (VDM). The observation of unexplained hypo-activity (ie, slowness) in the Tg-VDM group precluded comparison between-genotype (e.g., WT-VDM cf Tg-VDM), and also precluded inclusion of behavioural test results relating to speed. WT-control showed impaired learning in the water maze and y-maze as well as poorer performance in the probe trial of the Barnes maze when compared to WT-VDM and Tg-VDM groups. Tg-control mice also showed a clear impairment in learning in the Y-maze (where activity levels were not different between treatment groups). Furthermore, examining primary error score in the Barnes maze (which should not be dependent on activity) also revealed a learning deficit in the acquisition phase.

Nevertheless, there is sufficient evidence to conclude that vitamin D2 (and mushroom solids), in this case, was critical for learning and memory, an effect that was not specific to the AD model but observed consistently in both wild type and transgenic mice across all three behavioural tests. Other animal studies have investigated effects of Vitamin D3 oral supplementation, which is known to be metabolised slightly differently to Vitamin D2 [43-46], which can probably account for its higher bioavailability [12]. The relationship between mushroom vitamin D2 and cognitive performance in the APPswe/PS1 mice is consistent with a previous report where vitamin $D_3$ was found to be similarly effective in the same transgenic model [13].

A non-causal relationship between vitamin D and AD has been established for some time [47-49], with evidence for its specific protective biological role also emerging [50-53]. Vitamin D receptor (VDR) mRNA levels [54] are reduced in AD patients, and the gene encoding VDR has been highlighted as a risk factor in late-onset AD [55-57]. However, vitamin D levels are positively associated with cognitive performance in both AD [48, 52] and non-AD subjects [58-62] suggesting that AD-independent and AD-specific effects of Vitamin D may apply.

The APPswe/PS1 mouse is a well-characterised rodent model of AD, exhibiting both histological and behavioural hallmarks of the human condition. These mice initially present with amyloid deposits at

approximately 4 months of age, and overall amyloid burden accumulates with disease progression [63]. Previous studies in this strain consistently report learning deficits in the Morris water maze memory test [64-68], while spontaneous alternation behaviour and performance in the traditional Barnes maze appears to be intact [69, 70]. Extensive behavioural characterisation has demonstrated that APPswe/PS1 mice have no discernible impairments in motor coordination or general physical incapabilities compared to wild type littermates [70] and cannot explain the observed slowness of the Tg-VDM group in both the water and Barnes maze tests at the final timepoint. There was no genotype-based difference in Y-maze performance, which is in agreement with previous reportings, albeit using a novel arm rather than free spontaneous alternation approach [69, 70].

A possible explanation for the activity difference observed in the Tg-VDM group is the possibility of hypervitaminosis D. In this study, the dose of vitamin $D_2$ (~160 IU/g) contained in the food was approximately 80 times the amount in standard chow (2 IU/g) given in the first 6 weeks. However, as there was no evidence of increased plasma calcium levels nor elevation of liver function biomarkers, to suggest vitamin D toxicity [71, 72], this is unlikely. Furthermore, there was similarly no evidence of Vitamin D toxicity in the WT-VDM group, suggesting that the effect was specific to the AD genotype.

Alternatively, the slowness of the Tg-VDM group might be attributed to skeletal muscle atrophy caused by an increase in mitochondrial reactive oxygen species (ROS) [73]. Although the VDM dose should increase circulating levels of copper and zinc, it may reduce the half-life of the CuZn-superoxide dismutase (SOD) and/or increase mitochondrial ROS in skeletal muscle. In the VDM mice, it is possible that the induced AD state accelerated the effect of ROS on skeletal muscle compared with the WT. Further work is currently in progress to test this hypothesis.

Overall, the current study provides an unprecedented comparison of in vivo effects of Vitamin D2 (in the presence of mushroom solids) and D3 on cognition with benefits for memory and amyloid deposition, arising from the following alternative interpretations:

- The specific activity of Vitamin D2 was higher than Vitamin D3 and that additional mushroom solids had no effect
- The specific activity of Vitamin D2 was equivalent to Vitamin D3 and the relative benefits of the VDM diet was due to non-Vitamin D mushroom components.
- The Vitamin D2 was not active and the relative benefits of the VDM diet was due to non-Vitamin D mushroom components (probably not likely)
- The specific activity of Vitamin D2 was higher than Vitamin D3 and additional mushroom solids contributed additional synergistic benefits

Further studies are required to confirm the correct interpretation.

## 6.4 Regulation of Ab42 in brain and periphery and effects of Vitamin D

In the Ab42-overproducing APPswe/PS1dE9 transgenic mouse model, ratios of Ab42 to Ab40 strongly favour Ab42 in brain and conversely favour Ab40 in blood, measured at 6 and 12 months of age. The Ab peptide levels were accompanied by memory deficit that could be detected and increased from 6 months of age [74]. However, while levels of brain and CSF Ab42 are usually correlated, reported relationships between changes in brain Ab42 and peripheral Ab42 are more variable, and reflect the mechanistic basis of perturbation of production and clearance of Ab42. Useful studies fall into either of 2 categories: inverse correlation (most frequently reported effect), where decrease (or increase) in brain Ab42 is accompanied by increase (or decrease) in blood Ab42 and, positive correlation, where decrease (or increase) in brain Ab42 is accompanied by decrease (or

increase) in blood Ab42; and. The intervention with VDM produced a lowering of both brain and blood Ab42, ie, a positive correlation (Figures 7-9).

Inverse correlations between changes in brain and blood Ab42 levels (if both reported, frequently not) have been demonstrated in the following examples. In the absence of any intervention, the Tg2576 mouse model, like the APPswe/PS1 model, exhibits a strong inverse correlation between the brain load of Ab42 and levels in the CSF and blood [75], signifying that deposition of Ab42 as plaque is favoured over peripheral efflux, in advanced stages of plaque deposition in the brain. In the gene-targeted mouse model with Swedish familial AD mutation and humanised Ab domain, which also expressed endogenous APP, treatment with an agent that induced protein kinase C activity (PKC, phorbol 12-myristate 13-acetate), induced lowering of Ab42 in brain (effects on peripheral Ab42 not reported), and supported a role for PKC in promoting clearance of Ab42 [76]. In the APPswe/PS1dE9 mouse model, an inverse relationship between brain deposits of Ab42 and serum levels was induced by vaccination-mediated production of anti-Ab42 antibodies in the periphery [77]. The stimulation of Ab42 efflux producing an inverse correlation between brain and peripheral levels of Ab42 from brain to periphery using both anti-Ab42 antibodies and active vaccination, has been extensively demonstrated in mice [78]. Abramowski et al, 2008 showed that the use of gamma secretase inhibitors lowered both plaque load and CSF Ab42 in 3 genetically distinct APP transgenic mouse models (effects on blood Ab42 not reported). As for antibody therapy, levels of plasma Ab42 could be elevated by dosing either wild type or AD transgenic mice with glucose [79]. However, we cannot attribute similar effects to non-fibre sugars present in the VDM solids, where lowering of serum Ab42 was observed. In addition, a new drug for treating vascular complications of diabetes also lowered brain Ab42 while increasing plasma Ab42 in APPswe transgenic mice [80], as did NK-4, a cyanine dye in the Tg2576 mouse model [81]. These examples most likely reflect enhanced efflux of brain Ab42 without any effect on production rates of Ab42 in the brain.

In contrast, positive correlations between changes in brain and blood Ab42 levels in Ab42 over-expressing models necessarily reflect effects on Ab42 production rates *in addition to* perturbation of Ab42 deposition in and efflux from brain. Examples of this type of effect are much less frequently reported. For example, both brain and blood levels of Ab42 could be lowered using the combination of doxycycline (serum lowering effect) and anti-Ab42 antibody therapy (brain plaque lowering effect, Wang et al, 2011 [82]), whereas peripherally administered anti-Ab42 antibodies have been typically shown to elicit an *inverse* correlation in changes of brain and blood levels of Ab42 [78], as discussed above.

While Yu et al, 2011 [13] demonstrated that vitamin D3 produced a lowering of Ab42 peptide and plaque load in the brain, that was attributed to possible inhibition of beta secretase or enhanced clearance by nerve growth factor-stimulated astrocytes, the effects on blood Ab42 levels were not reported and it is not known if Vitamin D3 therapy also lowered blood Ab42 levels. However, we report that treatment of APPswe/PS1dE9 mice with VDM solids lowered *both* plaque load and peripheral Ab42, suggesting that Ab42 total production was lowered by treatment with VDM, possibly reflecting synergistic effects of Vitamin D2 and non-Vitamin D mushroom components. This is supported by the study of Wang et al, 2011 [82] which suggested that combination therapy was required to produce this type of unusual 'positive correlation' effect. The demonstrated lowering of cholesterol levels in rats fed standard Button mushrooms, attributed to the beta glucan fraction [30], and the important role of cholesterol-receptor type on Ab42 efflux from brain may infer a role of this pathway for Ab42 processing in the periphery, supporting that mushroom components in addition to Vitamin D2, might drive independent but synergistic effects with beneficial effects in AD. The results do not appear to support that the Zn dose associated with the VDM solids influenced Ab42 deposition in brain, which was reported to be enhanced by Zn overdose [83].

# 7 Summary

In this study, we have demonstrated a critical role for vitamin D2, and/or possibly other species in mushroom, in learning and memory in both wild type and AD transgenic mice. The results provide strong cumulative evidence that feeding a diet supplemented with Vitamin D2-rich mushroom solids versus a control diet containing trace levels of Vitamins D2 and D3, produced improvement in memory and cognitive performance in both WT and transgenic genotypes. The effects are expected to be attributed to differential specific activities of Vitamin D2 versus D3 and possibly, other unidentified components of the mushroom, which in previous *in vitro* studies were implicated to act specifically on the amyloid beta peptide toxicity pathway. The results indicated that Vitamin D2 and mushroom bioactives have potential to improve memory parameters in the absence and presence of amyloid beta peptide pathology. Also, the study has demonstrated the importance of Vitamin D on memory in both normal mouse brain and in the presence of amyloid beta peptide as present in Alzheimer's Disease. The results fully justify progression into human studies aimed at testing efficacy of Vitamin D2-rich mushrooms on cognition in healthy elderly and also in Alzheimer's Disease subjects. Since a large proportion of elderly are Vitamin D-deficient, if dietary intervention studies produce conclusive outcomes, this represents a significant potential opportunity for corrective supplementation and benefits for cognition. The only reported intervention study to date testing efficacy of high dose Vitamin D for improvement on cognition in Alzheimer's Disease did not demonstrate any benefit. However, we have shown for the first time that Vitamin D2-rich mushrooms were effective in rescuing memory deficit in AD mice and it is possible that the combination of Vitamin D2 *and* mushroom bioactives could produce important synergistic or inter-dependent effects, that are more effective than either Vitamin D2 or Vitamin D3 alone. Further studies are necessary to resolve the alternative interpretation of these results, in particular, to address the important question of Vitamin D2 versus D3 efficacy on cognition and other important health effects regulated by Vitamin D.

