THE UNIVERSITY OF QUEENSLAND

AUSTRALIA

The role of adult vitamin D deficiency in cognition and brain function in mice

Natalie Groves

Bachelor of Biomedical Science with First Class Honours

A thesis submitted for the degree of Doctor of Philosophy at

The University of Queensland in 2016

Queensland Brain Institute

Abstract

Vitamin D deficiency is prevalent throughout the world. Even in a temperate climate such as Australia, one-third of the adult population has insufficient levels of serum vitamin D (25-hydroxyvitamin D levels <50 nmol/L). Epidemiological studies have shown significant associations between vitamin D deficiency and an increased risk of various neuropsychiatric and neurodegenerative disorders, such as schizophrenia, depression, Alzheimer's disease and cognitive impairment. However, studies based on observational epidemiology cannot address questions of causality; they cannot determine if vitamin D deficiency is a causal factor leading to the adverse health outcome. The use of animal experiments can examine the biological plausibility of the relationship between vitamin D deficiency and adverse brain outcomes.

Vitamin D is a neurosteroid that has a wide range of functions within the brain including calcium maintenance, regulation of neurotrophic factors, neurogenesis, and neuroprotection. Using a model of adult vitamin D (AVD) deficiency in BALB/c mice, we have previously shown alterations in a range of behaviours and an imbalance between excitatory and inhibitory neurotransmission, which may be relevant to a range of neuropsychiatric disorders. Therefore, we have provided experimental evidence demonstrating that vitamin D deficiency in adulthood impacts on a range of brain functions, and is therefore a biologically plausible risk factor for the development of neuropsychiatric disorders. However, there is currently insufficient evidence to account for the mechanism(s) by which this occurs.

There were four main aims of the thesis; to determine whether AVD deficiency impacts on (1) cognition, (2) adult hippocampal neurogenesis, and (3) glutamate and GABA signaling in BALB/c mice; and to determine if AVD deficiency would (4) exacerbate the effects of a secondary exposure, in this case social stress, in BALB/c mice and in the more resilient C57BL/6J mice.

To address these aims I assessed attentional processing in BALB/c mice using the 5 choice serial reaction time task and found sex-dependent impairments in AVD-deficient male mice only. Structural and diffusion tensor imaging was assessed using MRI in male mice, however we found no significant alterations in gross brain structure or connectivity within the brain in the group exposed to AVD deficiency. I used immunohistological techniques to assess two measures of hippocampal neurogenesis, cell proliferation and survival of newborn neurons, and found that hippocampal neurogenesis was not affected by AVD deficiency at baseline or following wheel running stimulated neurogenesis.

Despite no cellular level change in neurogenesis, the analysis of the proteomics of the hippocampus, although preliminary, provided clues that AVD deficiency may have an effect on synapse formation and plasticity, and dendritic arborisation and that these changes may be responsible for impaired learning and memory. The proteomics also provided convergent evidence of an effect on glutamate and GABA signaling and an effect on glutathione synthesis that would lead to elevated levels of oxidative stress.

Based on the preliminary proteomics results, levels of glutathione were assessed in whole brain and showed that AVD-deficient mice had reduced glutathione levels, which would potentially leave them exposed to oxidative stress. I then used a specific hippocampal-dependent learning and memory task, active place avoidance, in male mice and showed that AVD-deficient mice took longer to learn the task than did controls. Although, AVD deficiency did not impact on numbers of newly born neurons, it is possible that the function of these neurons were altered through changes to dendritic arborisation, spine density or synaptic plasticity.

Finally, I examined a second-hit exposure, social stress, in both BALB/c and C57BL/6J strains to determine if AVD deficiency would exacerbate the effects of stress on behaviour. I found that AVD-deficient mice were more vulnerable to the effects of social stress using a social avoidance test, and this was dependent on strain.

Overall, the experiments covered in this thesis found that while AVD deficiency was not associated with changes in gross brain morphology or measures of adult hippocampal neurogenesis, we did find neurochemical alterations in glutathione synthesis, cognitive impairments, convergent evidence of an effect of AVD deficiency on glutamate and GABA signaling and preliminary proteomic results showing an effect of AVD deficiency on a number of proteins involved in synaptic plasticity.

Furthermore, the results from the two-hit study have provided evidence that vitamin D deficiency may exacerbate behavioural outcomes in mice vulnerable to stress, a finding that can help guide future studies. Importantly, the discoveries outlined in this thesis provide convergent evidence to support the epidemiological link between vitamin D deficiency and neuropsychiatric and neurodegenerative disorders; and has provided clues that can guide future studies related to unravelling the mechanisms of action linking adult vitamin D deficiency and adverse brain related outcomes.

Declaration by author

This thesis is composed of my original work, and contains no material previously published or written by another person except where due reference has been made in the text. I have clearly stated the contribution by others to jointly-authored works that I have included in my thesis.

I have clearly stated the contribution of others to my thesis as a whole, including statistical assistance, survey design, data analysis, significant technical procedures, professional editorial advice, and any other original research work used or reported in my thesis. The content of my thesis is the result of work I have carried out since the commencement of my research higher degree candidature and does not include a substantial part of work that has been submitted to qualify for the award of any other degree or diploma in any university or other tertiary institution. I have clearly stated which parts of my thesis, if any, have been submitted to qualify for another award.

I acknowledge that an electronic copy of my thesis must be lodged with the University Library and, subject to the policy and procedures of The University of Queensland, the thesis be made available for research and study in accordance with the Copyright Act 1968 unless a period of embargo has been approved by the Dean of the Graduate School.

I acknowledge that copyright of all material contained in my thesis resides with the copyright holder(s) of that material. Where appropriate I have obtained copyright permission from the copyright holder to reproduce material in this thesis.

Publications during candidature

Peer-reviewed publications

Groves, N.J., Bradford, D., Sullivan, R.K.P., Conn, K.A., Aljelaify, R.F., McGrath, J.J. & Burne, T.H.J (2016) Behavioural effects of adult vitamin D deficiency in BALB/c mice are not associated with proliferation or incorporation of adult born hippocampal neurons. *PLOS One*, 4(11)4, e0152328

Groves, N.J., Burne, T.H.J. (2016) Sex-specific attentional deficits in adult vitamin D deficient BALB/c mice. *Physiol Behav*, 157:94-101.

Cui, X., Gooch, H., **Groves, N.J.**, Sah, P., Burne, T.H., Eyles, D.W. & McGrath, J.J. (2014) Vitamin D and the brain: Key questions for future research. *J Steroid Biochem Mol Biol*, doi: 10.1016/j.jsbmb.2014.11.004.

Groves, N.J., McGrath, J.J. & Burne, T.H. (2014) Vitamin D as a Neurosteroid affecting the developing and adult brain. *Annu Rev Nutr*, 34:8.1-25.

Groves, N.J., Kesby, J.P., Eyles, D.W., McGrath, J.J., Mackay-Sim, A. & Burne, T.H. (2013) Adult vitamin D deficiency leads to behavioural and brain neurochemical alterations in C57BL/6J and BALB/c mice. *Behav Brain Res*, 241, 120-131.

Cui, X., **Groves, N.J.**, Burne, T.H., Eyles, D.W. & McGrath, J.J. (2013) Low vitamin D concentration exacerbates adult brain dysfunction. *Am J Clin Nutr*, doi: 10.3945/ajcn.113.061176.

Conference abstracts

Groves, N.J., Sullivan, R., Josh, P., McGrath, J. J. & Burne, T. H. J. (2015) Adult vitamin D deficiency does not alter adult neurogenesis but is associated with impaired performance on a hippocampal-dependent task. Proceedings of the Society for Neuroscience, 45. Poster Presentation.

Groves, N.J., McGrath, J. J. & Burne, T. H. J. (2015). Adult vitamin D deficiency is associated with impaired cognition, oxidative stress and vulnerability to second hit exposures. Proceedings of the Australian Neuroscience Society, 35. Poster Presentation.

Groves, N.J, McGrath, J.J. & Burne, T.H.J. (2014) Adult vitamin D deficiency exacerbated impairments caused by social stress in mice. Proceedings of Biological Psychiatry Australia. Poster Presentation.

Groves, N.J, Sullivan, R.K., McGrath, J. J. & Burne, T. H. J. (2014). Impact of adult vitamin D deficiency on hippocampal neurogenesis in BALB/c mice. Proceedings of the Australian Neuroscience Society, 34. Poster Presentation.

Groves, N.J, Eyles, D.W., McGrath, J.J. & Burne, T.H.J. (2013) Impaired attentional processing in adult vitamin D deficient BALB/c mice. Proceedings of Biological Psychiatry Australia. Poster Presentation.

Groves, N.J, Kesby, J.P., McGrath, J. J., Eyles, D. W., Mackay-Sim, A. and Burne, T. H. J. (2013). Adult vitamin D deficiency leads to behavioural and brain neurochemical alterations in C57BL/6J and BALB/c mice. Proceedings of the Australian Neuroscience Society, 33, 104. Poster Presentation.

Groves, N.J., Kesby, J.P., McGrath, J. J., Eyles, D. W., Mackay-Sim, A. and Burne, T. H. J. (2012). Adult vitamin D deficiency alters glutamatergic and GABAergic neurotransmission in BALB/c mice. Proceedings of Biological Psychiatry Australia. Poster Presentation.

Publications included in this thesis

Groves, N.J., McGrath, J.J. & Burne, T.H. (2014) Vitamin D as a Neurosteroid affecting the developing and adult brain. *Annu Rev Nutr*, 34:8.1-25 – incorporated as Chapter 1.

Contributor	Statement of contribution
Author Natalie Groves (Candidate)	Wrote the paper (70%)
Author John McGrath	Edited paper (10%)
Author Tom Burne	Edited paper (20%)

Groves, N.J., Burne, T.H.J. (2016) Sex-specific attentional deficits in adult vitamin D deficient BALB/c mice. *Physiol Behav*, 157:94-101 – incorporated as Chapter 2.

Contributor	Statement of contribution
Author Natalie Groves (Candidate)	Designed experiments (70%)

	Wrote the paper (80%)
Author Tom Burne	Designed experiments (30%) Edited paper (20%)

Groves, N.J., Bradford, D., Sullivan, K.P., McGrath, J.J. & Burne, T.H. (2016) Behavioural effects of adult vitamin D deficiency in BALB/c mice are not associated with proliferation or incorporation of adult born hippocampal neurons. *PLOS One* (Under review) – incorporated as Chapter 4.

Contributor	Statement of contribution
Author Natalie Groves (Candidate)	Designed experiments (70%) Performed behavioural experiments (100%) Helped with immunohistochemical experiments (30%) Performed microscopy and cell counts (100%) Analysis of data (90%) Wrote the paper (70%)
Author DanaKai Bradford	Helped edit paper for publication
Author Robert Sullivan	Helped with immunohistochemistry
Author John McGrath	Edited paper (10%)
Author Tom Burne	Designed experiments (30%) Analysis of data (10%) Edited paper (20%)

Contributions by others to the thesis

Associate Professor Tom Burne, as my principal supervisor, supported me throughout my PhD with design and supervision of my projects and was the prevalent contact person for the interpretation of obtained data. Professor John McGrath, as my associate supervisor, supported me during my PhD with writing and editing support. Dr. Nyoman Kurniawan was crucially involved in the experimental process and data analysis for Chapter 3. Dr. Robert Sullivan, Jane Ellis, Luke Hammond and Suzy Alexander assisted me with the immunohistochemistry and microscopy for Chapter 4. DanaKai Bradford helped me with the editing of Chapter 4 for publication. Peter Josh was essential for the proteomic experiment for Chapter 5 and the HPLC glutathione assay in Chapter 6. Michelle Sanchez and Suzy Alexander assisted with behavioural experiments in Chapter 2 and Chapter 7, respectively. Suzy Alexander assisted with tissue collection and perfusions. Henry Simila assayed blood samples for 25(OH)D levels.

Statement of parts of the thesis submitted to qualify for the award of another degree

None

Acknowledgements

I would like to express my appreciation to everyone who has supported me throughout my PhD and contributed to the development of this thesis. First and foremost, I would like to thank my principal supervisor, Associate Professor Tom Burne, who has always gone above and beyond in his encouragement, support, and teaching. Tom has been instrumental in my development as a behavioural neuroscientist and researcher. He has taught me how to develop and express ideas, to challenge the way I think, to develop research questions and design experiments to address them properly, and finally to cultivate my writing skills.

I would also like to express my gratitude towards my associate supervisor, Professor John McGrath, who has always believed in my abilities as a researcher and critical thinker, even when I was unsure myself. John was always willing to provide a listening ear, encouragement and advice when needed, and has provided invaluable help in the writing process.

To all the members of the lab, it has been a great experience being part of such an amazing and supportive group. Thank you to Pauline Ko and Henry Simila, who manage the lab and help with travel arrangements. In particular, I want to thank Suzy Alexander for all her help with my animals and for being a supportive co-worker and friend. A special thank you goes to Karly Turner and Emilia Lefevre, who have also been on their own PhD journeys. They have been awesome companions and supportive friends, and have helped keep me sane and on track.

I want to thank the staff of the animal house facilities for their constant care of the animals, Rob Sullivan and Jane Ellis for help with all things histology and Luke Hammond for help with the microscopes. I wish to thank Dr Nyoman Kurniawan for his help with the MRI study, Michelle Sanchez for help with the behavioural experiment for Chapter 2, Peter Josh for his work on the iTRAQ proteomics and the glutathione HPLC assay and Dana Bradford for helping with the manuscript for Chapter 4. I would also like to acknowledge the financial support I obtained from the Australian Postgraduate Award scholarship, as well as the top-up scholarships I received from both QBI and the lab.

My friends and family have been wonderfully supportive of my PhD journey. In particular, I want to thank my parents, Tony and Desley Ingram who have helped out whenever they can and my sister, Tammy Sooveere, who proofread my thesis at the final hour. Special thanks goes to Phil Christensen, who has put up with all my neurotic stress, late nights and weekend work, and provided never-ending positive affirmations. Finally, to my wonderful children, Alyssa and Riley Groves, who I dedicate this thesis to; I want to thank you for being so understanding and patient.