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**Table 1.** Summary of properties of dried Vitamin D-rich Button mushroom that was incorporated into the base mouse chow mix at 5% (w/w), for successive batches of feed.

| Feed Batch | Preparation Date (Storage Time at 4°C)* | Ratio of B1 and B2 | Vitamin D Content (µg/g dry weight) | Carbon, Hydrogen, Nitrogen Content (%) | Ash Content (%) | $IC_{50}$ for Fibril Inhibition by Aβ42 Assay (µg/ml) |
|---|---|---|---|---|---|---|
| Feed 1 (1.25 kg) | 12/04/11 (6 weeks) | 3:1 | 3.6 ± 0.3 | | | |
| Feed 2 (3.2 kg) | 05/05/11 (9 weeks) | 3:1 | 3.8 ± 0.24 | | | |
| Feed 3 (3.2 kg) | 06/06/11 (14 weeks) | 2:1 | 4.2 ± 0.3 | 40.3 ± 0.16<br>6.5 ± 0.06<br>5.7 ± 0.1 | 12.4 ± 0.3 | 17.9 ± 7.2 * |
| Feed 4 (3.2 kg) | 05/07/11 (18 weeks) | 2:1 | 3.8 ± 0.0 | | | |
| Feed 5 (2.0 kg) | 18/08/11 (24 weeks) | 2:1 | 5.1 ± 0.2 | | | 47.9 ± 16.9 |
| Feed 6 (3.2 kg) | 20/10/12 (33 weeks) | 2:1 | 3.6 ± 0.3 | | | >500 |
| Feed 7 (3.2 kg) | 10/11/11 (36 weeks) | 2:1 | 4.3 ± 0.5 | | | >500 |

* different supplier of Aβ42

**Table 2.** Summary of composition of mice chow base mix. The ingredients formulated to yield the defined composition included: wheat, wheat by-products, dehulled oats, meat meal, canola oil, full fat soyabean meal, skim milk powder, molasses, salt vitamins and minerals.

| Component | Concentration | Component | Concentration |
|---|---|---|---|
| Crude Protein | 22% | Vitamin K3 | 55 mg/kg |
| Crude fat | 9% | Vitamin B1 | 64 mg/kg |
| Crude fibre | 3.2% | Vitamin B2 | 48 mg/kg |
| Acid detergent fibre | 4.4% | Vitamin B6 | 30 mg/kg |
| Neutral detergent fibre | 10.4% | Vitamin B12 | 0.08 mg/kg |
| Digestible Energy-Horse | 13.2 MJ/kg | Niacin | 400 mg/kg |
| Ca | 1.2% | Panto | 220 mg/kg |
| P | 0.96% | Biotin | 1.48 mg/kg |
| Na | 0.35% | Folic Acid | 11 mg/kg |
| K | 0.89% | Fe | 51 mg/kg |
| Cl | 0.57% | Zn | 60 mg/kg |
| Mg | 0.25% | Mn | 120 mg/kg |
| Lysine | 1.22% | Cu | 10 mg/kg |
| Methionine | 0.38% | Se | 0.1 mg/kg |
| Linoleic Acid | 3.11% | Mo | 0.4 mg/kg |
| Vitamin A | 15IU/g | Co | 0.6 mg/kg |
| Vitamin D3 | 0 * | I | 1.4 mg/kg |
| Vitamin E | 260 mg/kg | Starch | 25% |

*Standard mouse chow contained 0.05 µg/g Vitamin D3

**Table 3.** Summary of mineral composition of Vitamin D-rich Button mushroom (that was incorporated into the base mouse chow mix at 5%, w/w) expressed in mg/100 g dry dried product, showing comparative contributions to daily intake from mushroom and base chow sources, and where possible, the percentage mineral intake 'boost' associated with VDM treatment groups.

| Element | Al | B | Ca | Cu | Fe | K | Mg | Mn | Mo | Na | P | S | Ti | Zn |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Dried Vit D mushroom (mg/100 g) | 2.08 | 1.09 | 13.75 | 3.41 | 3.51 | 3349 | 110.0 | 0.580 | 0.132 | 70.35 | 1080 | 259.3 | 0.200 | 5.98 |
| **Daily intake (mg/0.25 g VDM)** | **0.0052** | **0.0027** | **0.034** | **0.0085** | **0.0088** | **8.37** | **0.28** | **0.0014** | **0.0003** | **0.18** | **2.70** | **0.65** | **0.0005** | **0.015** |
| Barastoc base chow (mg/100 g)- | | | 1200 | 1.00 | 5.10 | 890.0 | 250.0 | 12.0 | 0.040 | 350.0 | 960.0 | | | 6.00 |
| **Daily intake * (mg/4.75 g chow)** | | | **57.0** | **0.0475** | **0.24** | **42.28** | **11.88** | **0.57** | **0.0019** | **16.63** | **45.60** | | | **0.29** |
| **Daily intake from all sources Barastoc+VDM (mg/5.0 g chow)** | **0.0052** | **0.0027** | **57.03** | **0.056** | **0.25** | **50.65** | **12.15** | **0.57** | **0.0022** | **16.8** | **48.3** | **0.65** | **0.0005** | **0.30** |
| **VDM-related intake boost-%** | | | **0.1** | **17.9** | **3.6** | **19.8** | **2.3** | **0.3** | **17.3** | **1.1** | **5.9** | | | **5.2** |

* Based on assuming average daily consumption of 5 g chow per day

**Table 4.** Plasma concentrations of selected cytokines after 3 months of intervention, determined in a random subset (N=6) from each treatment group, expressed as mean and standard error of the mean. Results designated 'ND' were below the analytical detection limit. None of the numerical results shown are significantly different to zero.

| Group | IFNg-pg/ml | IL2-pg/ml | IL4-pg/ml | IL10-pg/ml | IL17-pg/ml | TNFa-pg/ml |
|---|---|---|---|---|---|---|
| WT-control | 437.2±231.2 | 255.0±195 | ND | 86.9±78.9 | 25.3±17.3 | ND |
| WT-VDM | ND | ND | ND | ND | ND | ND |
| Tg-control | 38.2±30.2 | ND | ND | ND | ND | ND |
| Tg-VDM | ND | ND | ND | ND | ND | ND |

**Table 5.** Summary of behavioural test and implications for cognitive ability

| Behavioural Test | Stress Intensity associated with test | Parameter Measured | Description of Cognitive test | Summary of Statistical Analysis of Effects of feeds within Genotype |
|---|---|---|---|---|
| Morris Water Maze | High stress test also requiring motor skill competency. | Training - latency to platform | 9 days of training (4 trials per day) measuring time taken to discover the hidden platform in a water bath, on successive days for a maximum test period of 2 min. Repeat measure of rate of learning and memory/recall over 9 days (long term). | WT-VDM significantly improved learning at 9 month timepoint<br>2-way repeat measure ANOVA (feed, day) $F(1,19)=4.679$, $p=0.043$).<br><br>Tg-NS |
| | | Training - distance travelled to reach platform | 9 days of training (4 trials per day) measuring distance travelled to discover the hidden platform (or total distance travelled until maximum test period elapses) in a water bath, on successive days. Repeat measure of rate of learning and memory/recall over 9 days (long term). | WT - NS<br>Tg - NS |
| | | Training - speed | Repeat measure of change in speed (distance/latency) of mice over training period. | WT - NS<br>Tg–VDM significantly reduced activity at 9 month timepoint<br>2-way repeat measure ANOVA (feed, day) $F(1,23)=8.988$, $p=0.006$ |
| | High stress test also requiring motor skill competency, arguably higher stress than training. | Probe trial - home *quadrant* time | 1 min test performed the day after completing training (day 10) with platform removed so as to test memory of the platform location (ie, precise 'zone' versus general region 'quadrant') and therefore, how well the mouse has learned the training task. Probe home quadrant time is the time spent in the platform *quadrant* (very commonly performed in literature). | WT - NS<br>Tg - NS |
| | | Probe trial – latency to platform *zone* | Probe latency to platform zone is time elapsed before first entry to platform *zone*. | WT - NS<br>Tg - NS |
| | | Probe trial - platform *zone* entries | Probe platform zone entries is the number of entries into the platform *zone*, which is similar to time spent in platform *quadrant* but is more sensitive because of the smaller area included. | WT - NS<br>Tg - NS |
| Barnes | Moderately | Training – | 4 days of training (2 trials per | WT - no impairment |

| | | | | |
|---|---|---|---|---|
| Maze | stressful. Mice are encouraged to find the escape hole to avoid exposure to a loud buzzer and floodlights. Physically unchallenging. | primary latency to escape hole (time) | day) to find the target hole which provides an escape from the noise and lights. Trials were a maximum of 3 min long, and were terminated as soon as the mouse was successful in entering the escape hole. Primary latency measures time taken to *identify* the escape hole for the first time. | during learning the task<br>Tg - different activity levels means measure cannot be used. |
| | | Training – latency to escape hole (time) | 4 days of training (2 trials per day) to find the target hole which provides an escape from the noise and lights. Trials were a maximum of 3 min long, and were terminated as soon as the mouse was successful in entering the escape hole. Latency measures time taken before *entering* the escape hole. | WT - NS<br>Tg - different activity levels means measure cannot be used. |
| | | Training – distance | 4 days of training (2 trials per day) to find the target hole which provides an escape from the noise and lights. Trials were a maximum of 3 min long, and were terminated as soon as the mouse was successful in entering the escape hole (or total distance travelled until maximum test period elapses). Distance measures number of cm travelled before *entering* the escape hole. | WT - NS<br>Tg - different activity levels means measure cannot be used. |
| | | Training – speed | 4 days of training (2 trials per day) to find the target hole which provides an escape from the noise and lights. Trials were a maximum of 3 min long, and were terminated as soon as the mouse was successful in entering the escape hole. Speed represents a global measure of total distance travelled for the duration of the trial either 3 min or to sooner if hole was entered. | WT - NS<br>Tg-VDM have lower velocity than controls. 2-way repeat measure ANOVA (feed, day) $F(1,23)=10.760$, $p=0.003$. |
| | | Training – primary error score | For error scoring, each hole was designated a number between 0 (escape hole) and 18 (opposite escape hole) increasing with radial distance from the escape hole). Primary error score is a measure of 'correctness' of the first hole visited with 0 a perfect score. | WT - NS<br>Tg-VDM are more accurate than controls. 2-way repeat measure ANOVA (feed, day) $F(1,23)=13.092$, $p<0.001$ |
| | | Training – | Total error score is the total | WT - NS |

| | | | | |
|---|---|---|---|---|
| | | total error score | number of visits to all incorrect holes before entering the escape hole. | Tg - different activity levels means measure cannot be used. |
| | | Probe trial – latency (time) | After a rest on the fifth day, a 90 s duration probe trial was performed on the sixth day, where the escape box was removed and memory of the escape hole position tested. There are no survival consequences (unlike fear of drowning for MWM) for mis-identification of escape hole apart from inability to escape from the lights and noise. Probe latency measured the time taken before identifying the location of the escape hole for the first time. | WT - NS<br>Tg - different activity levels means measure cannot be used. |
| | | Probe trial - speed | Probe speed measured the mouse speed (total distance travelled per 90 s trial duration) as a global measure of activity. | WT - NS<br>Tg-VDM have lower velocity than controls. T-test, T=4.426, p<0.001 |
| | | Probe trial - primary error score | Probe primary error score is a measure of 'correctness' of the first hole visited in relation to the original location of the escape hole, with 0 a perfect score. | WT-VDM reduces in probe trial. T-test, t=2.186, p=0.042<br>Tg - NS |
| | | Probe trial – total error score | Total error score is the total number of visits to all incorrect holes. | WT-VDM reduces in probe trial. T-test, t=3.130, p=0.005.<br><br>Tg - different activity levels means measure cannot be used. |
| Y Maze | Low stress test and physically unchallenging. | Probe - time spent in *new* arm of maze | Short term memory test after a familiarisation period of 10 min with access to one arm of the Y maze blocked, followed by removal from the maze for next 2 hr. After re-introduction to the maze with all arms accessible, the test measures the time spent in the novel unexplored arm of the maze, thus measuring the natural exploratory instinct of the mouse. The test is a measure of memory and cognitive awareness. | WT-VDM exhibited significantly improved learning time spent in novel arm greater than other arms, Kruskal-Wallis One Way Analysis of Variance on Ranks H = 23.731 with 3 degrees of freedom. (P = <0.001) Controls shows similar time in all arms.<br><br>Tg-VDM exhibited significantly improved learning Kruskal-Wallis One Way Analysis of Variance on Ranks H = 21.324 with 3 degrees of freedom. (P = <0.001). Controls show similar |

| | | | | |
|---|---|---|---|---|
| | | | | time in all arms. |
| | | Probe – primary latency (time) | Time taken to enter the new arm of the maze | WT–VDM group shows improved learning. Lower latency to enter novel arm. Mann-Whitney Rank Sum Test U Statistic= 21.000, P = 0.018<br>TG-NS |

# 9 Figure Legends

## Figure 1.

(a) Calibration profile of Vitamin D2 content produced as a function of UV-C exposure time, fitted to the exponential function, $y = A \times (1\text{-}e^{-B.x})$, with A=2.325 and 28.62 and B=0.0158 and 0.0158 for curves fitted to fresh and dry weights of mushrooms, respectively. The fresh weight values of Vitamin D2 content were calculated using mean total solids content for fresh and dried mushrooms of 7.75 and 95.4%, respectively. The chi-squared value for the fit of both curves was 0.98. Images of freeze dried mushroom Batch 2 (b) and after incorporation into the Vitamin D-depleted mouse chow and extrusion into pellets, showing control and Vitamin D-mushroom preparations (c).

## Figure 2.

Concentration-dependent change in percentage inhibition of Aβ42 (30 µg/ml, 6.7 µM) fibril assembly monitored by ThT fluorescence assay, in the presence of increasing concentrations of batches of dried Vitamin D mushroom, representing storage at -15°C for 14, 18, 20 and 36 weeks, respectively. Data points represent the mean of triplicate analysis and standard deviations.

## Figure 3.

Schematic representations of the study design adopted for testing effects of dietary supplementation with Vitamin D mushroom solids at 5% (w/w) of Control chow, which did not contain Vitamin D, for the intervention period of 7.5 months, after weaning on Standard chow.

## Figure 4.

Body mass monitoring of Mouse weights during feed intervention showing averages within each treatment group. The Control feed cohort trial commenced 4 weeks after the Vitamin D cohort. Error bars have been omitted for clarity.

## Figure 5.

Results for analysis of liver toxicity biomarkers in bloods taken at mid timepoint of study.

## Figure 6.

Effects of dietary interventions on concentrations of plasma metabolites of (a) Vitamin D2 and D3 (25-OHD2 and 25, OH-D3) and (b) calcium in bloods taken after 7.5 months of feeding experimental diets. Results represent the averages of duplicate analysis of bloods from at least 2 animals per group, with error bars representing standard errors.

## Figure 7.

Concentrations of amyloid beta peptide in sera of transgenic mice taken at baseline (2 months), 6 and 10 months of feeding intervention. Results are the mean of triplicate analyses of each sample and shown as the average for the group (n=7-12) with standard errors at each timepoint.

## Figure 8.

Effects of dietary intervention on brain plaque load measured by immunohistochemical staining with IE8 antibody to Ab42 of brain sections, which were assessed for plaque load by systematic image segmentation analysis. Results show (a) fraction of plaque per total areas of cortex or hippocampus and (b) mean plaque area. Results are averaged across brains of n=12 or 13 mice and shown as the average for the group with standard deviations.

## Figure 9.

Quantifying plaque load. A) original RGB image of brain section showing significant plaque presence. B) Nuclear image obtained using color unmixing; plaques are mostly absent from this image C)

Plaque image obtained by color unmixing; nuclei are mostly absent from this image while plaques are clearly visible D) Masks for the cortex area and the hippocampus were produced manually and they are shown in dark grey shade. Plaques shown in white were segmented automatically from C) using intensity thresholding as described in the methods.

## Figure 10.

Results of Morris water maze training for wild type and APPswe/PS1d9 transgenic mice groups (*n* = 24 per genotype) at baseline (1.5 months of age), prior to the commencement of dietary intervention, showing (a) latency to platform, (b) distance travelled to reach platform and (c) the average speed during the test, on consecutive days for 9 days. Results represent the average for the group with standard error at each timepoint.

## Figure 11.

Results of probe trial testing on day 10, to assess memory retention in the Morris water maze after removing platform (a) at baseline, comparing genotype groups and (b) at completion of the study, comparing 4 treatment groups. WT and Tg represent wild type and APPswe/PS1d9 transgenic mice respectively and control and VDM represent control (Vit D–deficient) chow and Control chow containing mushroom solids. Results are shown for: amount of time spent in home quadrant; latency of first entry into the platform zone and number of entries into the platform zone. Results represent the average for the group with standard error. There were no significant differences between measures within genotype.

## Figure 12.

Results of Morris water maze training for wild type and APPswe/PS1d9 transgenic mice groups (*n* = 24 per genotype) at the endpoint of the study (9 months of age), after 7.5 months of dietary intervention, showing (a) latency to platform, (b) distance travelled to reach platform and (c) the average speed during the test, on consecutive days for 9 days. Results represent the average for the group with standard error at each timepoint. For latency to platform measure, learning rate for WT-VDM was significantly better than WT-control by 2-way repeat measure ANOVA (feed, day) $F(1,19)=4.679$, $p=0.043$). For speed measure, Tg–VDM groups was significantly slower than Tg-control group by 2-way repeat measure ANOVA (feed, day) $F(1,23)=8.988$, $p=0.006$.

## Figure 13.

Results of Barnes maze training for wild type and APPswe/PS1d9 transgenic mice groups at the endpoint of the study (7.5 months of age), after 7.5 months of dietary intervention, showing (a) primary latency to identify escape hole, (b) latency to enter escape hole, (c) the distance travelled before entering hole, and (d) the average speed during the test, on consecutive days for 4 days. Results represent the average for the group with standard error at each timepoint. For speed measure, Tg-VDM was significantly slower than Tg-control group by 2-way repeat measure ANOVA (feed, day) $F(1,23)=10.760$, $p=0.003$. Because of different activity levels, significant in other measures have not been considered.

## Figure 14.

Results of Barnes maze training for wild type and APPswe/PS1d9 transgenic mice groups at the endpoint of the study (7.5 months of age), after 7.5 months of dietary intervention, showing (a) primary error score and (b) total error score, on consecutive days for 4 days. Results represent the average for the group with standard error at each timepoint. For primary error score measure, Tg-VDM were more accurate than Tg-control group by 2-way repeat measure ANOVA (feed, day) $F(1,23)=13.092$, $p<0.001$. Because of different activity levels, significant in total error score measure has not been considered.

## Figure 15.

Results of probe trial testing on day 6, to assess memory retention of the Barnes maze after removing escape hole at completion of the study, comparing 4 treatment groups. WT and Tg represent wild type and APPswe/PS1d9 transgenic mice respectively and control and vdm represent control (Vit D–deficient) chow and Control chow containing mushroom solids. Results are shown for (a) latency of first entry into the escape hole zone, total distance travelled over trial duration of 90 s and average speed over the trial and (b) primary and total error scores. Results represent the average for the group with standard error. For speed measure, Tg-VDM was significantly slower than Tg-control group by T-test ($T=4.426$, $p<0.001$). For primary error score, WT-VDM group is significantly more correct than WT-control group by T-test ($t=2.186$, $p=0.042$). For total error score, WT-VDM group is significantly more correct than WT-control group by T-test ($t=3.130$, $p=0.005$). Because of different activity levels, significant in other measures for Tg groups has not been considered.

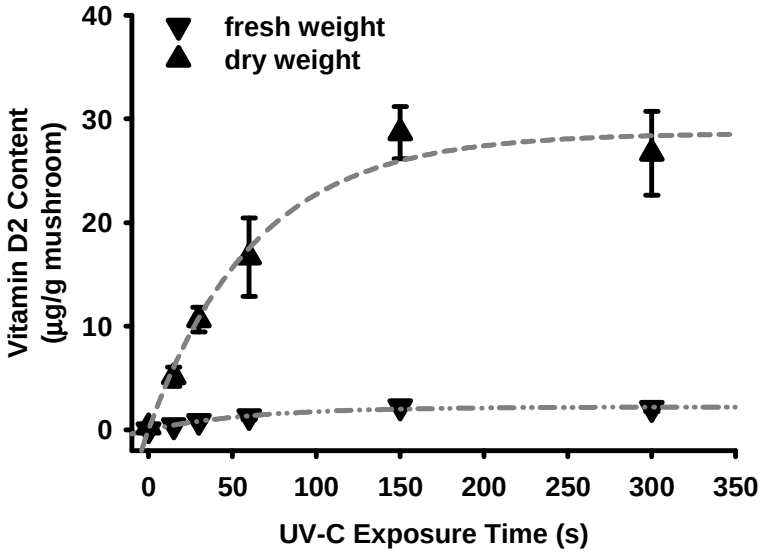
## Figure 16.

Results of Y maze probe trial after prior familiarisation period for mouse groups at the endpoint of the study (9 months of age), after 7.5 months of dietary intervention, showing (a) time spent in novel arm and (b) latency to novel arm. Results represent the average for the group with standard error at each timepoint. For time in novel arm measure, WT-VDM stayed significantly longer than WT-control group by Kruskal-Wallis One Way ANOVA on Ranks ($H = 23.731$ with 3 degrees of freedom, $P = <0.001$) Controls shows similar time in all arms. Likewise, Tg-VDM group stayed significantly longer than Tg-control group by Kruskal-Wallis One Way ANOVA on Ranks ($H = 21.324$ with 3 degrees of freedom, $P = <0.001$). WT-control and Tg-control groups show similar time in all arms. For latency to novel arm measure, WT–VDM group shows improved learning by Mann-Whitney Rank Sum Test (U Statistic= 21.000, $P = 0.018$).