Keywords

Animal models, behaviour, vitamin D, cognition, glutathione, proteomics, BALB/c, adult mice, MRI, neurogenesis

Australian and New Zealand Standard Research Classifications (ANZSRC)

ANZSRC code: 110903, Central Nervous System, 70%

ANZSRC code: 060103, Cell Development, Proliferation and Death, 10%

ANZSRC code: 060109, Proteomics and Intermolecular Interactions, 20%

Fields of Research (FoR) Classification

FoR code: 1109, Neurosciences, 70%

FoR code: 0601, Biochemistry and Cell Biology, 30%

Table of Contents

List of Figures

List of Tables

List of Abbreviations used in the thesis

% Cov	% Coverage with 95% confidence intervals
$1,25(OH)_2D$	1,25-dihydroxyvitamin D
25(OH)D	25-hydroxyvitamin D
5C-CPT	5 choice – continuous performance test
5C-SRT	5 choice – serial reaction time
6-OHDA	6-hydroxydopamine
Aβ	Amyloid β
AD	Alzheimer's disease
ANOVA	Analysis of variance
ANTs	Advanced normalisation tools
APA	Active place avoidance
APP	Amyloid beta A4 protein OR amyloid precursor protein
ASR	Acoustic startle response
AVD	Adult vitamin D
AxialD	Axial diffusivity
BDNF	Brain derived neurotrophic factor
BrdU	5-Bromo-2'-Deoxyuridine
CACNA1E	Voltage-dependent R-type calcium channel subunit alpha-1E
CYP24A1	24-hydroxylase
CYP27B1	1α hydroxylase
DABCO	1,4-diazabicyclo[2.2.2]octane
DTI	Diffusion tensor imaging
DVD	Developmental vitamin D
DWI	Diffusion-weighted images
E	Embryonic day
EAE	Experimental autoimmune encephalomyelitis

FA	Fractional anisotropy
FC	Fold change
FST	Forced swim test
GABA	γ-aminobutyric acid
GAD	Glutamic acid decarboxylase
GAT1	GABA membrane transporter 1
GDNF	Glial cell line-derived neurotrophic factor
HPLC	High performance liquid chromatography
HVA	Homovanillic acid
IGF-1	Insulin-like growth factor 1
IL	Interleukin
iNOS	Inducible nitric oxide synthesis
IPA	Ingenuity Pathway Analysis
iTRAQ	Isobaric tag for relative and absolute quantification
LTD	Long-term depression
LTP	Long-term potentiation
L-VGCC	L-type voltage-gated calcium channel
L-VSCC	L-type voltage-sensitive calcium channel
MD	Mean diffusivity
MECP2	Methyl-CpG-binding protein 2
MK-801	Dizocilpine
MMSE	Mini-mental state examination
MRI	Magnetic resonance imaging
MS	Multiple sclerosis
NeuN	Neuronal nuclei
NGF	Nerve growth factor
NGS	Normal goat serum
NMDA	*N*-methyl-D-aspartate

NO	Nitric oxide
NT-3	Neurotrophin 3
NT-4	Neurotrophin 4
$p75^{NTR}$	Low affinity neurotrophin receptor
PBS	Phosphate buffered saline
PBS azide	Sodium azide (0.05%) in 0.1M phosphate buffered saline
PD	Parkinson's disease
PFA	Paraformaldehyde
PFC	Prefrontal cortex
PPFIA3	Liprin alpha 3
PPI	Prepulse inhibition
PV	Parvalbumin
RadialD	Radial diffusivity
RASGRF1	Ras-specific guanine nucleotide-releasing factor 1
SEP	Separated housing
DEF	Social Defeat
TBSS	Tract-based spatial statistics
TH	Tyrosine hydroxylase
TNFα	Tumor necrosis factor-α
UVB	Ultraviolet B
VAMP3	Vesicle-associated membrane protein 3
VBM	Voxel based morphometry
VDR	Vitamin D receptor
VDREs	Vitamin D response elements
VGLUT1	Vesicular glutamate transporter 1
WMH	White matter hyperintensities
WSH	Within-session habituation

Chapter 1 General Introduction

1.1 Introduction

Vitamin D deficiency is prevalent throughout the world, particularly in high-risk groups including pregnant woman, infants, dark-skinned migrants and the elderly (Lips, 2010). In Australia, a recent study showed that 31% of the population has vitamin D deficiency, which is defined as a serum concentration of 25-hydroxyvitamin D [25(OH)D] below 50 nmol/L (Daly *et al.*, 2012). Although vitamin D is essential for calcium homeostasis and bone metabolism, it also has a role in other physiological functions, such as an immune modulator (Baeke *et al.*, 2010) and in cell proliferation and differentiation (Bikle, 2009). Research over the past 15 years has revealed many functions of vitamin D in brain development and adult brain function (Eyles *et al.*, 2011). More recently, evidence has accumulated to suggest that low vitamin D levels during adulthood may also be associated with adverse brain-related outcomes. A growing body of evidence from epidemiology and neuroscience links vitamin D deficiency with a range of neuropsychiatric disorders and neurodegenerative diseases (Eyles *et al.*, 2013). If low vitamin D is associated with adverse brain outcomes, this could have important public health implications because the treatment of vitamin D insufficiency (i.e. supplementation) is safe, cheap and publicly acceptable.

This review has three broad aims: (*a*) to summarise the role vitamin D plays within the healthy developing and adult brain, (*b*) to highlight the impact that vitamin D deficiency has on brain function in health and disease, and (*c*) to provide up-to-date evidence supporting the links between vitamin D deficiency and neuropsychiatric disorders and neurodegenerative diseases.

1.1.1 Vitamin D synthesis

Vitamin D is synthesized from 7-dehydrocholesterol within the skin via UVB radiation. For a review of the major pathway for the synthesis of vitamin D, see Figure 1.1. The amount of synthesis is dependent on a wide range of factors including latitude, season, atmospheric conditions, skin pigmentation and age; as well as personal habits including type of attire worn during exposure to sunlight (Holick, 1995), sunscreen use, and time spent outdoors (Chen *et al.*, 2007). Importantly, at high latitudes during winter and spring months, it is not possible to synthesize sufficient vitamin D from sunlight, and therefore dietary intake and supplementation are vital to maintain adequate levels of vitamin D (Engelsen *et al.*, 2005).

The conversion of 7-dehydrocholesterol in the skin of humans and animals forms vitamin D_3, whereas the conversion of ergosterol in plants, yeast and fungi forms vitamin D_2 (Lehmann & Meurer, 2010). Both forms can be converted to the biologically active vitamin, but they may not

have equal nutritional value in people (Heaney *et al.*, 2011), although this is likely to vary in different species, such as rodents (Horst *et al.*, 1982; Hohman *et al.*, 2011). Vitamin D_3 is reported to be 87% more potent in raising and maintaining serum 25(OH)D levels compared to vitamin D_2 in people, and it provides two- to threefold greater storage capacity of vitamin D in adipose tissue (Heaney *et al.*, 2011). Additionally, vitamin D_2 supplementation may even suppress endogenously formed vitamin D_3 (Stephensen *et al.*, 2012).

Vitamin D is converted to its biologically active form via two enzymatic steps, the first of which occurs in the liver. Vitamin D is hydroxylated to 25(OH)D via either the microsomal (CYP2R1) or the mitochondrial (CYP24A1) P450 25-hydroxylase enzymes (Schuster, 2011). 25(OH)D is then converted to the biologically active, 1,25-dihydroxyvitamin D [$1,25(OH)_2D$] in the kidney via 1α-hydroxylase (CYP27B1). 1α-hydroxylase is tightly controlled via feedback mechanisms from parathyroid hormone, calcium, phosphate, calcitonin, fibroblast growth factor 23, and vitamin D itself (Lehmann & Meurer, 2010).

Although the main expression of 1α-hydroxylase is within the kidney, a variety of other tissues also express this enzyme, including the skin, immune cells, placental tissue, and pancreas (Zehnder *et al.*, 2001; Eyles *et al.*, 2005). The presence of extra-renal 1α-hydroxylase suggests that an autocrine/paracrine mechanism plays a role in localized effects of vitamin D (Hewison *et al.*, 2007).

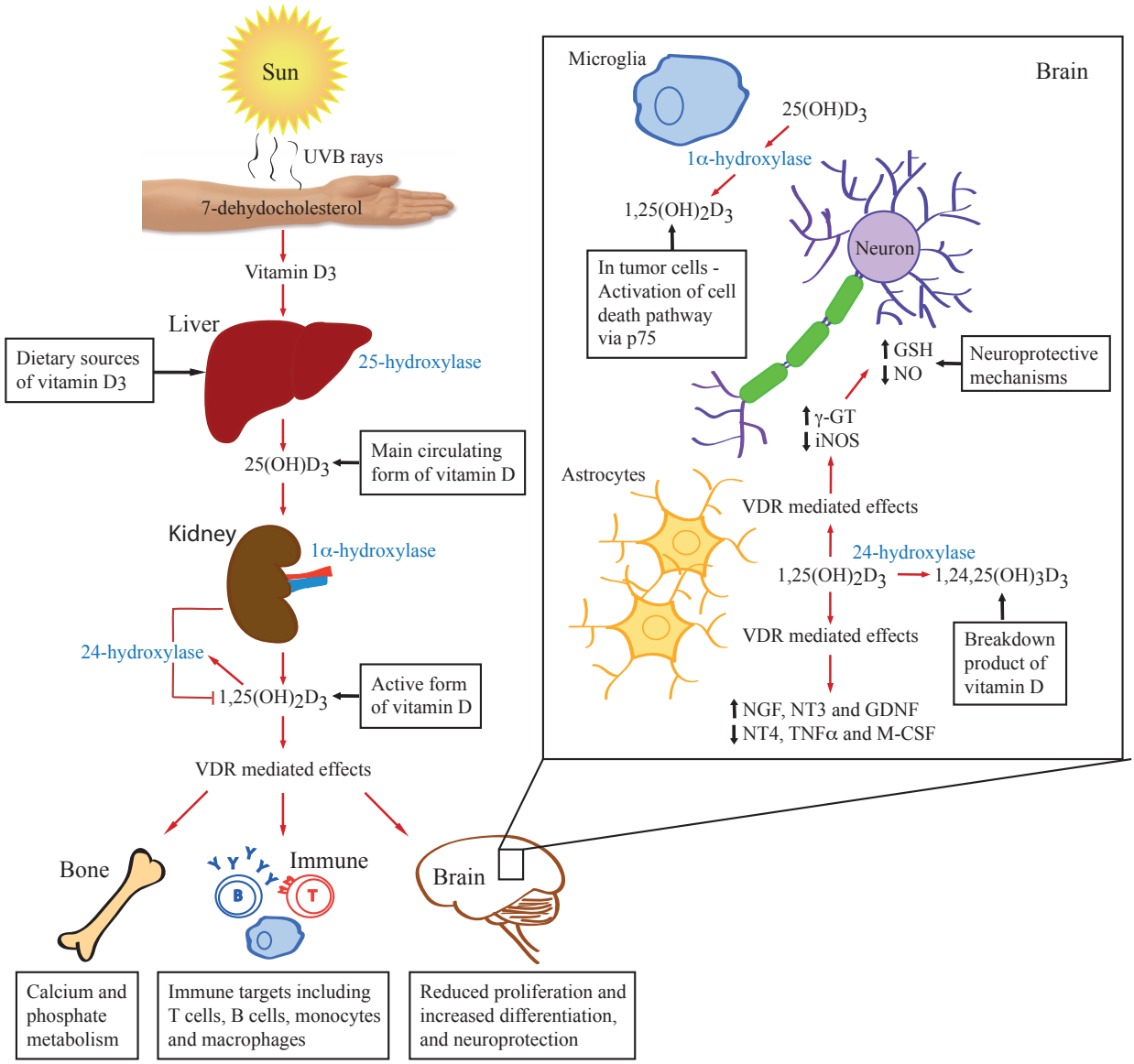

Figure 1.1 Synthesis, localisation and overview of known functions of vitamin D
The major pathway for the production of $1,25(OH)_2D$ is via UVB radiation of 7-hydrocholesterol in the skin, followed by two further hydroxylation steps in the liver and kidney respectively. Local production of the active form of vitamin D can be produced in other locations throughout the body including the brain. $1,25(OH)_2D$ has been shown to have a wide array of functions including calcium and bone homeostasis and immune function. There is also evidence for functional effects of $1,25(OH)_2D$ in the regulation of cell proliferation and differentiation as well as a role in neuroprotection. Within astrocytes, $1,25(OH)_2D$ regulates the synthesis of various neurotrophins and can upregulate γ-GT to increase GSH levels and inhibit iNOS to limit the production of NO, resulting in neuroprotection for neurons. Finally, there is also evidence that $1,25(OH)_2D$ can activate cell death pathways in tumour cells via p75. Abbreviations: NGF, nerve growth factor; NT3, neurotrophin 3; GDNF, glial cell line-derived neurotrophic factor; γ-GT, γ-glutamyl transpeptidase; NT4, neurotrophin 4; TNFα, tumour necrosis factor alpha; M-CSF, macrophage colony-stimulating factor; GSH, glutathione; iNOS, inducible nitric oxide synthase; NO, nitric oxide; p75, low affinity NGF receptor; VDR, vitamin D receptor. Figure modified from Garcion *et al.* (2002); Hart *et al.* (2011).

1.1.2 Genomic versus nongenomic pathways of action

Vitamin D exerts its effects via both genomic and nongenomic pathways (Bouillon *et al.*, 2008). The genomic pathway begins with vitamin D binding to the vitamin D receptor (VDR), which is a member of the steroid/thyroid superfamily of nuclear transcription factors (Tsai & O'Malley, 1994). VDR is present throughout the body in almost all tissue types (Eyles *et al.*, 2005; Barchetta *et al.*, 2012; Wang *et al.*, 2012b; Girgis *et al.*, 2013). Once vitamin D is bound to the receptor, VDR is phosphorylated to induce a change in conformation to release co-repressors and allow VDR to heterodimerise with the retinoid X receptor (Bouillon *et al.*, 2008). This heterodimer then recruits coregulatory protein complexes and binds one of many vitamin D response elements (VDREs) within the genome to influence gene transcription. The VDRE is composed of two hexameric binding sites on DNA, arranged as either direct repeats interspaced with a small but varying number of nucleotides or as inverted palindromes interspaced by nine nucleotides (Schrader *et al.*, 1997; Haussler *et al.*, 1998).