# 10 Figures

# Figure 1

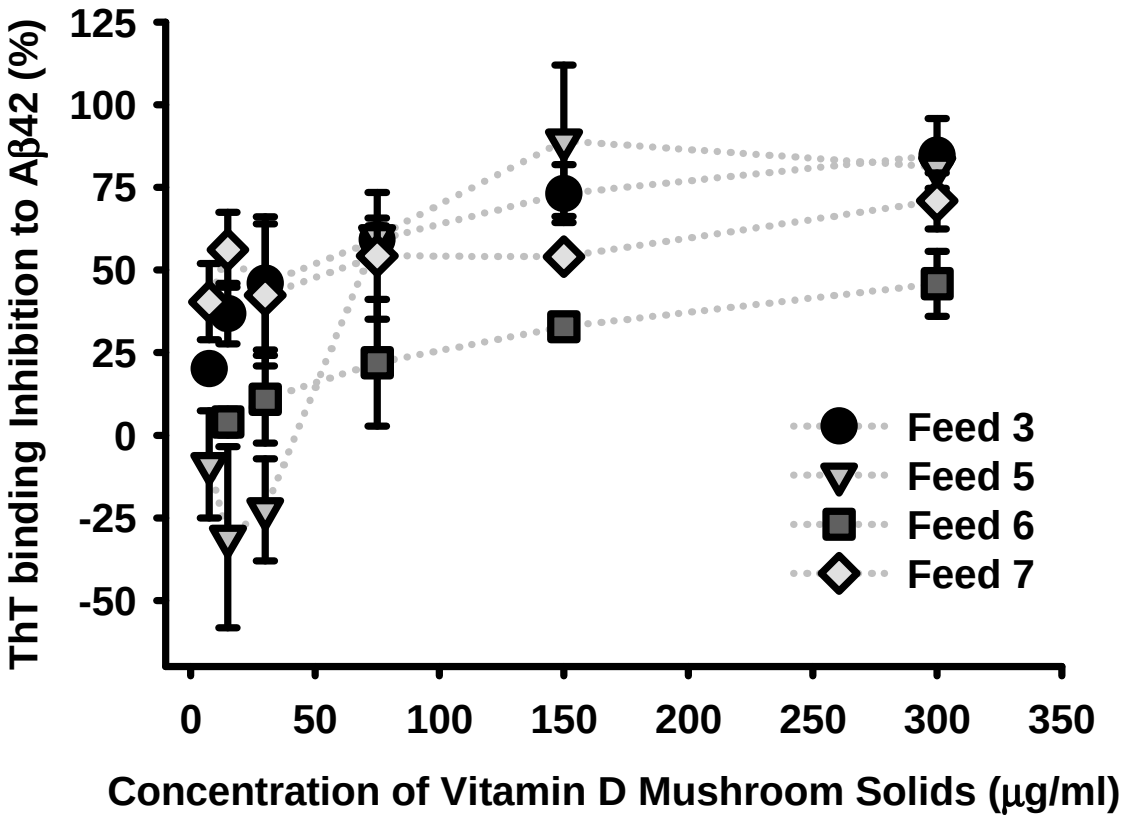
(a)


fresh weight
dry weight
Vitamin D2 Content (µg/g mushroom)
40
30
20
10
0
0
50
100
150
200
250
300
350
UV-C Exposure Time (s)

(b)

(c)

Figure 2.


ThT binding Inhibition to Aβ42 (%)
125
100
75
50
25
0
-25
-50
0
50
100
150
200
250
300
350
Concentration of Vitamin D Mushroom Solids (µg/ml)
Feed 3
Feed 5
Feed 6
Feed 7

## Figure 3.

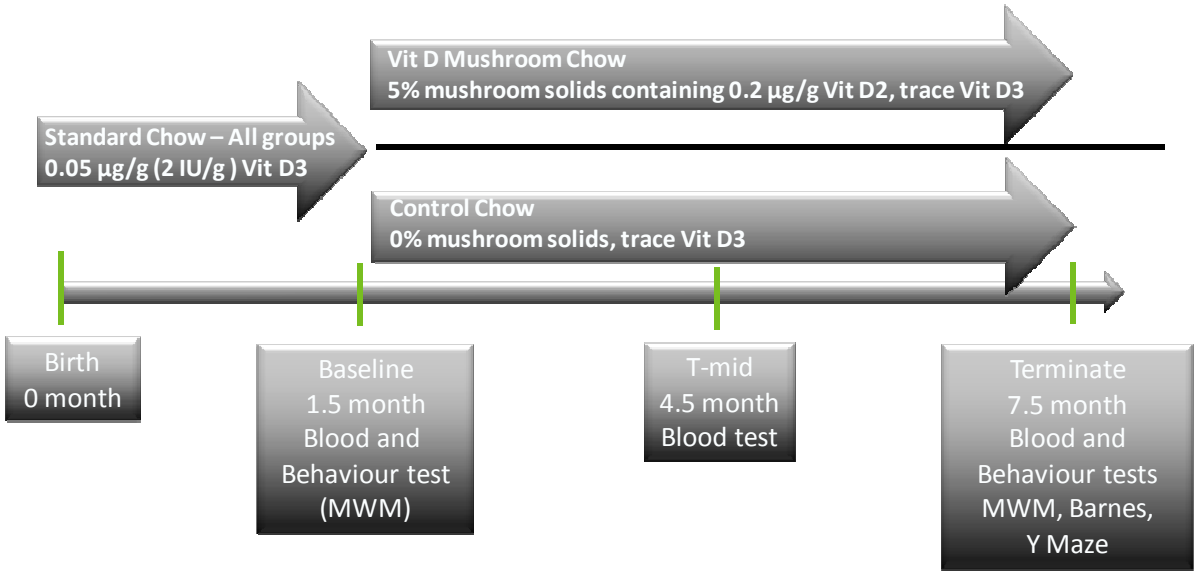

Vit D Mushroom Chow
5% mushroom solids containing 0.2 µg/g Vit D2, trace Vit D3
Standard Chow – All groups
0.05 µg/g (2 IU/g ) Vit D3
Control Chow
0% mushroom solids, trace Vit D3
Birth
0 month
Baseline
1.5 month
Blood and
Behaviour test
(MWM)
T-mid
4.5 month
Blood test
Terminate
7.5 month
Blood and
Behaviour tests
MWM, Barnes,
Y Maze

Figure 4.

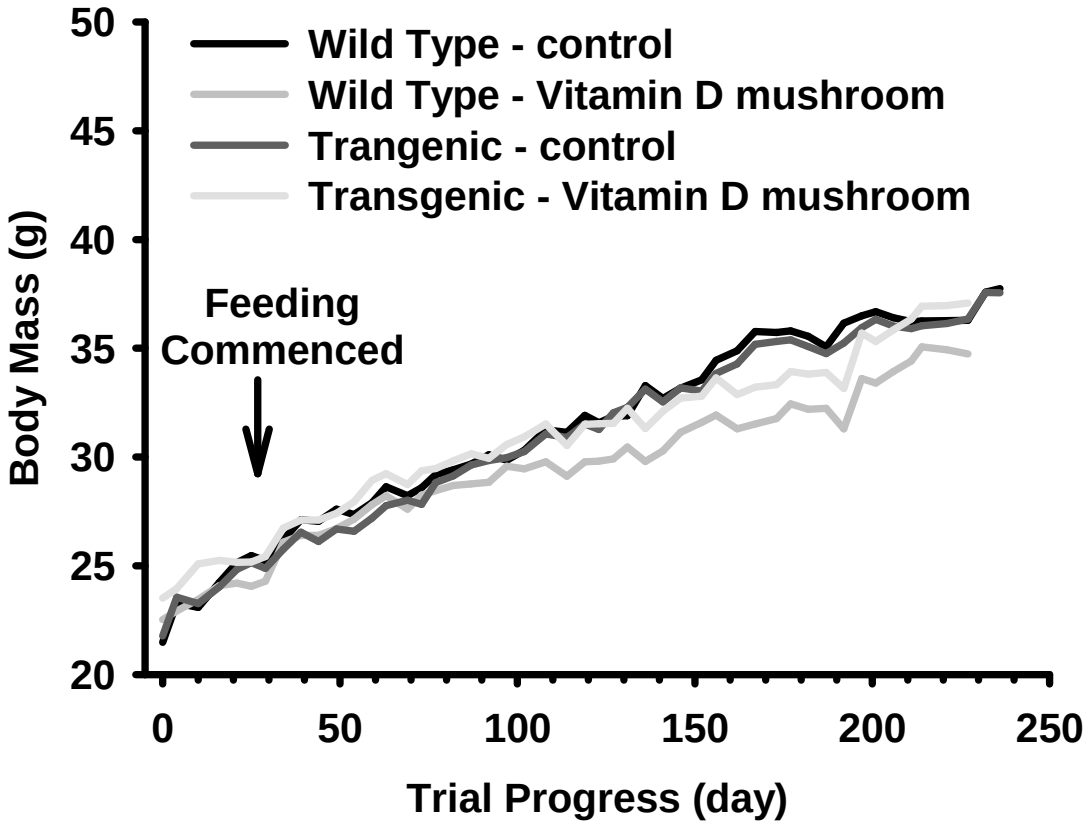
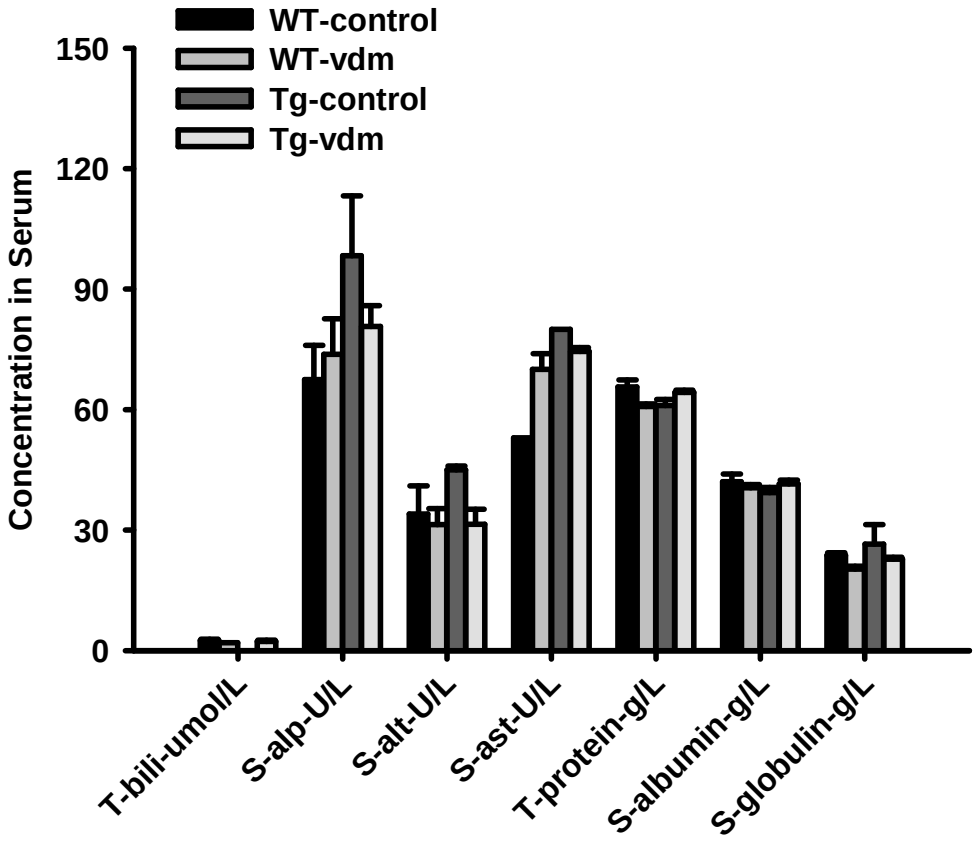
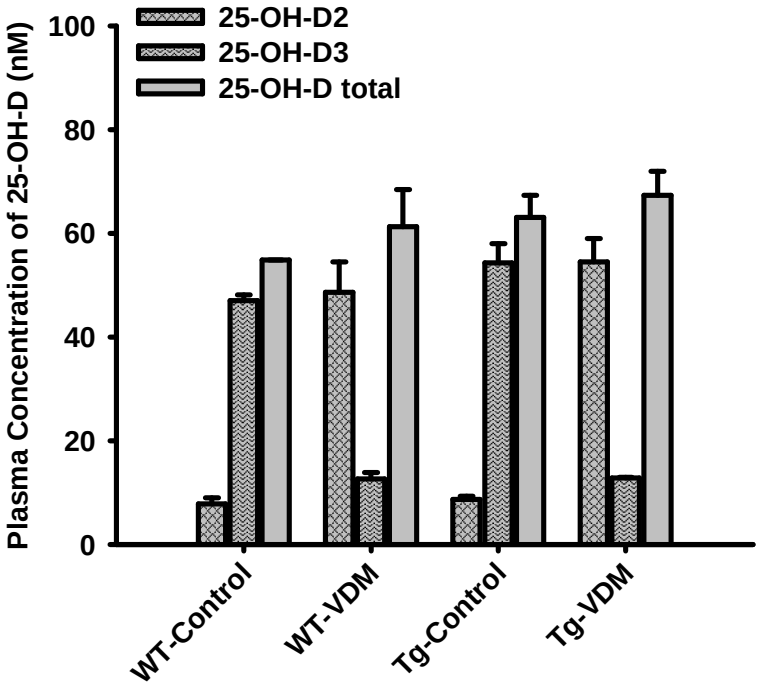