The ability of this heterodimer to influence gene transcription is dependent upon the range of co-regulatory protein complexes, such as steroid receptor co-activators and VDR-interacting protein, which determines whether repression or activation occurs (Fernandes de Abreu *et al.*, 2009). The recently established genome-wide map of VDR binding identified over 2,700 genomic positions occupied by the VDR, showing the pleiotropic nature of vitamin D (Ramagopalan *et al.*, 2010).

Like other neurosteroid hormones, vitamin D initiates nongenomic rapid responses via either a membrane-bound VDR (Menegaz *et al.*, 2011) or a protein-disulfide isomerase-associated 3 protein (PDIA3) (Chen *et al.*, 2010). The variety of signal transduction systems that are rapidly activated by vitamin D include influx of calcium; intracellular release of calcium stores; modulation of adenylate cyclase, phospholipase C, and protein kinases; and alteration of the phosphorylation states of cellular proteins (Falkenstein *et al.*, 2000; Chen *et al.*, 2010).

Rapid nongenomic effects of vitamin D may therefore play a role in a variety of cellular processes, with evidence supporting the role of vitamin D in proliferation and immune function (Khanal & Nemere, 2007). Importantly, both the VDR and PDIA3 receptors are present in the adult brain (Eyles *et al.*, 2007; Pendyala *et al.*, 2012).

1.1.3 Vitamin D metabolism in the brain

The activating enzyme of vitamin D, 1α-hydroxylase, is found in a wide variety of tissues throughout the body, including the brain (Zehnder *et al.*, 2001; Eyles *et al.*, 2005), along with 25-hydroxylase (Garcion *et al.*, 2002) and the enzyme required for the degradation of the biologically

active form of vitamin D, 24-hydroxylase (CYP24A1) (See Figure 1.1) (Balden *et al.*, 2012). Animal studies have shown that the VDR is also found within specific brain regions, including the hippocampus, amygdala, hypothalamus, thalamus, cortex, and cerebellum (Prufer *et al.*, 1999; Walbert *et al.*, 2001).

The distribution of the VDR and 1α-hydroxylase has also been elucidated in the adult human brain and is similar to that found in the rat (Eyles *et al.*, 2005). The VDR and the 1α-hydroxylase enzyme are co-localized and are found in both neurons and glial cells. The VDR seems to be exclusively nuclear in mature neurons, whereas 1α-hydroxylase is located within the cytosol (Eyles *et al.*, 2005). One recent study showed some immunohistochemical staining of VDR in the soma of mature dopaminergic cells; however, western blots confirmed that the VDR was restricted to the nucleus of both the developing and mature midbrain (Cui *et al.*, 2013b). In contrast, previous studies have shown that unliganded VDR constantly shuttles between nucleus and the cytoplasm (Prufer & Barsony, 2002).

The VDR is present early in the development of the rat, between embryonic day (E)12 and E15, with levels of VDR increasing until weaning (postnatal day 21) and still present in the adult brain (Veenstra *et al.*, 1998; Burket *et al.*, 2003; Cui *et al.*, 2013b). The time-dependent expression of VDR in the brain during fetal development supports a role for vitamin D in brain development. The initial VDR expression in brain corresponds within the appearance of dopaminergic neurons within the mesencephalon. Additionally, VDR is present within dopaminergic neurons in the adult substantia nigra (Cui *et al.*, 2013b).

1.2 The actions of vitamin D within the brain

Growing evidence shows that vitamin D has many functions in both the developing and adult brain, including maintaining calcium balance and signaling, regulating neurotrophic factors, providing neuroprotection, modulating neurotransmission, and contributing to synaptic plasticity.

1.2.1 Vitamin D and calcium signaling within the brain

A high level of calcium in the brain leads to neurotoxicity, and one action of vitamin D within the brain is associated with a reduction in calcium levels. Vitamin D has been shown to downregulate or modulate L-type voltage-gated calcium channels (L-VGCCs) (Zanatta *et al.*, 2012; Zhu *et al.*, 2012). This occurs through downregulation of L-type voltage-sensitive calcium channel (L-VSCC)-A1C subunit mRNA and protein, mediated by VDR mechanisms (Gezen-Ak *et al.*, 2011). Vitamin D treatment has also been shown to downregulate L-VSCC-A1D subunit mRNA, but this does not

occur via VDR (Gezen-Ak *et al.*, 2011). In mice lacking vitamin D, L-VGCCs are shown to be upregulated, leading to increased calcium influx (Zhu *et al.*, 2012). Evidence suggests that vitamin D can directly provide neuroprotection against excitotoxic insults in vitro by its downregulation of L-VGCCs (Brewer *et al.*, 2001).

Vitamin D also regulates the gene expression of a number of calcium-binding proteins, including parvalbumin and calbindin D28k (Van Cromphaut *et al.*, 2001; Dursun *et al.*, 2011), and proteins associated with calcium homeostasis (Eyles *et al.*, 2007). The evidence suggests that the effects of vitamin D on calcium occur via both genomic and nongenomic actions (Eyles *et al.*, 2007; Nemere *et al.*, 2012; Zanatta *et al.*, 2012).

1.2.2 Neurotrophic properties

The first evidence to support the role of vitamin D in neuronal differentiation, maturation, and growth came from in vitro studies showing that treatment with vitamin D led to changes in several neurotrophic factors, with increased synthesis of nerve growth factor (NGF) (Wion *et al.*, 1991; Neveu *et al.*, 1994b), glial cell line-derived neurotrophic factor (GDNF) (Naveilhan *et al.*, 1996b) and neurotrophin 3 (NT-3), and with decreased synthesis of NT-4 (Neveu *et al.*, 1994a). Vitamin D treatment was also shown to increase levels of the low-affinity neurotrophin receptor ($p75^{NTR}$) in vitro (Naveilhan *et al.*, 1996a). Depletion of vitamin D during rat fetal development leads to a reduction in NGF, GDNF and $p75^{NTR}$ in newborn pups (Eyles *et al.*, 2003).

NGF is essential for the survival and differentiation of sensory and sympathetic neurons as well as the cholinergic basal forebrain neurons (Korsching *et al.*, 1985) and GDNF is integral to the development of dopaminergic (Tomac *et al.*, 1995) and noradrenergic systems (Quintero *et al.*, 2004). Although NT-3 stimulates the production of neurons and has widespread effects on their function and survival (Maisonpierre *et al.*, 1990), the $p75^{NTR}$, along with NGF, is essential for necessary programmed cell death in the brain (Chao, 1994).

Initial in vitro work in several cancer cell lines including mouse myeloid leukemia cells and melanoma cells, showed that the addition of vitamin D inhibited cell growth, led to a reduction in proliferation and increased differentiation (Pols *et al.*, 1990). Vitamin D's ability to induce differentiation was shown to be extended to normal bone marrow progenitor cells in vitro (Miyaura *et al.*, 1982), and its antiproliferative effects were confirmed in vivo against malignant cancers (Eisman *et al.*, 1987). Furthermore, the addition of vitamin D to cultured embryonic hippocampal cells was shown not only to increase NGF but also to increase neurite outgrowth and decrease mitosis (Brown *et al.*, 2003).

1.2.3 Neuroprotection

Evidence indicates that vitamin D provides neuroprotection by regulating NGF and GDNF. In vitro studies have shown that NGF protects against glutamate toxicity and calcium ionophore and nitric oxide (NO) donor toxicity (Kume *et al.*, 2000). Animal studies have shown that NGF is able to protect against excitotoxic injury (Frim *et al.*, 1993), and in an animal model of Parkinson's disease, GDNF is neuroprotective against ischemia (Wang *et al.*, 2002b), 6-hydroxydopamine (6-OHDA) toxicity (Kearns *et al.*, 1997) and injury (Gash *et al.*, 1998).

Vitamin D itself has also been shown to provide neuroprotection against excitotoxic injury from 6-OHDA, both in vitro and in vivo (Wang *et al.*, 2001), which may occur by its downregulation of L-VGCCs (Brewer *et al.*, 2001). Pretreatment with vitamin D ameliorated the locomotor deficits seen with 6-OHDA lesions into the medial forebrain bundle. In addition, pretreatment protected against 6-OHDA-mediated depletion of dopamine and metabolites within the substantia nigra (Wang *et al.*, 2001). Injection of vitamin D into adult rats leads to an increase in GDNF mRNA and protein expression within the striatum (Sanchez *et al.*, 2002), and pretreatment with vitamin D has also been shown to significantly increase GDNF protein expression and tyrosine hydroxylase (TH) immunoreactivity in the substantia nigra after 6-OHDA-lesioning (Sanchez *et al.*, 2009).

It is well known that vitamin D has an effect on the immune system and directly affects immune cells (Mora *et al.*, 2008). Within the CNS, vitamin D exerts immunomodulatory effects directly on infiltrating macrophages and parenchymal microglia (Nataf *et al.*, 1996). Treatment of microglia in vitro with vitamin D inhibits the production of tumor necrosis factor-α (TNFα), interleukin-6 (IL-6), and NO by activated microglia, which suggests an anti-inflammatory role for vitamin D within the brain (Lefebvre d'Hellencourt *et al.*, 2003). Vitamin D has been shown to downregulate the expression of inducible nitric oxide synthesis (iNOS) (Garcion *et al.*, 1998) and to regulate the expression of gamma glutamyl transpeptidase (Garcion *et al.*, 1999), an enzyme important in the glutathione pathway; these findings suggest that vitamin D has an important role in antioxidant metabolism. Vitamin D-deficient aged (22-month-old) male Fischer 344 rats have elevated inflammatory proteins in the brain, including TNFα and IL-6, indicating that baseline brain inflammation may be increased even without injury (Cekic *et al.*, 2011). Furthermore, in middle-aged (9-11-month-old) female Sprague-Dawley rats, a vitamin D-deficient diet produced elevated IL-6 levels within the brain and reduced insulin-like growth factor 1 (IGF-1) levels within plasma and brain following ischemia (Balden *et al.*, 2012).

A recent animal study showed elevated levels of vitamin D metabolism enzymes, VDR, and 25(OH)D in the hippocampus following a chronic unpredictable mild stress paradigm in rats when

compared to controls (Jiang *et al.*, 2013). Vitamin D is known to be neuroprotective, and therefore it may be that upregulation of vitamin D can protect against the damaging effects of stress within otherwise-healthy subjects.

1.2.4 Neurotransmission

Vitamin D has been shown to regulate a number of neurotransmitter systems. For example, vitamin D treatment leads to increased choline acetyltransferase activity in specific brain regions, which may impact on cholinergic neurotransmission (Sonnenberg *et al.*, 1986). In rats, vitamin D has been shown to protect against methamphetamine-induced reductions in dopamine and serotonin in both the striatum and accumbens (Cass *et al.*, 2006). Vitamin D treatment has also been shown to increase both potassium- and amphetamine-evoked overflow of striatal dopamine as well as increase substantia nigra tissue levels of dopamine and its main metabolites (Cass *et al.*, 2012).

Not only can vitamin D act to transiently alter neurotransmitters upon exposure, but there is also evidence to suggest that hormonal imprinting that occurs during the neonatal period permanently alters biogenic amine levels in adulthood. For example, male rats treated with vitamin D at birth showed altered brain stem dopamine and striatal and hypothalamus homovanillic acid (HVA) levels three months later (Tekes *et al.*, 2009a). In addition, hormonal imprinting with vitamin D that occurred during the neonatal period in female rats was shown to alter biogenic amine levels in two-month-old offspring. Alterations included increased norephinephrine, dopamine, and serotonin levels in the brainstem; decreased serotonin levels in the hippocampus; and decreased serotonin and HVA levels in the frontal cortex. These changes most likely occurred as a result of epigenetic mechanisms (Tekes *et al.*, 2009b).

1.2.5 Synaptic plasticity

Long-term potentiation (LTP) is a long-lasting enhancement of signal transmission between neurons. LTP is one of the mechanisms underlying synaptic plasticity and is important in learning and memory. Prenatal vitamin D deficiency has been shown to alter many genes involved in synaptic plasticity (Eyles *et al.*, 2007), and evidence suggests that prenatal vitamin D deficiency induces a long-lasting enhancement of hippocampal LTP, in freely moving adult rats. Treatment with haloperidol, a high-affinity dopamine D_2 receptor antagonist, reverses the enhanced LTP (Grecksch *et al.*, 2009).

Optimal levels of vitamin D were shown to be required for the induction of LTP within the adult rat brain (Salami *et al.*, 2012). However in that study, vitamin D-deficient adult rats also showed a

reduction in serum calcium, which may have led to the impaired LTP (Salami *et al.*, 2012), because increased postsynaptic intracellular calcium is necessary for the induction of LTP (Brown *et al.*, 1988).

1.3 Rodent models of vitamin D deficiency

In rodents, vitamin D deficiency from weaning, during adulthood, or throughout life has produced a range of significant impairments, including reduced body weight, musculoskeletal deficits, impaired prepulse inhibition of the acoustic startle response, and spatial learning deficits (Altemus *et al.*, 1987; Burne *et al.*, 2004b; Taghizadeh *et al.*, 2013). Neurochemical changes have also been noted, with significant increases in norephinephrine, dopamine, dihydroxyphenylacetic acid (a breakdown product of dopamine), and γ-aminobutyric acid (GABA) (Baksi & Hughes, 1982; Tenenhouse *et al.*, 1991). However, all of these studies (Baksi & Hughes, 1982; Altemus *et al.*, 1987; Tenenhouse *et al.*, 1991; Burne *et al.*, 2004b; Taghizadeh *et al.*, 2013) found a reduction in serum calcium levels with vitamin D deficiency, which suggests that the impairments may have been due to altered calcium metabolism. This demonstrates the importance of maintaining normal serum calcium levels and appropriate musculoskeletal function.