Wild Type - control
Wild Type - Vitamin D mushroom
Trangenic - control
Transgenic - Vitamin D mushroom
Feeding Commenced
Body Mass (g)
50
45
40
35
30
25
20
0
50
100
150
200
250
Trial Progress (day)

Figure 5.


WT-control
WT-vdm
Tg-control
Tg-vdm
Concentration in Serum
150
120
90
60
30
0
T-bili-umol/L
S-alp-U/L
S-alt-U/L
S-ast-U/L
T-protein-g/L
S-albumin-g/L
S-globulin-g/L

## Figure 6.

(a)


25-OH-D2
25-OH-D3
25-OH-D total
Plasma Concentration of 25-OH-D (nM)
100
80
60
40
20
0
WT-Control
WT-VDM
Tg-Control
Tg-VDM

(b)

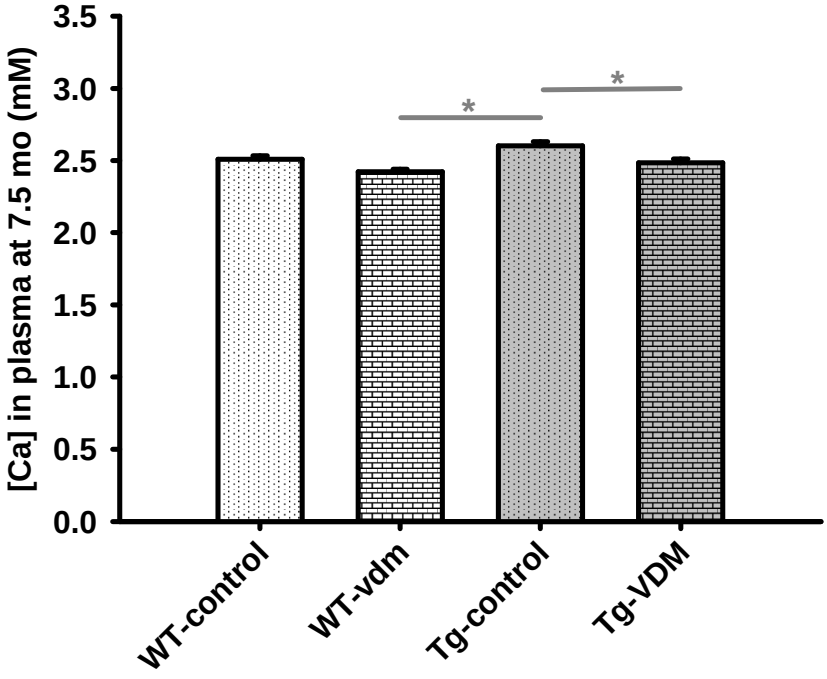
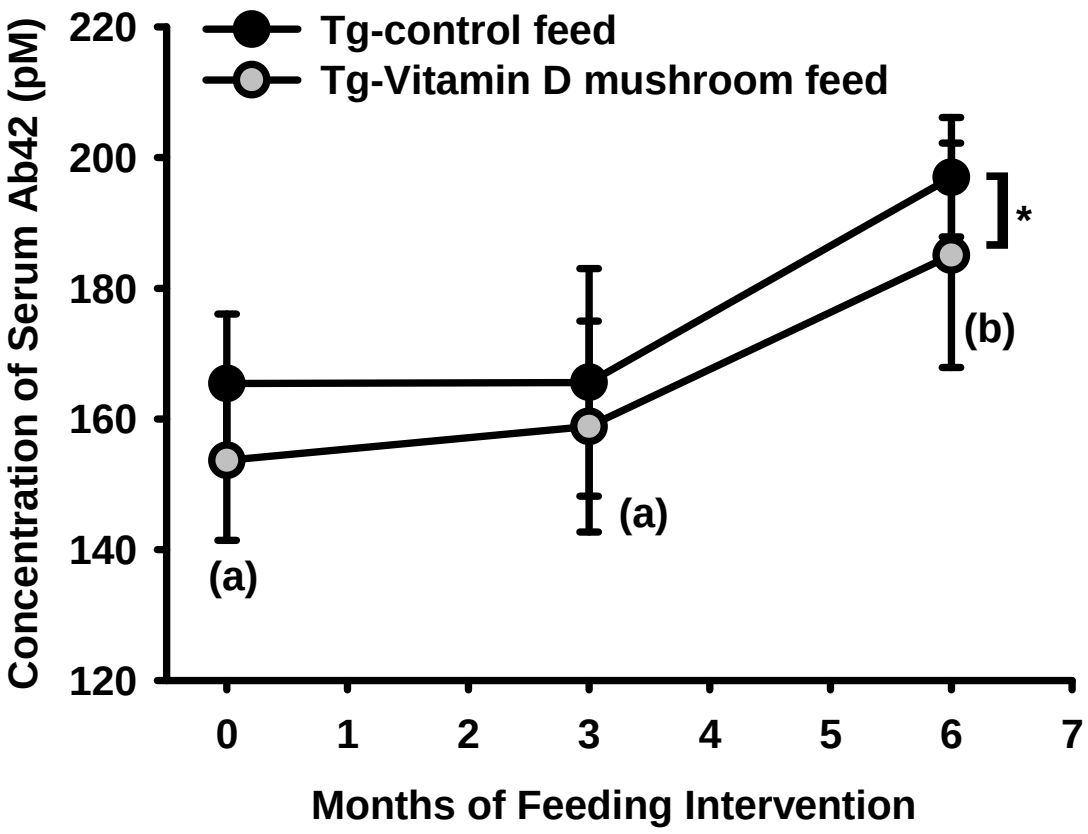
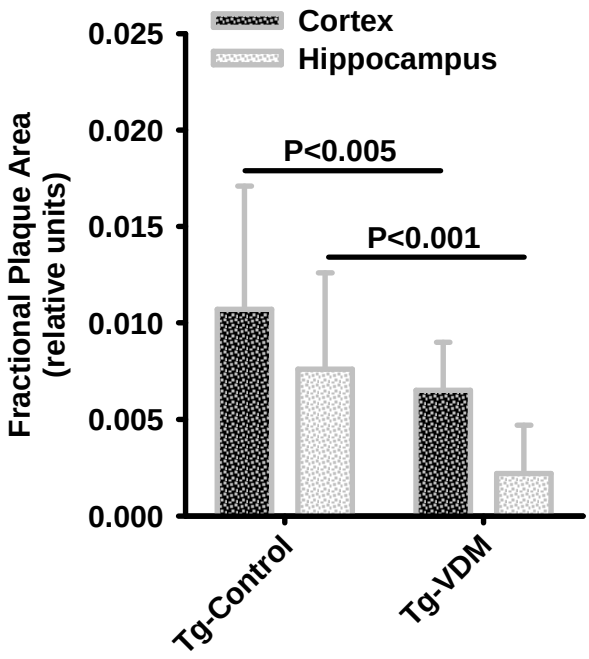

[Ca] in plasma at 7.5 mo (mM)
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
*
WT-control
WT-vdm
Tg-control
Tg-VDM

Figure 7.

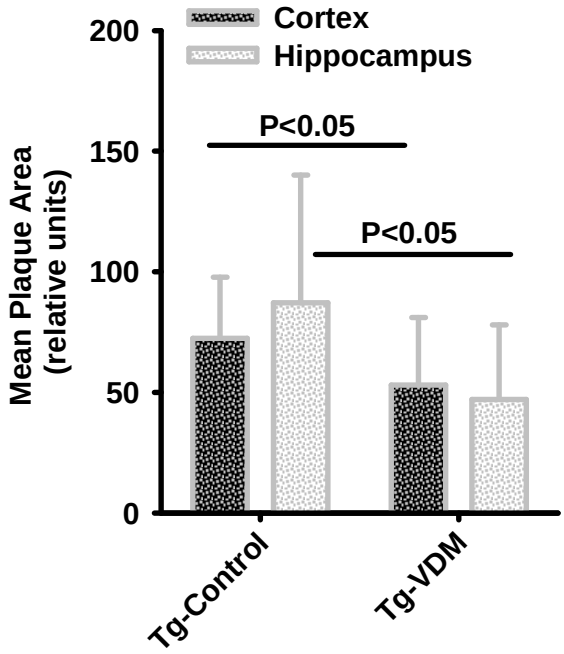

Tg-control feed
Tg-Vitamin D mushroom feed
Concentration of Serum Ab42 (pM)
220
200
180
160
140
120
0
1
2
3
4
5
6
7
Months of Feeding Intervention
(a)
(a)
(b)
*