1.3.1 Developmental vitamin D deficiency

A developmental vitamin D (DVD)-deficient model was first created in Sprague-Dawley rats, in which vitamin D is removed from the diet for 6 weeks prior to and throughout conception. Under these conditions, dams have a serum concentration of 25(OH)D at the lower limit of detection (<5 nmol/L), which represents a frank vitamin D deficiency seen in less than 4% of the Australian population (Daly *et al.*, 2012). However, dams are placed back on normal rat chow at the birth of the pups, and 25(OH)D concentrations return to control levels within 2 weeks (Eyles *et al.*, 2013). Use of this model of transient prenatal vitamin D deficiency in rodents has provided compelling proof-of-principle evidence for the association between DVD deficiency and a wide range of alterations in neuroanatomical, neurochemical, and behavioural measures while normal serum calcium levels are maintained. The DVD-deficient rodent model is reviewed in detail elsewhere (Harms *et al.*, 2011; Kesby *et al.*, 2013).

1.3.1.1 Neurogenesis

Vitamin D deficiency has been shown to alter the gene expression of many cell cycle genes and apoptotic genes during foetal development, leading to changes in cell proliferation and apoptosis (Ko *et al.*, 2004). Cells dissociated from neonatal rat subventricular zone following vitamin D

deficiency during gestation showed increased neurosphere production, which suggests that the absence of vitamin D leads to greater proliferation of neuroprogenitor cells (Cui *et al.*, 2007). One study looked at the effects of maternal vitamin D deficiency on adult hippocampal neurogenesis and found that the prenatal vitamin D deficiency resulted in decreased neurogenesis in adult rats and that the decrease in neurogenesis could be reversed by treatment with haloperidol, a dopamine inverse agonist (Keilhoff *et al.*, 2010).

The 1α-hydroxylase knockout mouse lacks the ability to make $1,25(OH)_2D$, and this is associated with increased cell proliferation in the hippocampal dentate gyrus and a reduction in the survival of newborn neurons at 8 weeks of age (Zhu *et al.*, 2012). The $1,25(OH)_2D$ deficiency also significantly increased apoptosis in the hippocampal dentate gyrus, which suggests that this deficiency may be responsible for the loss of the newborn neurons. These results were independent of extracellular calcium (Zhu *et al.*, 2012).

1.3.1.2 Proliferation, differentiation, and apoptosis

In the rat model of DVD deficiency, the vitamin D-depleted pups had brains that were larger and longer, with larger ventricular volume and a thinner neocortex, than brains from control pups. It was shown that the changes in brain morphology were at least in part due to an increase in cell proliferation (Eyles *et al.*, 2003). Moreover, analysis of genes involved in the regulation of apoptosis found that the DVD-deficient pups had a significant reduction in apoptosis during gestation compared with control pups (Ko *et al.*, 2004). Lack of vitamin D during development also led to a multitude of changes in gene expression of pro-apoptotic and cell cycle genes, which corresponded to observed changes in apoptosis and increased cell proliferation (Ko *et al.*, 2004). These studies confirmed that loss of vitamin D leads to alterations in cell proliferation, differentiation and apoptosis during critical periods of brain development.

1.3.1.3 Vitamin D and dopaminergic pathways

A consistent finding from the DVD-deficient rat model is altered dopamine signaling. In neonatal rats, DVD deficiency decreases dopaminergic turnover by a reduction in the expression of catechol-O-methyl transferase enzyme, which is responsible for the breakdown of a dopamine metabolite (Kesby *et al.*, 2009). DVD deficiency has also been shown to significantly reduce factors crucial for specifying dopaminergic phenotype, such as Nurr1 and p57Kip2, during fetal development (Cui *et al.*, 2010). Nurr1 knockout animals have complete agenesis of dopamine neurons (Zetterstrom *et al.*, 1997), and p57Kip2 knockouts have no TH-positive mesencephalic cells at E18.5 (Joseph *et al.*, 2003).

Adult female DVD-deficient rats have a significantly increased dopamine transporter density in the caudate putamen and binding affinity in the nucleus accumbens compared with controls and are more sensitive to amphetamine, a dopamine-releasing agent (Kesby *et al.*, 2010). It was recently confirmed that the VDR is present in the nucleus of TH-positive neurons in both human and rat substantia nigra (Cui *et al.*, 2013b).

1.3.1.4 DVD-deficient model and schizophrenia

A wide range of epidemiological findings have pointed to developmental vitamin D deficiency as a risk factor for the development of schizophrenia (McGrath, 1999); these findings are discussed in more detail in section 1.4.4 Schizophrenia. In this section we discuss findings in the DVD-deficient animal model that are relevant to schizophrenia.

The enlarged lateral ventricles and reduced cortical thickness seen in DVD-deficient rat pups are frequently reported in schizophrenia patients (Harrison, 1999). Adult DVD-deficient 129svJ mice (Harms *et al.*, 2008) and adult DVD-deficient rats had greater spontaneous hyperlocomotion (Burne *et al.*, 2004a) compared with controls. DVD-deficient rats also showed increased locomotion in response to dizocilpine (MK-801), a noncompetitive *N*-methyl-D-aspartate (NMDA) receptor antagonist, and a reduction in both MK-801-induced and spontaneous hyperlocomotion with haloperidol, a dopamine receptor antagonist, which was selective for DVD-deficient rats (Kesby *et al.*, 2006). DVD-deficient adult rats also showed impaired latent inhibition (Becker *et al.*, 2005), another feature of schizophrenia (Williams *et al.*, 1998).

DVD deficiency also led to cognitive impairments in mice (Fernandes de Abreu *et al.*, 2010b) and impaired response inhibition on the rodent version of the continuous performance task in rats (Turner *et al.*, 2013), a key feature of the cognitive deficits seen with schizophrenia. These impairments were reversed by acute treatment with clozapine, an atypical antipsychotic (Turner *et al.*, 2013). Although the DVD-deficient model does not replicate all of the features of schizophrenia [sensorimotor gating [prepulse inhibition (PPI)] is normal (Kesby *et al.*, 2006)], and the effects are dependent on type of rodent and strain; it is a plausible model that can be used to explore the neurobiological mechanisms in schizophrenia.

1.3.2 Adult vitamin D deficiency in rodents

Vitamin D deficiency has recently been investigated in adult Sprague-Dawley rats to determine whether similar disruptions occur in both the developing and adult brain. In the adult vitamin D (AVD)-deficient model, rats were placed on a vitamin D-deficient diet at 10 weeks of age and at 16 weeks began behavioural testing. They were tested on a wide range of behavioural domains, and

overall, AVD deficiency was not associated with an altered phenotype. In a cognitive test of attention and vigilance, the AVD-deficient rats had no learning or attentional deficits but showed a mildly impulsive phenotype. The AVD-deficient rats had increased levels of GABA and an increased dihydroxyphenylacetic acid/HVA ratio in the striatum. The AVD-deficient rats were shown to be vitamin D deficient and importantly, had normal calcium levels after eight to ten weeks on the diet (Byrne *et al.*, 2013).

The impact of AVD deficiency on brain function and behaviour was also investigated in two strains of inbred mice, C57BL/6J and BALB/c. The mice were placed on a vitamin D-deficient diet at 10 weeks of age for a period of 10 weeks prior to behavioural testing. This procedure resulted in serum calcium levels and body weight in AVD-deficient mice that were not different from those of controls. AVD deficiency was found to result in spontaneous hyperlocomotion in both strains (Groves *et al.*, 2013). The C57BL/6J strain showed no other behavioural effects of AVD deficiency. However, the BALB/c AVD-deficient mice showed altered behaviour on the elevated plus maze, a test used to measure anxiety levels, as well as altered responses to heat, shock, and sound. (Groves *et al.*, 2013).

Brain neurochemistry was also analysed, and the effects of AVD deficiency in the two strains differed markedly. In the C57BL/6J strain, dopamine and 5-hydroxytryptamine turnover was increased by AVD deficiency, whereas the BALB/c strain showed decreases in levels of glutamate and glutamine and increased levels of GABA and glycine. Of particular interest, both strains showed a small but significant decrease in the level of an enzyme involved in GABA synthesis, glutamic acid decarboxylase (GAD65/67) (Groves *et al.*, 2013).

These studies show the importance of background strain, with BALB/c mice more susceptible to AVD deficiency than C57BL/6J mice or Sprague-Dawley rats. These general bodies of research also indicate that the timing of exposure to low vitamin D has different impacts on brain outcomes. The absence of vitamin D during development alters the orderly cascade of brain development, which results in a range of neurobiological outcomes as discussed above and reviewed by (Kesby *et al.*, 2013). In contrast, low vitamin D during adulthood is associated with only subtle changes in some behaviours and selective changes in neurochemistry that may be related to excitatory/inhibitory systems.

1.3.3 Two-hit animal models

1.3.3.1 Vitamin D and experimental autoimmune encephalomyelitis: an animal model of multiple sclerosis

The experimental autoimmune encephalomyelitis (EAE) animal model is a model of multiple sclerosis (MS). Using this model, a number of studies have looked at the effects of both vitamin D treatment and a vitamin D deficient diet on EAE outcomes (Lemire & Archer, 1991; Cantorna *et al.*, 1996; Nataf *et al.*, 1996).

Treatment with vitamin D before or during the induction of EAE is effective in preventing EAE, and treatment with vitamin D after the induction of EAE is effective in decreasing the clinical signs of EAE (Lemire & Archer, 1991; Cantorna *et al.*, 1996; Nataf *et al.*, 1996). In addition, vitamin D deficiency increases the susceptibility to EAE and increases the clinical signs of EAE (Cantorna *et al.*, 1996; Garcion *et al.*, 2003). Recently, an animal model of MS pretreated with high-dose vitamin D was shown to have a reduction in demyelination and attenuated microglia activation and macrophage infiltration (Wergeland *et al.*, 2011).

Vitamin D is known to be an immune modulator with immunosuppressant activity. Its actions in the EAE animal model and in human MS are most likely to involve, in part, the regulation of inflammatory cytokines. For example, vitamin D decreases the production of pro-inflammatory cytokines and increases the production of anti-inflammatory cytokines (Manolagas *et al.*, 1985; Cantorna *et al.*, 1998). One study showed that vitamin D treatment for MS patients significantly increased serum levels of transforming growth factor-β1, which is an anti-inflammatory cytokine (Mahon *et al.*, 2003). Vitamin D also directly affects cellular immunity. It has been shown to inhibit Th1 cell development in EAE, as well as dendritic cell maturation to suppress inflammatory activity (Lemire & Adams, 1992; Griffin *et al.*, 2001) and regulate the actions of other T cells (Mayne *et al.*, 2011).

Recently, studies have shown that vitamin D inhibits T-cell proliferation, inhibits the development of IL-6- and IL-17-producing cells, and enhances IL-10 production and the number of Tregs, all mechanisms to promote anti-inflammatory actions (Correale *et al.*, 2009). It seems clear that vitamin D can alter the immune response, which is vitally important in autoimmune disorders such as MS.

Unexpectedly, adult offspring of a DVD mouse model showed milder and delayed EAE when compared to control offspring (Fernandes de Abreu *et al.*, 2010a). One hypothesis proposed was that mice deprived of vitamin D in utero and subsequently placed on a vitamin D diet from birth

actually grew up in an enriched-like environment (Fernandes de Abreu *et al.*, 2010a). This hypothesis is supported by epidemiological data showing a reduced risk of MS is associated with higher sun exposure in children (van der Mei *et al.*, 2003; Kampman *et al.*, 2007). In mice it was shown that offspring born to either a DVD-deficient mother or father displayed early and more severe EAE when compared to control mice (Fernandes de Abreu *et al.*, 2012). More studies are required to examine the molecular basis for the discordant effects between first and second generations.

1.3.3.2 Vitamin D deficiency and stroke

Studies with animal models of ischemic stroke have also looked at the effects of vitamin D on stroke severity and prognosis (Yasuhara *et al.*, 2008; Balden *et al.*, 2012). For example, a study examined the impact of vitamin D deficiency on stroke severity in the adult rat and found that vitamin D-deficient animals had greater infarct volumes compared to controls, and this corresponded with greater impairments, post-stroke, in sensorimotor behavioural testing. Investigations into the mechanism showed that vitamin D-deficient animals had significantly lower plasma, brain, and liver levels of IGF-1 compared to controls. IGF-1, a neuroprotectant that is usually elevated after injury to protect the tissue, has been attenuated with vitamin D deficiency, which indicates that lower IGF-1 levels may contribute to the greater infarct volume seen with vitamin D deficiency (Balden *et al.*, 2012).

The inflammatory response was also altered in the vitamin D-deficient rats compared to controls with a reduction in the levels of a variety of cytokines/chemokines including IL-1β, IL-10, and interferon-γ and with an increase in IL-6. These changes could also contribute to the greater infarct volume seen. The same study (Balden *et al.*, 2012) also looked at the effects of an acute treatment with vitamin D immediately following stroke injury and found no effects on infarct volume or functional capabilities.

1.3.3.3 Vitamin D deficiency and traumatic brain injury

Vitamin D-deficient rats with traumatic brain injury show increased inflammation and greater open field test behavioural deficits in comparison with controls (Cekic *et al.*, 2011). Although progesterone, a neurosteroid that has been beneficial as a treatment in traumatic brain injury in recent clinical trials (Wright *et al.*, 2007; Xiao *et al.*, 2008), was beneficial in injured control animals, there was no improvement with treatment in vitamin D-deficient animals. This suggests that vitamin D deficiency exacerbates traumatic brain injury and diminishes the benefits of progesterone treatment (Cekic *et al.*, 2011).

A combination treatment of progesterone and low-dose vitamin D after brain injury in vitamin D-sufficient animals was found to preserve spatial and reference memory in comparison with controls, and the combination was more effective than progesterone treatment alone (Hua *et al.*, 2012). The combination treatment also stimulated astrocytic activity around the injury site, which suggests that the neuroprotective effects are mediated through activated astrocytes (Hua *et al.*, 2012).