## Figure 8.

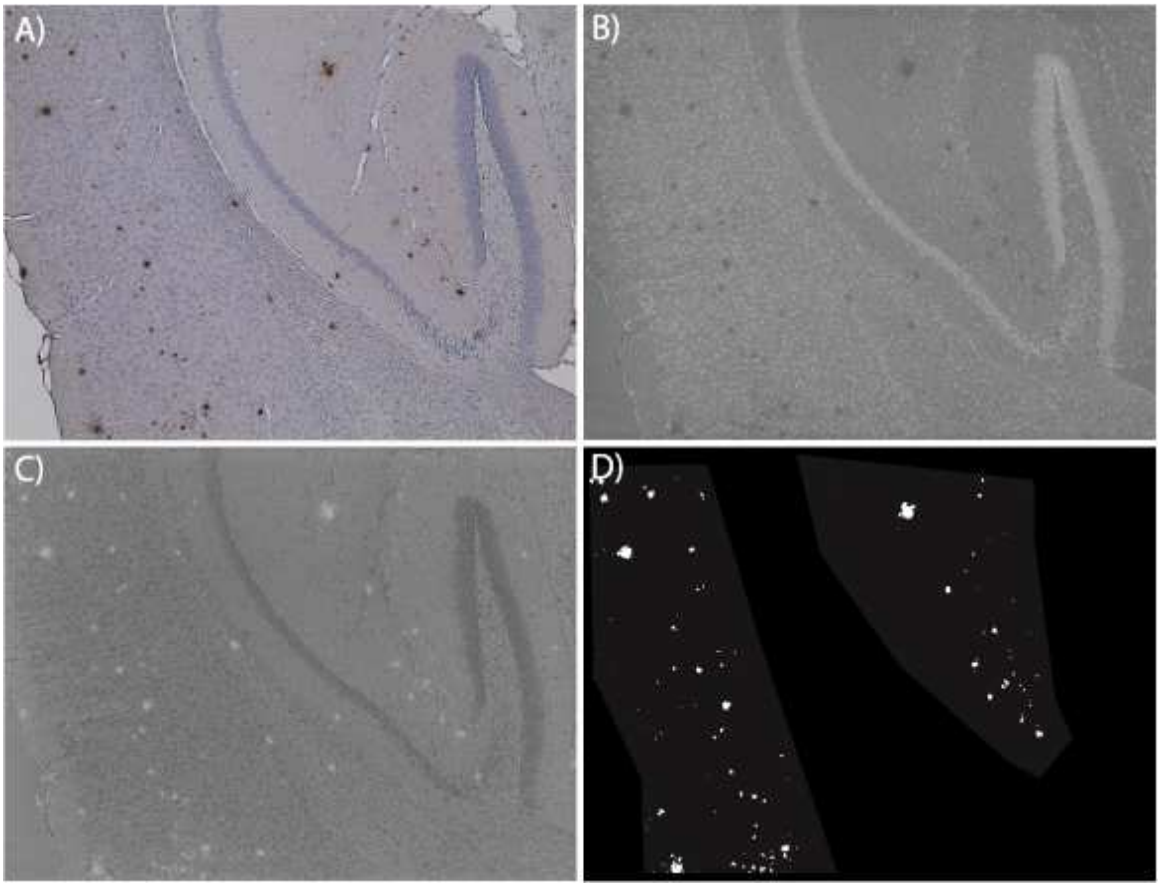
(a)


Cortex
Hippocampus
Fractional Plaque Area (relative units)
0.025
0.020
0.015
0.010
0.005
0.000
P<0.005
P<0.001
Tg-Control
Tg-VDM

(b)

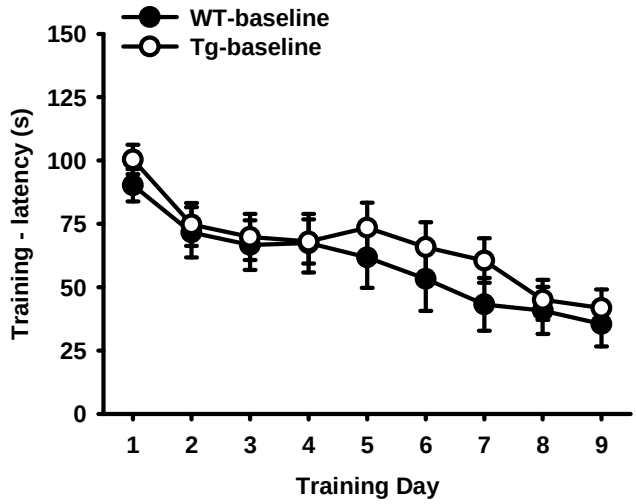

Cortex
Hippocampus
Mean Plaque Area (relative units)
200
150
100
50
0
P<0.05
P<0.05
Tg-Control
Tg-VDM

## Figure 9.

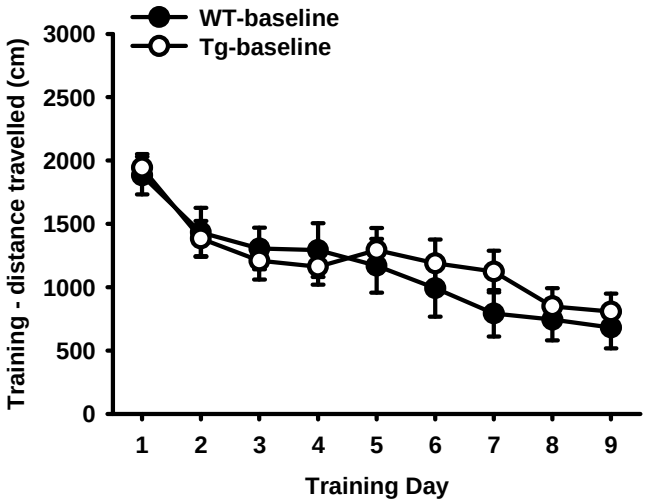


## Figure 10.

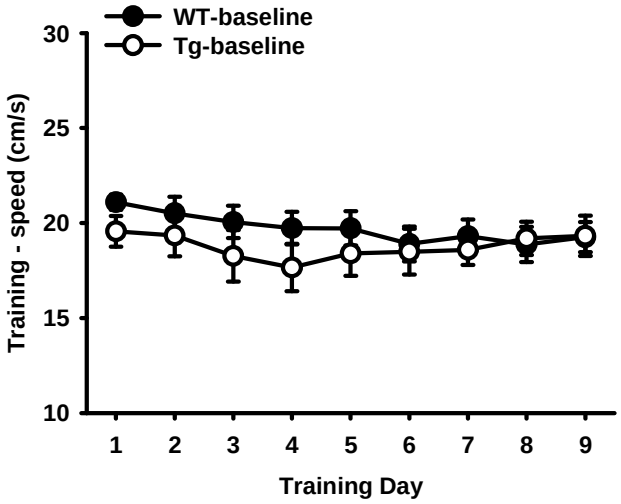
(a)




(b)

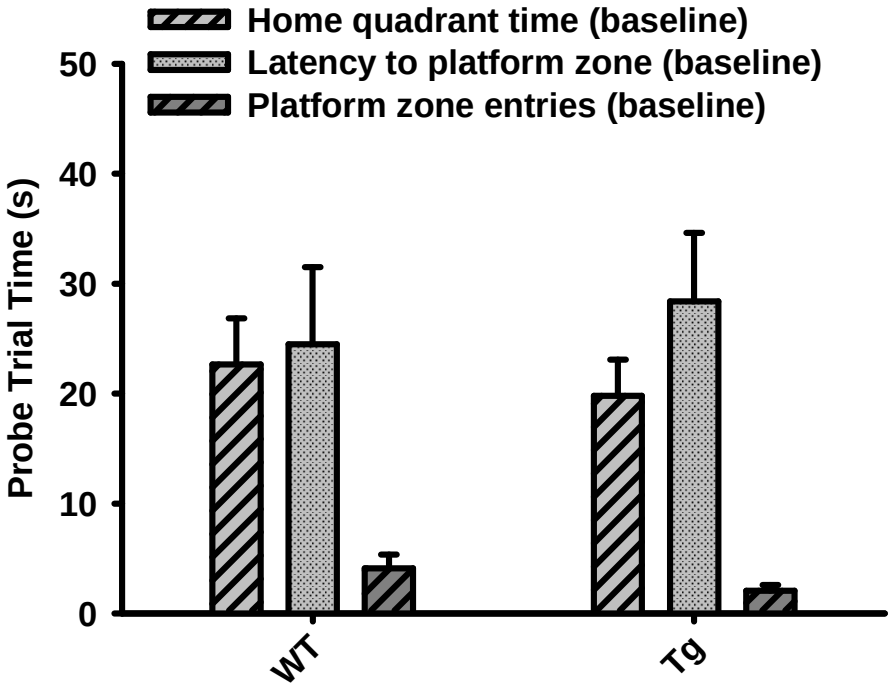



(c)

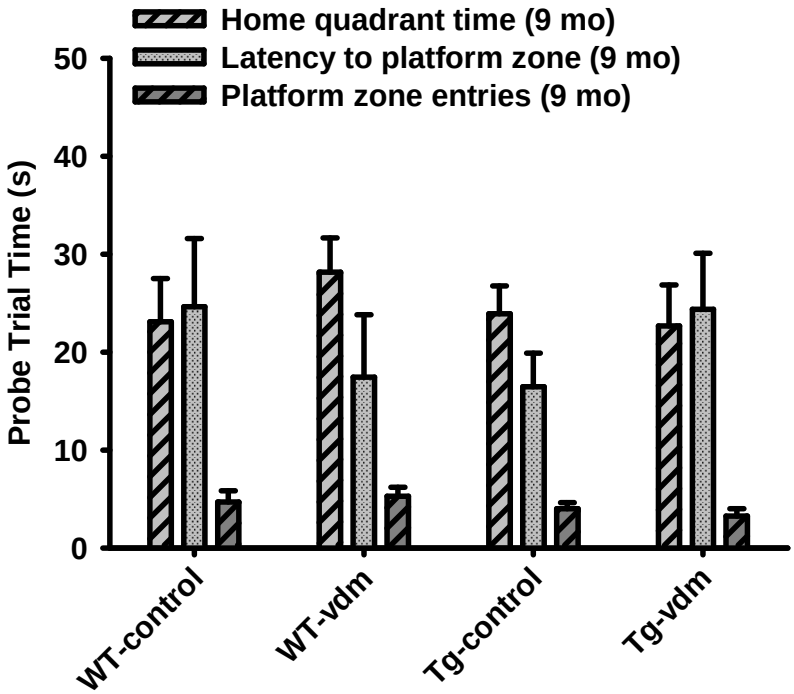


## Figure 11.

(a)

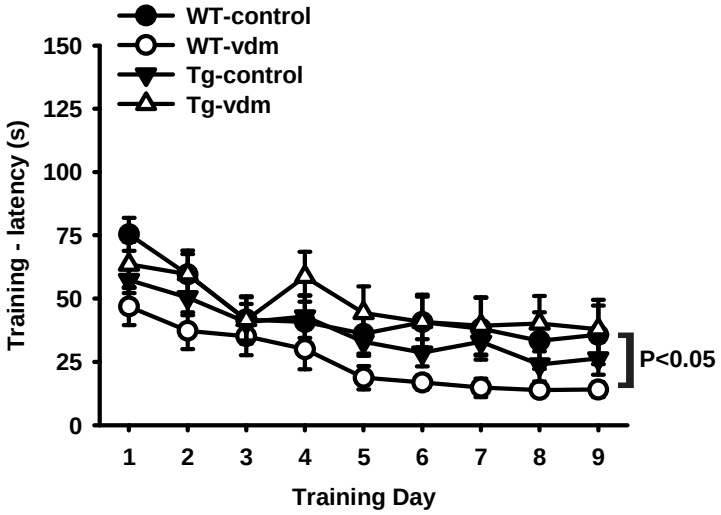

Home quadrant time (baseline)
Latency to platform zone (baseline)
Platform zone entries (baseline)
Probe Trial Time (s)
50
40
30
20
10
0
WT
Tg

(b)