1.3.3.4 Vitamin D deficiency, ageing, and cognition

Aged rats (20 months old) were analysed for the effects of vitamin D treatment on a spatial memory task and on a spontaneous object recognition task, inflammatory state, and amyloid-β (Aβ) load and clearance. Aged controls demonstrated significant learning and memory impairment compared to young control animals. However, vitamin D treatment significantly improved this age-related decline (Briones & Darwish, 2012). Age-related changes in inflammatory state were also mitigated by vitamin D treatment, with increased expression of the anti-inflammatory IL-10 and decreased expression of the inflammatory mediator IL-1β after treatment with vitamin D. The aged control animals showed an increase in amyloid burden compared to young controls. However, this was reduced by vitamin D treatment (Briones & Darwish, 2012).

1.3.3.5 Vitamin D deficiency and amyloid-β toxicity

The introduction of Aβ into cortical neuron culture leads to neurodegeneration via upregulation of L-VGCCs and suppression of VDR, whereas additional treatment of vitamin D protected the neurons from cytotoxicity by downregulating L-VGCCs and upregulating VDR (Dursun *et al.*, 2011). Alzheimer's disease (AD) believed to progress in part from inflammatory processes, including oxidative damage and elevated levels of NO, that occur via iNOS induction. In vitro studies using cortical neurons show that iNOS is elevated following Aβ treatment, whereas vitamin D treatment prevents Aβ-induced cytotoxicity and iNOS upregulation, via VDR (Dursun *et al.*, 2013). Previous studies have shown that vitamin D regulates the expression of iNOS (Garcion *et al.*, 1998). Therefore, vitamin D supplementation could lead to a reduction in NO-mediated inflammation in AD, a possibility that should be further investigated.

Furthermore, treatment with a PDIA3 receptor agonist has been shown to significantly improve performance of object recognition memory, reduce amyloid plaques and neurofibrillary tangles, and reduce degenerated axons and presynaptic terminals in a mouse model of AD (Tohda *et al.*, 2012). Vitamin D, a known endogenous agonist of PDIA3, may therefore be important for anti-AD therapy (Tohda *et al.*, 2012).

Polymorphisms in the VDR gene have been shown to be associated with the risk of AD (Beecham *et al.*, 2009; Gezen-Ak *et al.*, 2012). A recent genetic and functional study found that an AD risk allele was associated with lower VDR promoter activity and that overexpression of VDR or vitamin D treatment suppressed amyloid precursor protein transcription in vitro (Wang *et al.*, 2012a). An analysis of mRNA expression following vitamin D treatment of mixed neuron-glia cell cultures showed upregulation of genes related to neurodegenerative disorders, including 10 genes that encode proteins that could possibly limit AD development (Nissou *et al.*, 2013). Growing evidence supports a protective role of vitamin D against the progression of AD, which is highly relevant owing to endemic vitamin D deficiency, particularly in the elderly. Randomised controlled trials that examine the benefits of vitamin D supplements in AD subjects are needed.

1.4 Links with neuropsychiatric and neurodegenerative disorders

Recent convergent evidence indicates that vitamin D deficiency has an impact during brain development and on the adult brain, and that it is biologically plausible for vitamin D deficiency to affect human health in terms of neuropsychiatric and neurodegenerative disorders. These disorders tend to have a complex aetiology, with both gene and environmental influences. However, vitamin D deficiency seems to be a common risk factor. In this section, we provide the evidence from epidemiology, prospective studies, and clinical trials that links vitamin D deficiency to a range of disorders involving the central nervous system. The type of evidence that connects vitamin D deficiency to the range of disorders is shown in Error! Reference source not found..

1.4.1 Cognitive impairment

Many epidemiological studies have found an association between serum 25(OH)D and cognitive function, including memory and orientation (Llewellyn *et al.*, 2009) and executive function (Buell *et al.*, 2009; Lee *et al.*, 2009). In older adults living independently, low serum 25(OH)D concentrations were significantly associated with cognitive impairment (Peterson *et al.*, 2012). In a recently published systematic review and meta-analysis, lower vitamin D concentrations were significantly associated with poorer cognitive function (Balion *et al.*, 2012). However, an association is not always found (McGrath *et al.*, 2007; Slinin *et al.*, 2010) and moreover, from epidemiological studies it is not clear if low vitamin D levels precede the development of cognitive impairments or are a result of poor diet and disability.

A prospective study found that patients identified as vitamin D deficient at initial assessment had greater impairment of cognitive function at baseline and during follow-up, three and six years later (Llewellyn *et al.*, 2010). Cognition was measured using the Mini-Mental State Examination

(MMSE), a widely used neuropsychological test of cognitive function, and Trail-Making Tests A and B. At initial assessment, scores on all three tests were significantly worse in subjects who were vitamin D deficient or severely deficient compared to those who were vitamin D sufficient. At the six-year follow-up, subjects who were severely 25(OH)D deficient at baseline were more likely to experience substantial later cognitive decline as assessed by the MMSE and the Trail B, which measures executive functioning, but not on the Trail A, which measures attention (Llewellyn *et al.*, 2010).

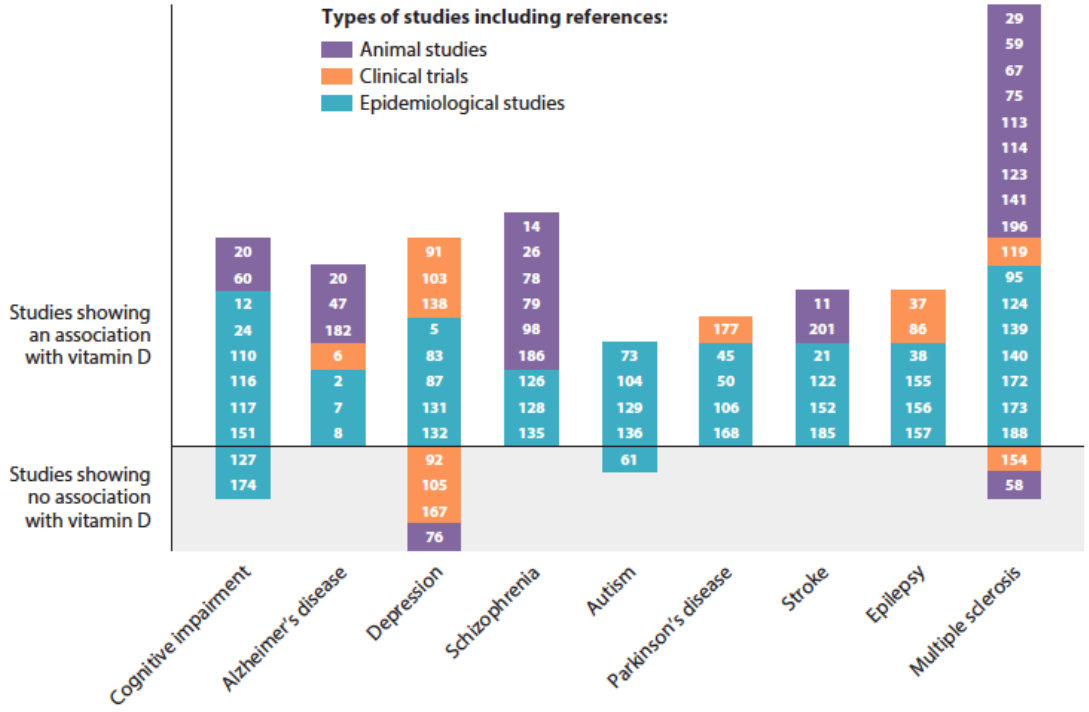

Figure 1.2 Representation of studies testing an association between vitamin D deficiency and various disorders
Animal studies, clinical trials and epidemiological studies that show evidence of an association between vitamin D and various disorders, and studies that show no association between vitamin D and various disorders. Numbers in boxes correspond to reference numbers in the published review (Groves *et al.*, 2014).

1.4.2 Alzheimer's disease

AD is a neurodegenerative disorder characterized by progressive and irreversible cognitive deficits and behavioural alterations. The most common symptom is that of memory impairment and loss of spatial memory. A recent meta-analysis (Annweiler *et al.*, 2013) looked at the association between low serum 25(OH)D and AD and found that serum 25(OH)D concentrations were overall significantly lower in AD cases than in controls. The meta-analysis revealed a large association of low 25(OH)D concentration with AD. A recent prospective study on the risk of AD in the general population showed an increasing risk of AD with decreasing levels of vitamin D (Afzal *et al.*, 2013).

One study showed an association between higher dietary vitamin D intake and a lower risk of developing AD among older women (Annweiler *et al.*, 2012b). A small pilot study found that patients who took memantine, an NMDA receptor antagonist, plus vitamin D for six months had a statistically and clinically relevant gain in cognition, whereas those who took memantine or vitamin D alone showed no effect. This suggests that there may be a synergistic effect in combining the treatments (Annweiler *et al.*, 2012a).

1.4.3 Depression

Clinical depression is characterized by an all-encompassing low mood and loss of interest in normally enjoyable activities (Lorr *et al.*, 1967). It is generally associated with significant disability, due to an inability to function normally, and with a decreased health status (Moussavi *et al.*, 2007). Epidemiological studies have shown a number of risk factors for depression, including gender (higher incidence in females), prior depression, low socioeconomic status, psychiatric comorbidity, medical illness, and major adverse life events (Kaelber *et al.*, 1995) and, more recently, low vitamin D levels (Anglin *et al.*, 2013).

Many observational and prospective studies suggest an association between low vitamin D levels and depression, particularly in the elderly (Hoogendijk *et al.*, 2008; Milaneschi *et al.*, 2010; Hoang *et al.*, 2011; Milaneschi *et al.*, 2011). A recent large systematic review and meta-analysis found that low vitamin D was significantly associated with an increased risk of depression (Anglin *et al.*, 2013); however, from these types of studies, it is not clear whether low vitamin D levels precede depressive symptoms or are a result of having depression.

A randomized, double-blind placebo-controlled trial examined the effects of vitamin D supplementation on depressive symptoms in overweight and obese subjects. At the start of the trial there was an association between low serum 25(OH)D and symptoms of depression. Treatment with 20,000 or 40,000 IU vitamin D per week for 1 year, but not placebo, resulted in significant improvement in depressive symptoms. This study suggests a possible causal link between low vitamin D and depression, at least in the overweight and obese (Jorde *et al.*, 2008).

Treatment with fluoxetine, a serotonin selective reuptake inhibitor, is known to improve depressive symptoms; however, a recent study showed that combining vitamin D treatment with fluoxetine improved depressive symptoms significantly more than fluoxetine alone did (Khoraminya *et al.*, 2013). Another recent clinical trial (Mozaffari-Khosravi *et al.*, 2013) was undertaken in adults who were vitamin D deficient and suffering from depression. Participants were given either a single dose of 150,000 IU or 300,000 IU vitamin D or no treatment and were tested again for depression three

months later. The single dose of 300,000 IU vitamin D not only proved safe but was also effective at significantly improving depression. This study shows that correcting vitamin D deficiency can improve the depression state (Mozaffari-Khosravi *et al.*, 2013).

However, in subjects who were not vitamin D deficient, high-dose vitamin D treatment did not have the same benefits (Jorde *et al.*, 2013), nor did all studies find improvement of depression with vitamin D treatment (Kjaergaard *et al.*, 2012). Randomised controlled studies based on general population samples have also not found an association between vitamin D supplementation and scores on measures of depression (Sanders *et al.*, 2011).

1.4.4 Schizophrenia

Schizophrenia is a group of disorders with symptoms including hallucinations, delusions, thought disorder, blunted affect, social withdrawal, and cognitive impairments (Frith, 1996; Pearlson, 2000). It is most likely a neurodevelopmental disorder and is characterized by alterations in brain morphology and abnormal laminar organisation as well as altered expression of proteins related to the early migration of neurons and glia, cell proliferation, formation of neural circuitry, and apoptosis (Fatemi & Folsom, 2009). Risk factors for the development of schizophrenia include both genetic factors and environmental influences. Some environmental risk factors include pregnancy and birth complications, maternal infection, immigration, adverse life events, and substance abuse (Davis *et al.*, 2016; Janoutova *et al.*, 2016).

Developmental vitamin D deficiency was first suggested as a risk factor for schizophrenia in 1978 because people with schizophrenia tend to be born in winter (Moskovitz, 1978). Additional epidemiological findings, including increased schizophrenia in dark-skinned migrants to cold climates and in the urban versus rural setting and an increased risk of schizophrenia with prenatal famine, led McGrath (McGrath, 1999) to propose that vitamin D deficiency during development could adversely affect the developing brain and lead to an increased risk of adult-onset schizophrenia (McGrath, 1999).

Recently, a case-controlled study analysed neonatal vitamin D status and risk of schizophrenia. It was found that low neonatal vitamin D is significantly associated with an increased risk of schizophrenia (McGrath *et al.*, 2010b). A recent genome-wide analysis comparing genes involved in schizophrenia and genes related to vitamin D, found a significant overlap of 70 genes (Amato *et al.*, 2010).

1.4.5 Autism

Autism is a neurodevelopmental disorder characterized by impaired social interaction, communication, and stereotypical behaviour. Although it is well known that autism has a strong genetic component, research has also shown that environmental factors are likely to contribute to the development of autism (Abrahams & Geschwind, 2008). Epidemiological data have shown a number of factors that are associated with autism, including prenatal exposure to mutagens and advanced paternal age. A number of the other exposures can be linked to vitamin D deficiency. These include regions at higher latitudes (especially for dark-skinned individuals), urban residence, and regions with high precipitation rates (Kinney *et al.*, 2010).

Studies have shown that autistic children have lower serum 25(OH)D levels compared to healthy controls (Meguid *et al.*, 2010; Mostafa & Al-Ayadhi, 2012). For example, a cross-sectional study in Egypt showed that children with autism have significantly lower serum 25(OH)D, $1,25(OH)_2D$ and calcium levels compared to controls (Meguid *et al.*, 2010). Other studies have found no significant association between serum 25(OH)D and autism (Fernell *et al.*, 2010). Recently, autism prevalence was shown to be inversely correlated with solar UVB doses in an ecological study, which suggests that vitamin D deficiency during fetal brain development or early life could be relevant to the development of autism (Grant & Cannell, 2013).

1.4.6 Parkinson's disease

Parkinson's disease (PD) is a progressive neurodegenerative disease. It is characterized by slow, selective dopaminergic neuronal loss. Symptoms include dyskinesia, rigidity, and tremor as well as postural instability and gait disorders (Bonnet & Houeto, 1999).

Epidemiological evidence from cross-sectional studies provides some support for a link between vitamin D deficiency and PD incidence (Sato *et al.*, 2005; Evatt *et al.*, 2008). Furthermore, the first longitudinal study investigating the association between vitamin D status and subsequent occurrence of PD showed that low serum vitamin D levels predicted an elevated risk of PD (Knekt *et al.*, 2010). Subsequent studies have shown that vitamin D deficiency is also associated with more advanced severity of disease (Ding *et al.*, 2013).

A recently published study related to PD has lent support for the neuroprotective properties of vitamin D. Using a placebo-controlled, randomised trial, Suzuki *et al.* (2013) examined the impact of vitamin D supplementation (1,200 IU per day, for one year) on various Parkinson's Disease related outcomes. Those on placebo had a steady worsening of PD-related outcomes. In contrast,

those on vitamin D supplements had no change in PD outcomes over the year. The results strongly suggest that low vitamin D status exacerbates disease progression of PD (Cui *et al.*, 2013a).

1.4.7 Stroke

Studies in humans have revealed that low levels of serum vitamin D are independently predictive for the occurrence of strokes (Marniemi *et al.*, 2005; Pilz *et al.*, 2008), and a large population-based prospective study showed stepwise increases in the risk of ischemic stroke with decreasing serum 25(OH)D (Brondum-Jacobsen *et al.*, 2013). A further study in China not only showed that patients with acute ischaemic stroke had significantly lower vitamin D levels compared to controls, but also that vitamin D levels were a prognostic marker of short-term functional outcome and death in stroke patients (Tu *et al.*, 2014).

1.4.8 Epilepsy

Epilepsy is a brain disorder characterized by recurrent and unpredictable interruptions in normal brain function (epileptic seizures) (Fisher *et al.*, 2005). Epidemiological studies indicate that epilepsy is another brain disorder that shows seasonal variation of birth, with an excess of those with epilepsy born in winter compared to summer (Procopio *et al.*, 1997; Procopio & Marriott, 1998; Procopio *et al.*, 2006). Additionally, epileptic seizures themselves show seasonal variation, with a reduction in seizures during summer (Clemens *et al.*, 2013). A very early small controlled pilot study showed a reduction in the number of seizures following treatment with vitamin D compared to placebo (Christiansen *et al.*, 1974). Nearly 40 years later this study was followed up with another pilot study showing a median reduction in seizures of 40% following vitamin D supplementation (Hollo *et al.*, 2012).

1.4.9 Multiple sclerosis

MS is a slow progressive disorder of the central nervous system that is characterized by demyelination of the brain and spinal cord. Although its aetiology is unclear, it seems to be multidimensional, with environmental factors, genetic factors, and dysregulation of the immune response all playing a part (VanAmerongen *et al.*, 2004). Environmental risk factors include infection, cigarette smoking, and low vitamin D (Pugliatti *et al.*, 2008; O'Gorman *et al.*, 2012). A significant positive association exists between MS prevalence and latitude globally, which supports the role of ultraviolet radiation and vitamin D in its development (Simpson *et al.*, 2011). Additionally, studies show that vitamin D intake is inversely associated with the risk of MS (Munger *et al.*, 2004; Munger *et al.*, 2006) and serum 25(OH)D levels are significantly lower in

patients with MS compared to healthy subjects (Mazdeh *et al.*, 2013). Furthermore, vitamin D concentrations correlate with the severity of MS (Shahbeigi *et al.*, 2013). Genetic studies have shown links between MS susceptibility and both CYP27B1 and CYP24A1, vitamin D metabolism enzymes (Ramagopalan *et al.*, 2011; Sawcer *et al.*, 2011). However, not all studies show a significant effect of vitamin D on MS, and more work is required (Pozuelo-Moyano *et al.*, 2013).

1.5 Future directions

Accumulating evidence supports the need for optimal vitamin D levels both during development and throughout adulthood for proper brain function. However, it is still unknown what the optimal vitamin D level is for the brain or how the timing or length of vitamin D deficiency can alter the risk of disease. Recommendations for optimal vitamin D concentrations are usually based on bone outcomes (e.g. parathyroid concentrations) (Holick *et al.*, 2012). More research is required to determine if vitamin D treatment of brain diseases is an effective tool or if prevention of vitamin D deficiency is the only method to lower risk.

The results from recent animal and human studies suggest that vitamin D deficiency during adulthood may exacerbate underlying brain disorders and/or worsen recovery from brain stressors. Therefore, research is required to determine the molecular mechanism behind this possible vulnerability. For example, the direct regulation of calcium by vitamin D within the brain may be a key molecular mechanism to protect against the neurotoxicity that can occur in disease and aging, or it may be vitamin D's immunomodulatory and neurotrophic effects providing neuroprotection to maintain a healthy brain. Therefore, both animal experiments and in vitro research are required to explicate these mechanisms within the brain, such as electrophysiology of calcium transport. Research in animals that combines vitamin D deficiency with relevant animal models of neuropsychiatric and neurodegenerative disorders is also required.

Additionally, with the extensive links between vitamin D deficiency and a wide range of neuropsychiatric, neurodegenerative, and other brain disorders now evident, there is a need for large, well-controlled clinical trials.

1.6 Conclusion

This review has shown that vitamin D is a neurosteroid that exerts a multitude of effects that are important in both the correct development of the brain and the proper functioning of the adult brain. In addition, mounting evidence suggests that maintaining optimal vitamin D levels may lower the risk of developing a wide range of brain disorders. With vitamin D deficiency widespread

throughout the world, it is no wonder that research is focusing on elucidating the mechanisms of vitamin D's actions within the brain. In light of the advantage that vitamin D supplementation is readily available and affordable, this review highlights the need for further research.

1.7 Gaps in the research addressed by this thesis

There is a large body of evidence supporting the need for optimal vitamin D levels during development. However, despite the growing epidemiological data showing an association between vitamin D deficiency and a wide range of adverse brain outcomes in adulthood, animal studies of adult vitamin D deficiency so far have been mostly restricted to models of MS. There are only a limited number of animal studies that have addressed the effects of vitamin D deficiency in healthy adults and even fewer that have maintained normal calcium levels in the animals. In addition to the lack of animal models, most studies addressing possible mechanisms of action of vitamin D in the adult brain have been performed using vitamin D treatment; or the addition of vitamin D in vitro, and have failed to address the consequences of the absence of vitamin D in the adult brain.

We have previously developed a model of AVD deficiency in mice, in which ten-week-old animals from two strains of mice were placed on a diet deficient in vitamin D for a ten-week period, following which a range of behavioural domains as well as neurochemical outcomes were studied (see Adult vitamin D deficiency in rodents section) (Groves *et al.*, 2013). I have continued to use this model to study the effects of AVD deficiency on brain function in this thesis. One important issue about the use of vitamin D deficient models relates to possible confounding factors such as hypocalcemia (i.e. adverse brain impacts may only emerge in animals with more severe physiological disruption). Because hypocalcemia is less common in healthy adults with vitamin D deficiency (due to release of calcium from the bones and/or access to adequate dietary calcium), researchers need to develop rodent models that more closely approximate that seen in clinical and community-based epidemiological studies. Using this model, AVD-deficient mice had normal serum calcium levels and at the time of behavioural testing were around six months of age, depending on exact experimental protocol.

1.8 Aims and outline of thesis

The four main aims of the research covered in this thesis included determining if AVD deficiency in mice would impact on (1) cognition, (2) adult hippocampal neurogenesis, and (3) glutamate and GABA signaling; and to determine if AVD deficiency would (4) exacerbate the consequences of a secondary exposure, in this case social stress.

The first aim of this research was to determine the impact of AVD deficiency on cognitive processes. Based on the epidemiological association between vitamin D deficiency and cognitive impairments (Balion *et al.*, 2012), it was hypothesised that AVD deficiency would lead to impairments on cognitive tasks, with relevance to attentional processes and executive function in humans. This was examined in Chapter 2. These results were followed up using diffusion tensor MRI to study connectivity in the brain. Vitamin D deficiency in elderly, cognitively impaired patients has been previously associated with disruption in neuronal integrity as measured by diffusion tensor imaging in conjunction with worse cognitive impairment (Moon *et al.*, 2015). This was detailed in Chapter 3.

A second aim of this research was to determine if adult hippocampal neurogenesis was altered by AVD deficiency. There is a large body of evidence supporting a role for vitamin D in cell proliferation, survival and differentiation, and based on this research it was hypothesised that AVD deficiency would alter hippocampal neurogenesis. This was assessed in Chapter 4.

A third aim of the research presented in this thesis was to explore the proteomic correlates of AVD deficiency, with a preliminary proteomic study based on hippocampal tissue. It was hypothesised that proteins involved in glutamate and GABA signaling and metabolism would be altered following AVD deficiency. This was explored in Chapter 5. Based on some of the results from the proteomic analysis, we further hypothesised that AVD deficiency would deplete glutathione levels and impair hippocampal-dependent learning and memory and this was examined in Chapter 6.

The final aim of this research was to explore if AVD-deficient mice were more vulnerable to a secondary exposure. Recent studies have suggested that vitamin D deficiency in adulthood will exacerbate the effects of a secondary insult or comorbid disorder or disease (Balden *et al.*, 2012; Suzuki *et al.*, 2013). Therefore, it was hypothesised that combining AVD deficiency with social stress would lead to greater detrimental outcomes compared to either treatment alone and this was examined in the final experimental chapter, Chapter 7.

Chapter 2 Sex-Specific Attentional Deficits in Adult Vitamin D Deficient BALB/c Mice

2.1 Introduction

Epidemiological studies have shown an association between vitamin D deficiency and a range of neurodegenerative and neuropsychiatric disorders, such as AD (Annweiler *et al.*, 2013), depression (Anglin *et al.*, 2013), schizophrenia (Yuksel *et al.*, 2014), autism (Kocovska *et al.*, 2014), and MS (Munger *et al.*, 2006). Many of these disorders either contain a cognitive component or are found to be co-morbidly associated with cognitive impairments. There are a large number of epidemiological studies that have examined links between vitamin D deficiency and cognitive disorders. For example, an observational study with independently living older adults, found lower serum 25(OH)D concentrations were significantly associated with cognitive impairment (Peterson *et al.*, 2012).

Cross-sectional studies have shown significant associations between low serum 25(OH)D in adults and impairments in memory and orientation (Wilkins *et al.*, 2006; Przybelski & Binkley, 2007; Llewellyn *et al.*, 2009) and executive function (Buell *et al.*, 2009; Lee *et al.*, 2009). Moreover, prospective studies have also shown greater cognitive decline over time, to be independently associated with low 25(OH)D levels in elderly individuals (Llewellyn *et al.*, 2010; Toffanello *et al.*, 2014). However, the majority of these studies involve elderly populations, which is not representative of the general population. Furthermore, an association between vitamin D deficiency and cognitive disorders are not always found (McGrath *et al.*, 2007; Slinin *et al.*, 2010). Therefore, epidemiological studies are unable to address causality and have been inconclusive in determining a link between vitamin D deficiency and cognitive impairment.

Recent studies in adult rats have shown significantly lower performance on cognitive tasks only in vitamin D-deprived rats that were also hypocalcemic (Briones & Darwish, 2012; Byrne *et al.*, 2013; Taghizadeh *et al.*, 2013). However, in aged (20 month old) rats vitamin D treatment has significantly improved performance on cognitive tasks, although serum calcium levels were not measured in that study (Briones & Darwish, 2012).

The effect of longer-term exposure (6 months) to diets containing varying amounts of vitamin D on cognitive performance was recently tested in middle-aged (5-6 month old) F344 rats (Latimer *et al.*, 2014). Rats on high vitamin D (10,000 IU/kg food) significantly outperformed low (100 IU/kg food) and medium vitamin D (1,000 IU/kg food) groups in a challenging memory task, demonstrating a causal link between vitamin D status and cognitive function in aging animals. Importantly in this study, all groups were normocalcemic (Latimer *et al.*, 2014). However, another study in ageing C57BL/6J mice, showed no effect on cognitive performance of a long-term vitamin

D deficient diet (on diet at 10 months and tested at 22-23 months) (Brouwer-Brolsma *et al.*, 2014), although the task design varied between studies (Latimer *et al.*, 2014) (Brouwer-Brolsma *et al.*, 2014).

We have previously developed a model of AVD deficiency in which mice are placed on a vitamin D deficient diet at 10 weeks of age and maintained on the diet for a further 10 weeks prior to and during behavioural testing and were shown to be normocalcemic (Groves *et al.*, 2013). The controls were given the recommended amount of vitamin D (National Research Council, 2015) and the vitamin D deficient mice had serum levels at the lower level of detection, which is seen in clinically deficient humans (Chel *et al.*, 1998; Saaf *et al.*, 2011).

Two strains of mice have been examined using this model, BALB/c and C57BL/6J mice, and significant differences between strains were observed. BALB/c mice showed significant effects of AVD deficiency across a range of behaviours while C57BL/6J mice had a subtle behavioural phenotype. The BALB/c AVD-deficient mice exhibited an imbalance between excitatory and inhibitory neurotransmitters, with reductions in glutamate and glutamine levels, elevated levels of GABA and a reduction in the levels of both GAD65 and GAD67 enzymes in the brain (Groves *et al.*, 2013).

There is convergent evidence suggesting that altered GABA and glutamate neurotransmission in the prefrontal cortex (PFC) is involved in cognitive disturbances in schizophrenia, as reviewed by Lewis and Moghaddam (2006). Although the alterations seen in the AVD-deficient BALB/c mouse model were opposite to those reported in schizophrenia, the neurotransmitter levels were measured in whole brain, not specifically in the PFC (Groves *et al.*, 2013). Furthermore, the whole brain reduction in the levels of the GAD enzymes were consistent with post-mortem brain tissue from schizophrenia patients showing reductions in GAD67 levels in the PFC (Hashimoto *et al.*, 2003) and impairments in working memory have been linked to deficits in GABAergic signaling in schizophrenia patients (Lewis *et al.*, 2005).