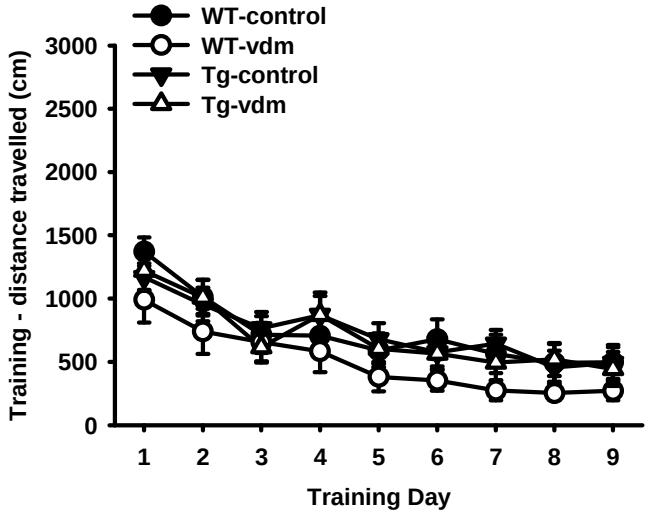

Home quadrant time (9 mo)
Latency to platform zone (9 mo)
Platform zone entries (9 mo)
Probe Trial Time (s)
50
40
30
20
10
0
WT-control
WT-vdm
Tg-control
Tg-vdm

## Figure 12.

(a)




(b)




(c)

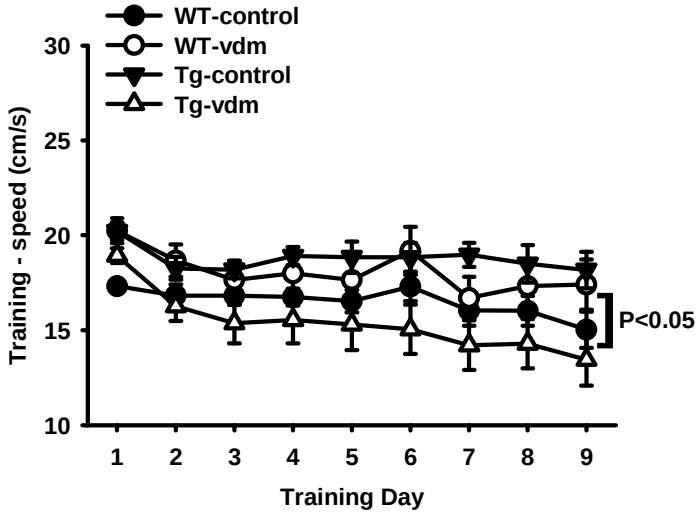


Figure 13.

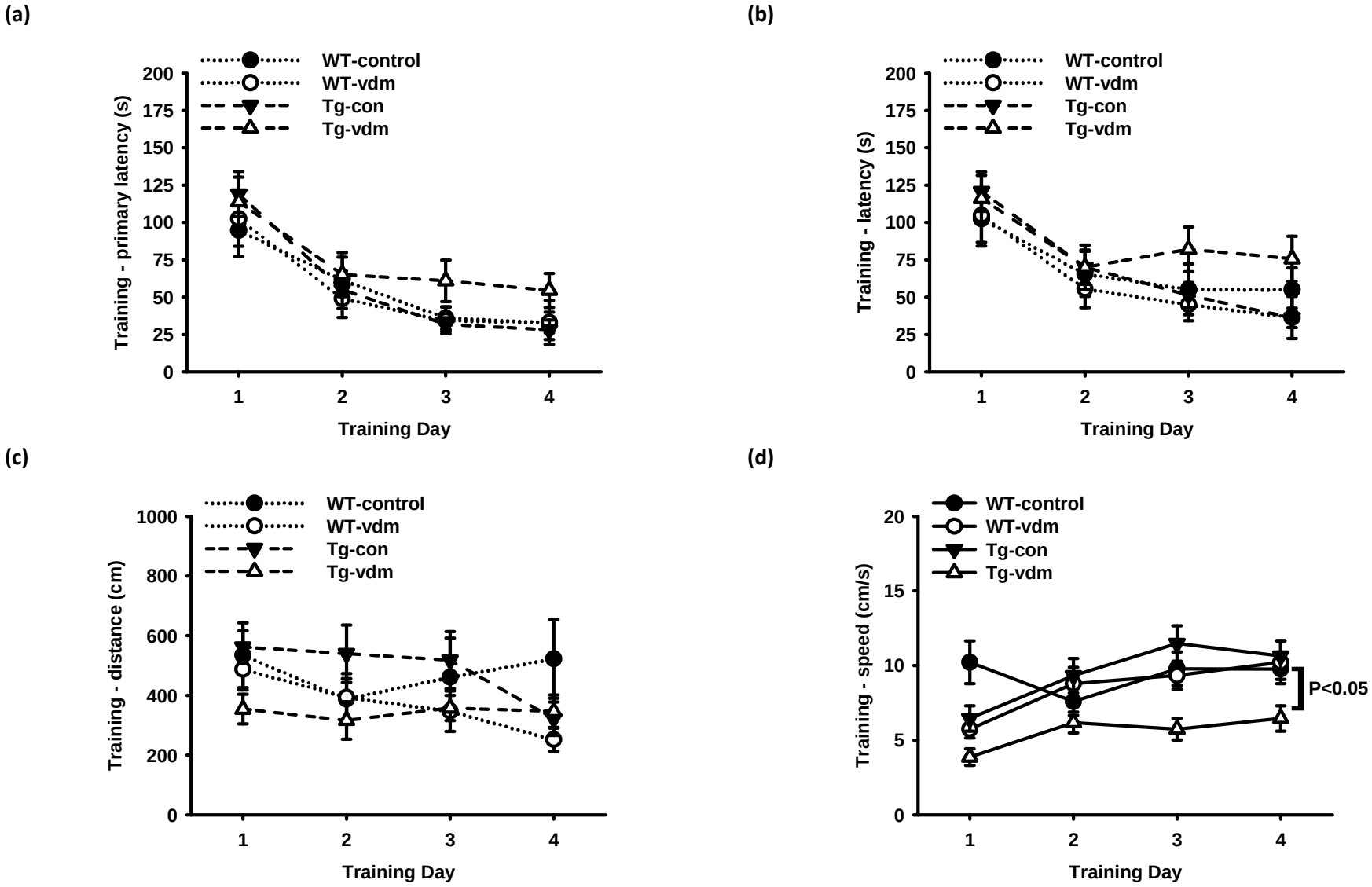
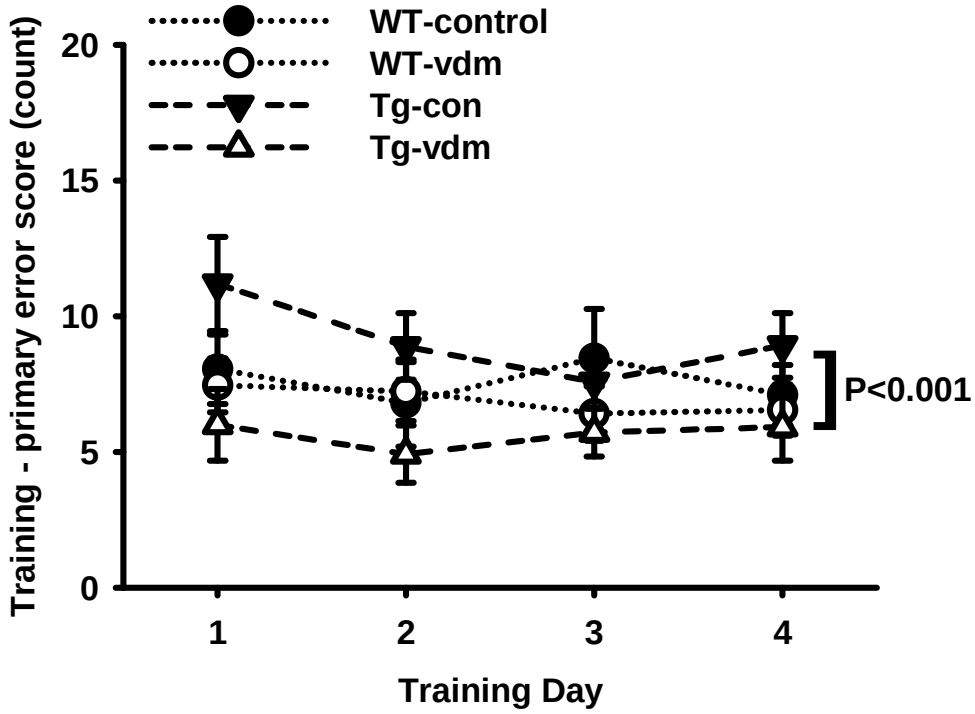

(a)
WT-control
WT-vdm
Tg-con
Tg-vdm
Training - primary latency (s)
Training Day
(b)
Training - latency (s)
(c)
Training - distance (cm)
(d)
Training - speed (cm/s)
P<0.05

Figure 14.

(a)


WT-control
WT-vdm
Tg-con
Tg-vdm
Training - primary error score (count)
20
15
10
5
0
1
2
3
4
Training Day
P<0.001

(b)

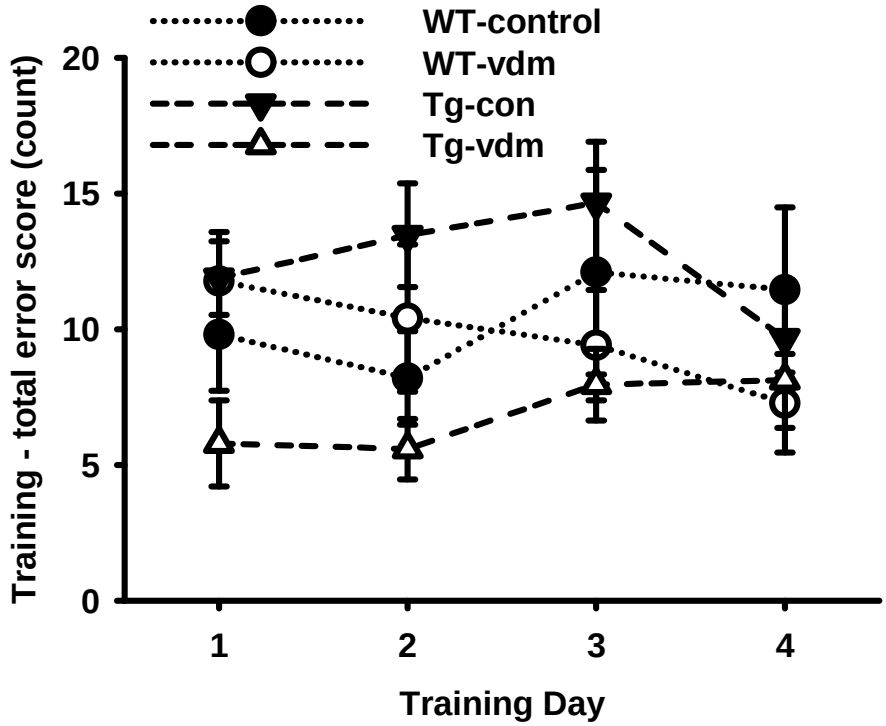
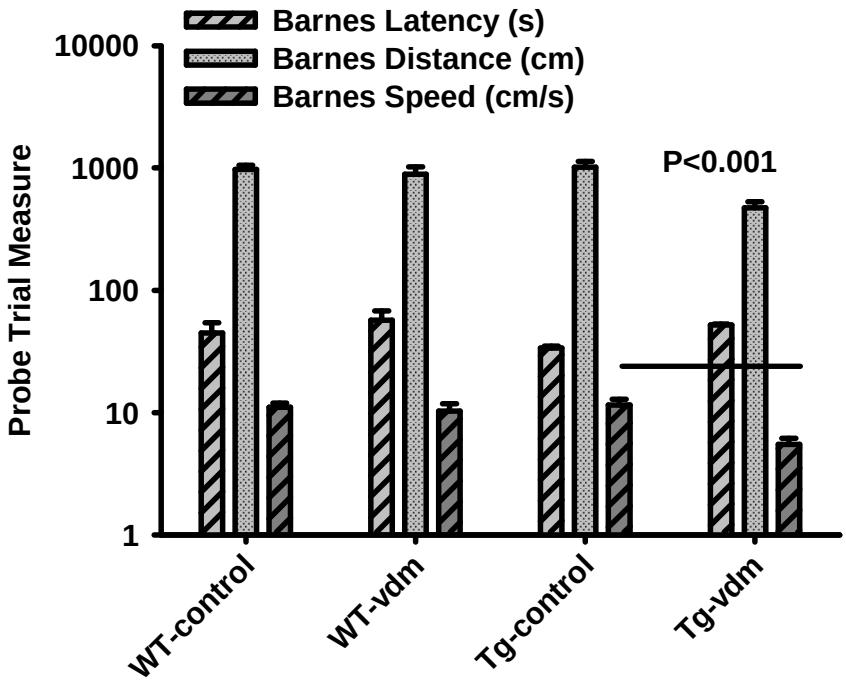