The 5 choice - serial reaction time (5C-SRT) task is a widely used rodent test that assesses attentional processing and can also be used to analyse impulsivity and perseverance (Robbins, 2002; Young *et al.*, 2009b). In addition to the standard 5C-SRT, further testing in the 5 choice - continuous performance test (5C-CPT) can be performed to assess additional aspects of attentional processing as the rodent has to also learn to withhold a response to a new stimulus in order to receive a reward. The addition of the withhold condition assesses response inhibition and sustained vigilance (Young *et al.*, 2009a). Both of these rodent tests mirror tests that can be performed in humans to analyse deficits in cognitive function. Therefore, the aim of this study was to determine

if AVD deficiency results in impairments in attentional processing using the 5C-SRT task and the 5C-CPT.

2.2 Materials and methods

2.2.1 Animals and housing

A total of 27 BALB/c mice (14 males and 13 females) were used in this study. Ten-week old BALB/c mice (Animal Resources Centre, Canning Vale, WA, Australia) were obtained and housed in groups of up to four, in individually ventilated OptiMICE cages (Animal Care Systems, CO, USA). All cages were given bedding (Sanichips, Harlan Laboratories, USA) and nesting material and housed at the Queensland Brain Institute Animal House Facility, University of Queensland.

The mice were maintained on a 12-hour light-dark cycle (lights on at 07:00 h) and were housed under incandescent lighting free from UVB radiation. All mice were housed with *ad libitum* access to food and water except during testing of the 5C-SRT task and 5C-CPT, during which they were food restricted to ~90% of their free feeding body weight. All experimental work was performed with approval from the University of Queensland Animal Ethics Committee (QBI/121/10/NHMRC), under the guidelines of the National Health and Medical Research Council of Australia.

The mice were assigned to either a control diet (Standard AIN93G Rodent diet with 1,500 IU vitamin D_3/kg (prior to irradiation with 25 kGy), Specialty Feeds, WA, Australia, **Appendix 1**) or a vitamin D-deficient diet (irradiated with 25 kGy) (Vitamin D Deficient AIN93G Rodent diet, Specialty Feeds, WA, Australia, **Appendix 1**) for 10 weeks prior to the start of behavioural testing; and for the entire duration of the experimental procedures.

2.2.1.1 Food restriction during 5C-SRT and 5C-CPT

Three days before the start of the 5C-SRT task mice were weighed and placed on a restricted feeding schedule to decrease body weight to ~90 % of their original free-feeding weight. Mice were given 0.075 g of food/g of starting body weight/mouse/day at1600 h. The mice were also given 1 ml of liquid reinforcer (Breaka strawberry milk, Parmalat, QLD, Australia) in their home cage for the 3 days prior to testing to habituate them to the liquid reward used during operant testing. Mice were weighed daily throughout behavioural testing and the amount of food provided each day was adjusted to maintain weight ~90 % at all times, ensuring that their weight did not fall below 85 %. Mice had *ad libitum* access to water in the home cage throughout testing. Mice were housed in groups of three to four mice, unless weight loss between the mice became uneven, possibly from

more dominant mice in the cage consuming more food than the others, in which case the groups of four were split into pairs.

2.2.1.2 Body weight and food intake

The percentage of the original free-feeding body weight of the mice during training and testing was continuously monitored in order to balance the amount of food the mice were given each day to maintain appropriate body weights. The amount of food given to the mice each day was recorded.

2.2.2 Behavioural testing

After 10 weeks on the diet when the mice were 20-weeks old, 14 males (7 control and 7 AVD-deficient) and 13 females (7 control and 6 AVD-deficient) were tested in the 5C-SRT task and 12 of the females (6 control and 6 AVD-deficient) continued on to perform the 5C-CPT. There were inadequate numbers of male AVD-deficient mice that passed criteria during training for 5C-CPT and therefore males were not tested on the second test.

2.2.2.1 Operant chambers

Training and testing for the 5C-SRT task and 5C-CPT was performed in four nine-hole nose poke mouse operant chambers (Med Associates, VT, USA). Only five of the nine holes were used in experiments (first, third, fifth, seventh and ninth). Each chamber was placed into a larger sound and light attenuated chamber to minimize exposure to external stimuli. A central device that was connected to a computer running MED-PC IV controlled the chambers. For all experiments, modified versions of the programs for the standard nine-choice serial reaction time task supplied with the system were used (Harms *et al.*, 2012b).

2.2.2.2 5 choice - serial reaction time task

Mice were trained to perform the 5C-SRT task according to a standard protocol (Bari *et al.*, 2008). Each mouse was trained or tested for one session per day for 5 to 7 days per week. Each session consisted of up to 100 trials or 30 min. To initiate a session, a reward was offered in the reward magazine and the reward light was illuminated. When the mouse nose poked into the reward magazine to drink the reward, the session began. In the first training level (Level 0) the mice needed to nose poke any hole while the reward light was lit, to receive a reward. Mice needed to nose poke 80 times in one session to progress to the next training level.

For the next level (Level 1), mice had to respond to a light presented in one of the five holes by nose poking in the lit hole, to receive a reward. This stimulus was presented for 30 sec during this

level. If the mouse failed to respond with a nose poke, this was recorded as an omission and if the mouse nose poked in an unlit hole, this was recorded as an incorrect response. Both omissions and incorrect responses were penalised by a timeout, a period of 5 s in which the house light was lit and no reward offered, before a new trial would begin. Premature responses (a response during the inter-trial interval) and perseverant responses (repeated nose pokes into the correct hole) were also recorded, as were the number of head entries into the reward magazine during each session.

With each level, the stimulus duration was shortened until Level 7 in which the stimulus was presented for only 1 s. To reach criteria for the next level (for Levels 1-6), the mice had to perform with at least 80 % correct responses and no more than 20 % omissions. On Level 7, the mouse had to perform with at least 70 % correct responses and no more than 20 % omissions. Once the mice had passed level 7 on two consecutive days, they were tested for their response to multiple stimulus durations (Level 8) within a session (random stimulus durations between 0.2 and 1 s). Mice were tested for 3 consecutive days, regardless of performance and their results were averaged. After two further days on Level 7, the mice were then tested for their response to altering the inter-trial intervals (Level 9) within a session (random presentation of intervals between 1 s and 9 s). Mice were again tested for 3 consecutive days regardless of performance and their results were averaged.

2.2.2.3 5 choice – continuous performance task

After testing was completed for the 5C-SRT task, the mice were placed on training for 5C-CPT. Training consisted of 120 trials per session, within a 30 min time period, and random variable inter-trial intervals of 3, 4, 5, 6 and 7 s (Level 10). To pass criteria, the mice were required to achieve 80 % correct and have no more than 20 % omissions within a session. Once they passed criteria for level 10, they began testing on the 5C-CPT. The 5C-CPT consisted of 80 target trials and 40 non-target trials, with variable inter-trial intervals as for Level 10. For the 40 non-target trials, the mouse had to withhold a response in order to receive a reward. For these trials, all nine holes were lit and the mouse had to withhold from responding for 3 s. If the mouse did nose poke during this time, it was counted as a false alarm and was penalised by a timeout. As for the 5C-SRT task, premature and perseverant responses were recorded. Mice were tested on the 5C-CPT for 20 consecutive days regardless of performance.

2.2.3 Serum 25(OH)D levels

We have shown in separate mice under the same conditions that there is no significant difference between males and females in serum 25(OH)D levels in BALB/c mice (Control males = 33.51 ± 2.37, Control females = 31.56 ± 2.51, AVD-deficient males = 2.94 ± 0.56, AVD-deficient females

= 3.17 ± 0.55; $F_{1,19} = 0.25$, $p = 0.623$), and that mice are deficient in serum 25(OH)D after 10 weeks on the Diet ($F_{1,19} = 294.50$, $p < 0.001$).

2.2.4 Statistical analysis

Data were analysed using SPSS version 20. All data were analysed for the main effects of Diet (control or AVD-deficient) and Sex using analysis of variance (ANOVA) or, where appropriate, repeated measures ANOVA. Significant differences ($p<0.05$) were followed up with post-hoc t-tests. In the 5C-CPT, the following parameters were also calculated, as described previously (Young *et al.*, 2009a), hit rate, *p*(Hit) as the proportion of target trials correctly detected; false alarm rate, *p*(FA) as the proportion of non-target trials with an incorrect response; discrimination index, d' as a parametric measure of the ability to discriminate between target and non-target trials; and perceptual bias, B" a measure of the amount of signal required to generate a response, and these are outlined in Table 2.1.

Table 2.1 Calculations for the additional variables used to analyse the 5C-CPT results

Variable	
***p*(Hit) – probability of a hit response**	$\frac{\text{No. of correct responses}}{\text{Total no. of target trials}}$
***p*(FA) – probability of a false alarm**	$\frac{\text{No. of false alarm responses}}{\text{Total no. of non-target trials}}$
***d'* – discrimination index**	Z score of *p*(Hit) – Z score of *p*(FA)
B" – perceptual bias index	$\frac{\{p(\text{Hit})[1-p(\text{Hit})]\} - \{p(\text{FA})\{1-p(\text{FA})]\}}{\{p(\text{Hit})[1-p(\text{Hit})]\} + \{p(\text{FA})[1-p(\text{FA})]\}}$

2.3 Results

2.3.1 Mice weights and food intake

The percentage of the original free-feeding body weight of the mice during training and testing was analysed and there was no significant effect of Diet ($F_{1,23} = 0.08$, $p = 0.780$) or Sex ($F_{1,23} = 3.26$, $p = 0.084$) on body weight and no interaction ($F_{1,23} = 0.53$, $p = 0.473$). The main effect of Sex ($F_{1,23} = 4.10$, $p = 0.055$) failed to reach significance for the amount of food given to the mice each day and there was no significant effect of Diet ($F_{1,23} = 0.16$, $p = 0.696$) and no interaction ($F_{1,23} = 0.15$, $p = 0.702$). Therefore, we conclude that the dietary manipulation did not affect body weight or food intake during training and testing.

2.3.2 5 choice – serial reaction time task

2.3.2.1 Days to criteria and training

There was a main effect of Sex ($F_{1,27}$ = 11.68, p = 0.002) but not of Diet ($F_{1,27}$ = 0.70, p = 0.410) on days to criteria for all training levels (Figure 2.1), with female mice completing training in a shorter period of time. When analysing separately for Sex, there was no significant effect of Diet ($F_{1,13}$ = 0.62, p = 0.445) in males when analysing days to pass all training levels. However, there was a significant effect of Diet on the number of days required to pass Level 0 ($F_{1,13}$ = 4.88, p = 0.046) and Level 2 ($F_{1,13}$ = 5.20, p = 0.040). In female mice, there was no effect of Diet ($F_{1,13}$ = 0.44, p = 0.836) on the number of days to reach criteria. Throughout training, the AVD-deficient female mice had a significantly shorter latency to receive reward ($F_{1,13}$ = 6.41, p = 0.025) compared to control female mice.

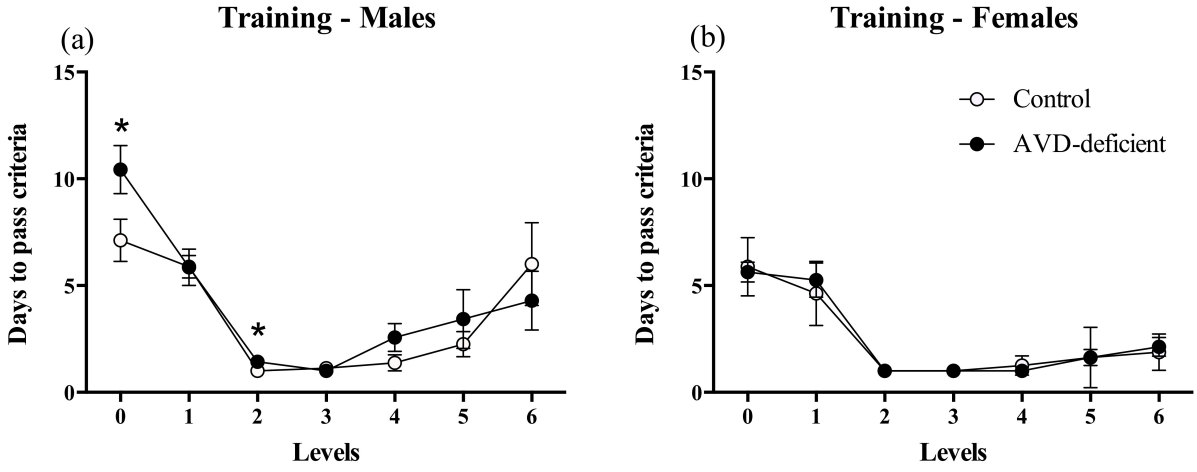

Figure 2.1 Days to pass criteria during training for the 5C-SRT task
Days to pass criteria during each training level (0-6) as detailed in section 2.2.2.2 for the 5C-SRT task for males **(a)** and females **(b)**. Mean ± SEM (n = 6-7/group, * $p < 0.05$)

2.3.2.2 Multiple Stimulus Durations

The effect of varying the stimulus duration during testing (0.2-1.0 s) was analysed and averaged over three days of testing. At low stimulus durations (0.2-0.4 s), all mice made more omissions ($F_{1,23}$ = 163.38, $p < 0.001$) and were less accurate ($F_{1,23}$ = 129.83, $p < 0.001$) when responding compared with longer stimulus durations (0.5-1.0 s). There was a main effect of Sex on the number of correct responses ($F_{1,23}$ = 5.86, p = 0.024) and omission rate ($F_{1,23}$ = 5.96, p = 0.023), but not on accuracy ($F_{1,23}$ = 0.01, p = 0.952), the number of incorrect responses ($F_{1,23}$ = 0.60, p = 0.446), premature responses ($F_{1,23}$ = 3.21, p = 0.086) or perseverative responses ($F_{1,23}$ = 0.04, p = 0.847). There were no main effects of Diet on any of these measures, but there was a Sex x Diet interaction ($F_{1,23}$ = 4.44, p = 0.046) on the number of correct responses. In males, the AVD-deficient mice

made significantly fewer correct responses ($F_{1,12} = 4.85$, $p = 0.048$, Figure 2.2a) compared to the control mice. They were also less accurate ($F_{1,12} = 5.34$, $p = 0.039$) and made significantly more omissions ($F_{1,12} = 5.87$, $p = 0.032$, Figure 2.2c) during the longer stimulus durations (0.6-1.0 s) compared to controls.