WT-control
WT-vdm
Tg-con
Tg-vdm
Training - total error score (count)
20
15
10
5
0
1
2
3
4
Training Day

Figure 15.

(a)


Barnes Latency (s)
Barnes Distance (cm)
Barnes Speed (cm/s)
Probe Trial Measure
10000
1000
100
10
1
P<0.001
WT-control
WT-vdm
Tg-control
Tg-vdm

(b)

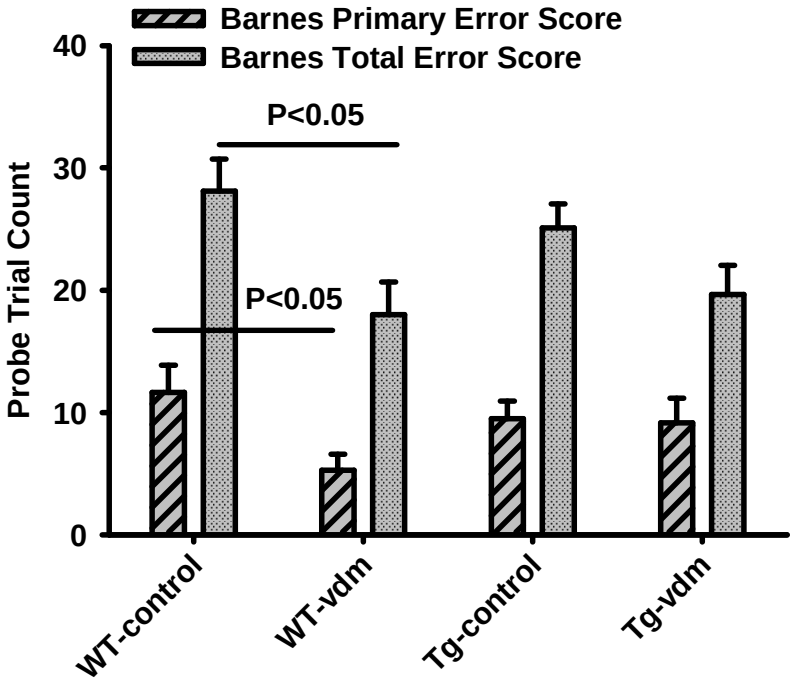
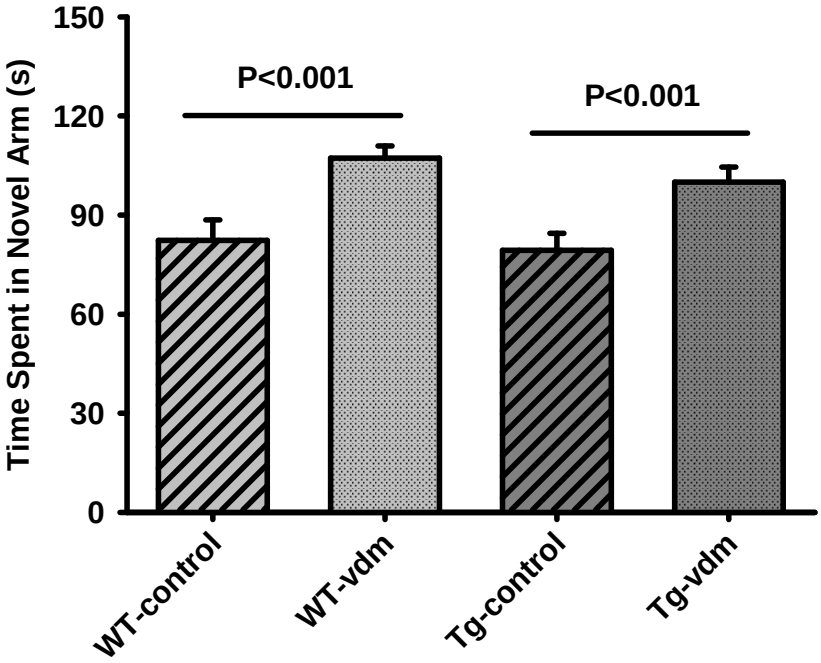
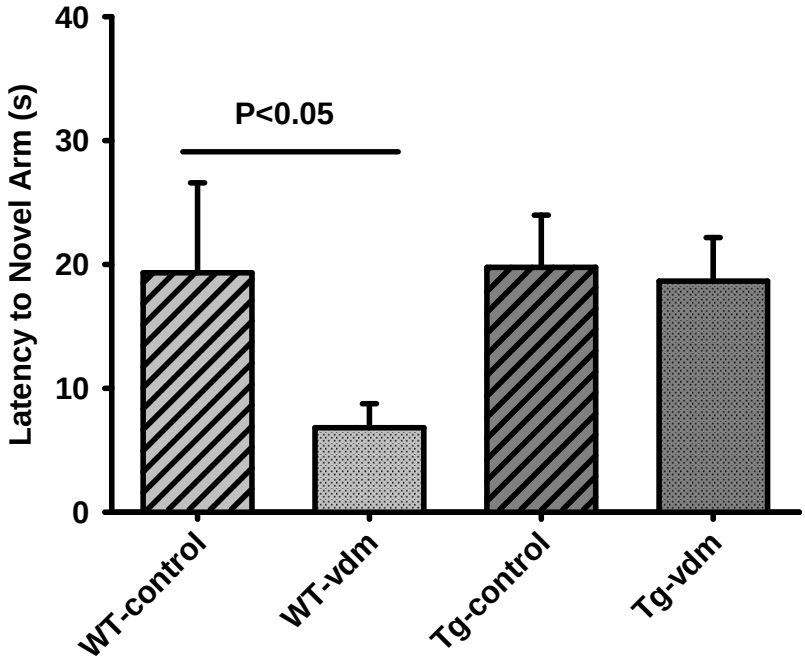

Barnes Primary Error Score
Barnes Total Error Score
Probe Trial Count
40
30
20
10
0
P<0.05
P<0.05
WT-control
WT-vdm
Tg-control
Tg-vdm

Figure 16.

(a)


150
120
90
60
30
0
Time Spent in Novel Arm (s)
P<0.001
P<0.001
WT-control
WT-vdm
Tg-control
Tg-vdm

(b)


40
30
20
10
0
Latency to Novel Arm (s)
P<0.05
WT-control
WT-vdm
Tg-control
Tg-vdm

# 11 Technology Transfer

Communication Activities

Poster presentation given at Alzheimer's Association International Conference, Vancouver, British Columbia, Canada, July, 2012.

**Title:** Vitamin D2 in mushroom is bioavailable and improves memory of wild type and $APP_{Swe}$/PS1dE9 transgenic mice

Invited oral presentation to be given at Benefiq 2012, International Rendezvous on Health Ingredients, Quebec, Canada, September, 2012.

**Title:** Vitamin D2-rich mushroom provides a bioavailable source of Vitamin D and improves memory of wild type and Alzheimer's Disease transgenic mice

Participation of Professor Manny Noakes and Dr Louise Bennett in a video interview arranged for Australian Mushroom Growers Communications by Chris Rowley.

2 manuscripts planned for submission to peer-reviewed journals arising from final report:

1. White Button mushrooms enriched in Vitamin D2 rescue memory impairment associated with Vitamin D deficiency in wild type and $APP_{Swe}$/PS1dE9 transgenic mice
2. Effects on inflammatory cytokines of supplementing Vitamin D-deficient wild type and $APP_{Swe}$/PS1dE9 transgenic mice with Vitamin D2-enriched White Button mushroom

3 manuscripts planned for submission to peer-reviewed journals arising from previous project, adapted from original single collective manuscript:

1. A heat-stable component of Wood Ear (*Auricularia polytricha*) mushroom inhibits *in vitro* activity of beta secretase
2. *In vitro* fibril inhibition properties of White Button and Shiitake mushrooms
3. *In vitro* anti-inflammatory properties of Wood Ear, White Button, Shiitake and Oyster mushrooms present potential neuroprotective role

# 12 Recommendations

## 12.1 Vitamin D2-rich mushrooms for maintaining healthy cognition in aging populations

The research outcomes justify progression of further research studies as described in the new proposal (MU12003), now approved for funding, and to commence in July, 2012. Objectives of the new project are:

1. to explore the relationship between vitamin D status and cognition by evaluating the comparative efficacy of Vitamin D-enriched mushrooms against agreed controls, for improving cognition, mood and depressive symptoms in healthy, aged people.
2. to undertake a staged research program that will provide for the preparation of at least 3 manuscripts suitable for submission to peer-reviewed journals, and to support other forms of public communication.

## 12.2 Developing versatile food ingredients from Vitamin D2-rich mushrooms

The industry should consider the potential competition from Vitamin D2 enrichment of Bakers and Brewers yeast and their potential uses in fortifying the large consumer product categories of bread and beer, respectively. Mushrooms appear to have an advantage at present based on a growing research evidence base however, can be expected to meet competition from other fungi-based food ingredients, which can also be enriched in Vitamin D.

The potentially unique neuroprotective properties of mushroom depend on determining if bioactive components other than Vitamin D2 are present. If so, then the development of food ingredients and dietary supplements may be justified, based on the 'package' of Vitamin D and other bioactives, for which the ratio of Vitamin D to non-Vitamin D bioactives can be controlled and optimised during processing. Non-Vitamin D bioactives in mushroom are expected to be distinct from those in yeasts.

A parallel project is therefore proposed that might be supported by a consortia of partners representing the commercial supply chain. The project could be conducted within a 12 month timeframe and would involve a further mouse feeding study to resolve effects of Vitamin D2-specific from non-Vitamin D2-related effects on memory, from White Button mushroom. The results would inform the decision to develop a range of versatile food ingredients from Vitamin D2-enriched mushrooms. Further elaboration of this project rationale and budget can be supplied on request.

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