With regard to speed of responding, there was a Sex x Diet interaction on the latency to make a correct response ($F_{1,23} = 7.99$, $p = 0.010$) and on reward latency ($F_{1,23} = 4.89$, $p = 0.037$), but no significant effects on the latency to make an incorrect response. In males, AVD-deficient mice had a significantly increased latency to a correct response ($F_{1,12} = 5.26$, $p = 0.041$, Figure 2.3a) when compared to controls. This effect was seen without a change in reward latency ($F_{1,12} = 1.31$, $p = 0.275$, Figure 2.3c). In female mice, there was a significant effect of Diet on reward latency ($F_{1,11} = 6.98$, $p = 0.023$, Figure 2.3d), with the AVD-deficient mice responding quicker to the presentation of reward than controls.

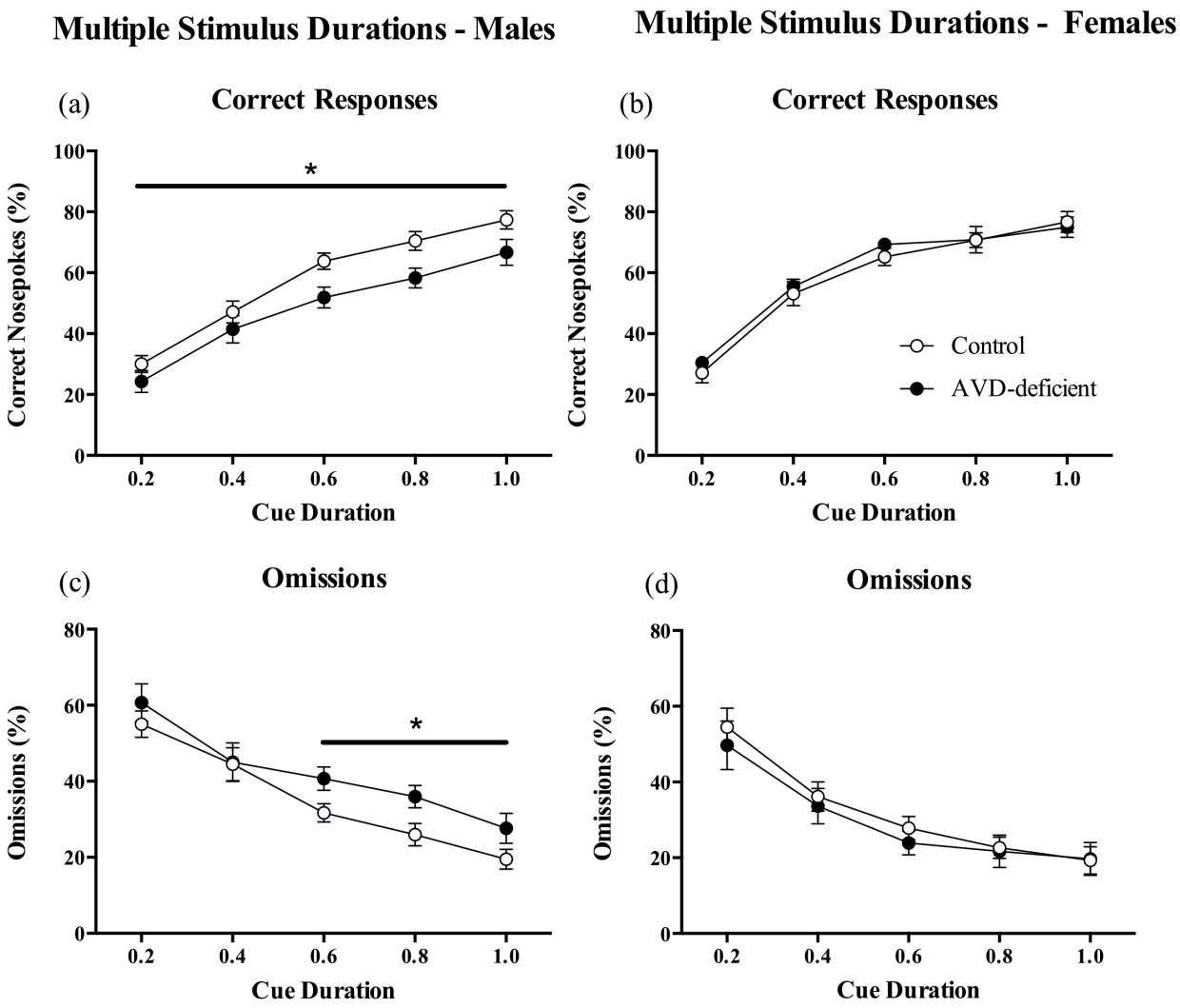

Figure 2.2 Results of manipulating stimulus duration in the 5C-SRT task
Male results are shown in **a, and c**, with female results shown in **b, and d**. The percentage of correct responses are shown in **a** and **b**, with AVD-deficient male mice making significantly fewer correct responses compared to control males. The percentage of omissions is shown in **c** and **d**, with AVD-deficient male mice making significantly more omissions in the longer (0.6-1.0 s) stimulus durations, compared to control males. Mean ± SEM (n = 6-7/group * p <0.05)

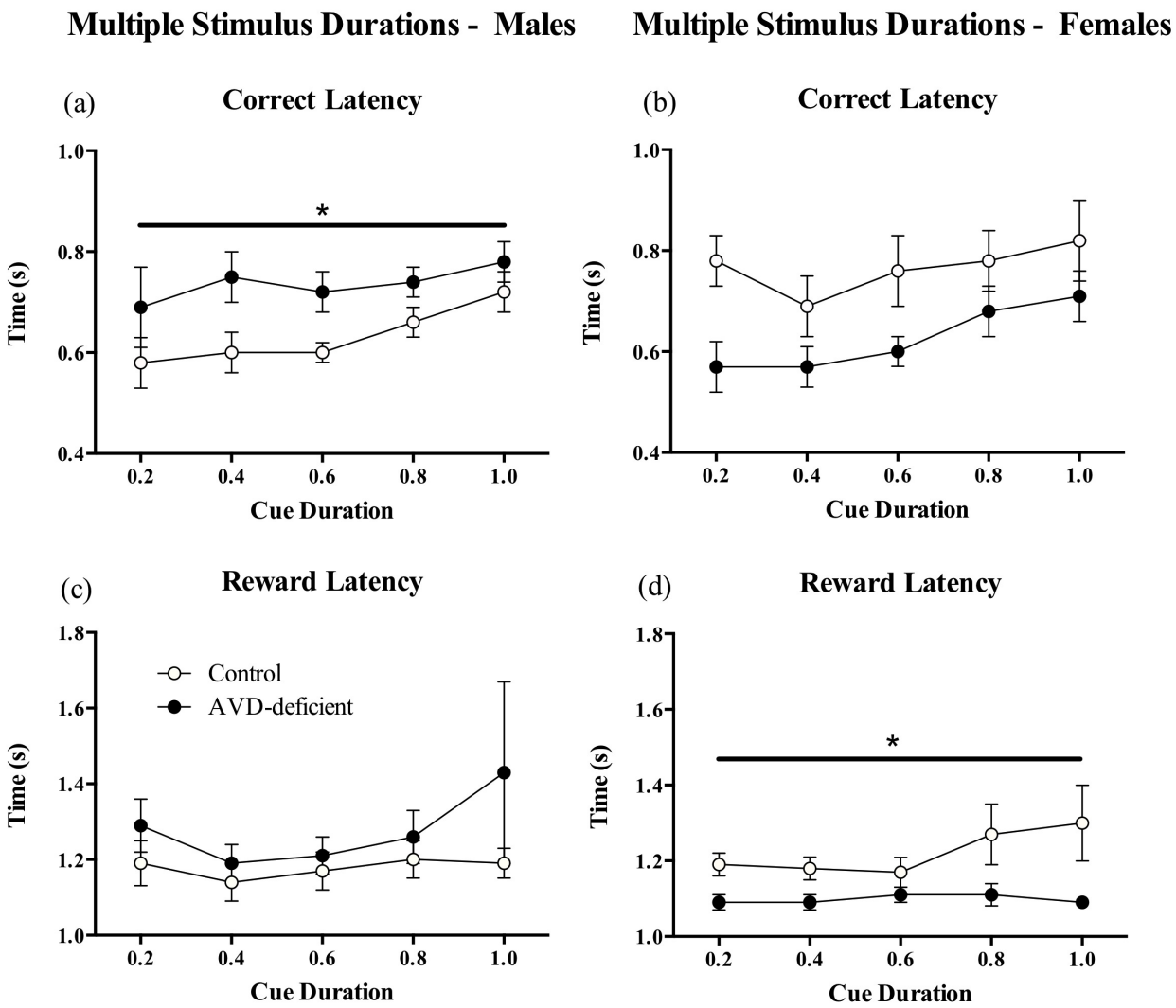

Figure 2.3 Latency results from manipulating stimulus duration in the 5C-SRT task
Male results are shown in **a, and c**, with female results shown in **b, and d**. The latency to make a correct response is shown in **a and b**, with AVD-deficient male mice taking longer to make correct responses compared to control males. The latency to collect reward is shown in **c and d**, with AVD-deficient female mice making quicker responses to collect reward compared to control females. Mean ± SEM (n = 6-7/group * p <0.05)

2.3.2.3 Multiple Inter-trial Intervals

The effects of varying the inter-trial interval during testing (1-9 s) was also analysed and averaged over three days of testing. Over the different inter-trial intervals, the omission rate and accuracy was relatively stable except for an increase in omissions ($F_{1,23} = 130.08$, $p < 0.001$) and a decrease in accuracy ($F_{1,23} = 123.51$, $p < 0.001$) after a 1 s inter-trial interval. There was no main effect of Diet or Sex on the number of correct responses, incorrect responses, accuracy or omission rates when varying the inter-trial interval, or on speed of responding. In male mice, there was a significant effect of Diet on the latency to make a correct response ($F_{1,12} = 5.93$, $p = 0.031$), with the AVD-deficient mice taking longer to make a response. In female mice, there was no significant effect of Diet of any measure, including reward latency ($F_{1,11} = 1.56$, $p = 0.237$).

2.3.2.4 Combined Data

When analysing both multiple stimulus durations and inter-trial intervals together, there was an overall significant effect of Diet in males for the number of correct responses ($F_{1,12} = 5.43$, $p = 0.038$) and latency to a correct response ($F_{1,12} = 6.64$, $p = 0.024$), while in females there was an overall significant effect of Diet on latency to receive a reward ($F_{1,11} = 5.61$, $p = 0.037$).

2.3.3 5 choice – continuous performance task

2.3.3.1 Training for 5 choice – continuous performance task

There was a main effect of Sex ($F_{1,23} = 5.72$, $p = 0.025$) but not of Diet ($F_{1,23} = 0.59$, $p = 0.451$) on the days to pass training for 5C-CPT, with females taking less time to pass compared to males (Figure 2.4).

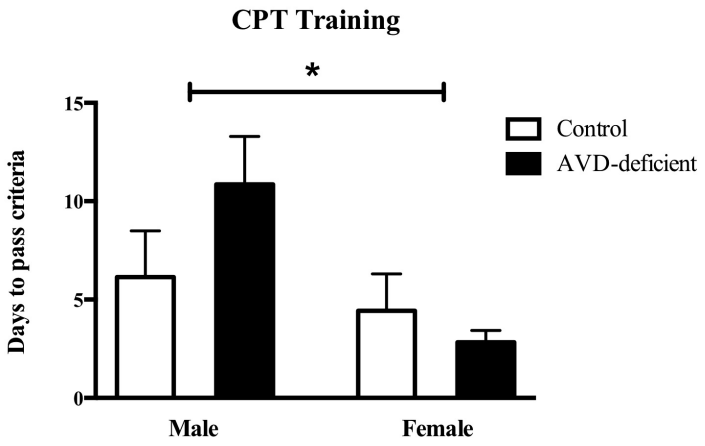

Figure 2.4 Days to pass criteria during training for 5C-CPT
There was a significant main effect of Sex, with females passing training for 5C-CPT in less time than males. Mean ± SEM (n = 6-7/group, * p <0.05)

Training for the 5C-CPT involved increasing trials to 120 per session with variable inter-trial intervals (3 -7 s). During the first two days of training, the ability of the mice to adapt to the change in protocol was assessed. However, during the first two days of training, there was a significant main effect of Diet ($F_{1,17} = 6.17$, $p = 0.024$) on the number of correct responses, with the AVD-deficient mice making significantly fewer correct responses compared to controls. When analysed separately for Sex, there was no significant difference in females for any measure during training and all female mice except one reached criteria for 5C-CPT and went on to testing. In male mice, there was a significant effect of Diet on the number of correct responses ($F_{1,8} = 5.45$, $p = 0.048$, Figure 2.5a) during the first two days of training with the AVD-deficient mice made significantly fewer correct responses. The AVD-deficient male mice made less head entries over these two days compared to controls ($F_{1,8} = 5.92$, $p = 0.041$, Figure 2.5c). They also made more omissions but this failed to reach significance ($F_{1,8} = 5.22$, $p = 0.052$, Figure 2.5e). 71% (n=5 out of 7) of male control mice reached criteria but only 29% (n=2 out of 7) of AVD-deficient mice reached criteria. Due to the inability of enough AVD-deficient male mice to reach criteria during training, the male mice were not tested on the 5C-CPT.

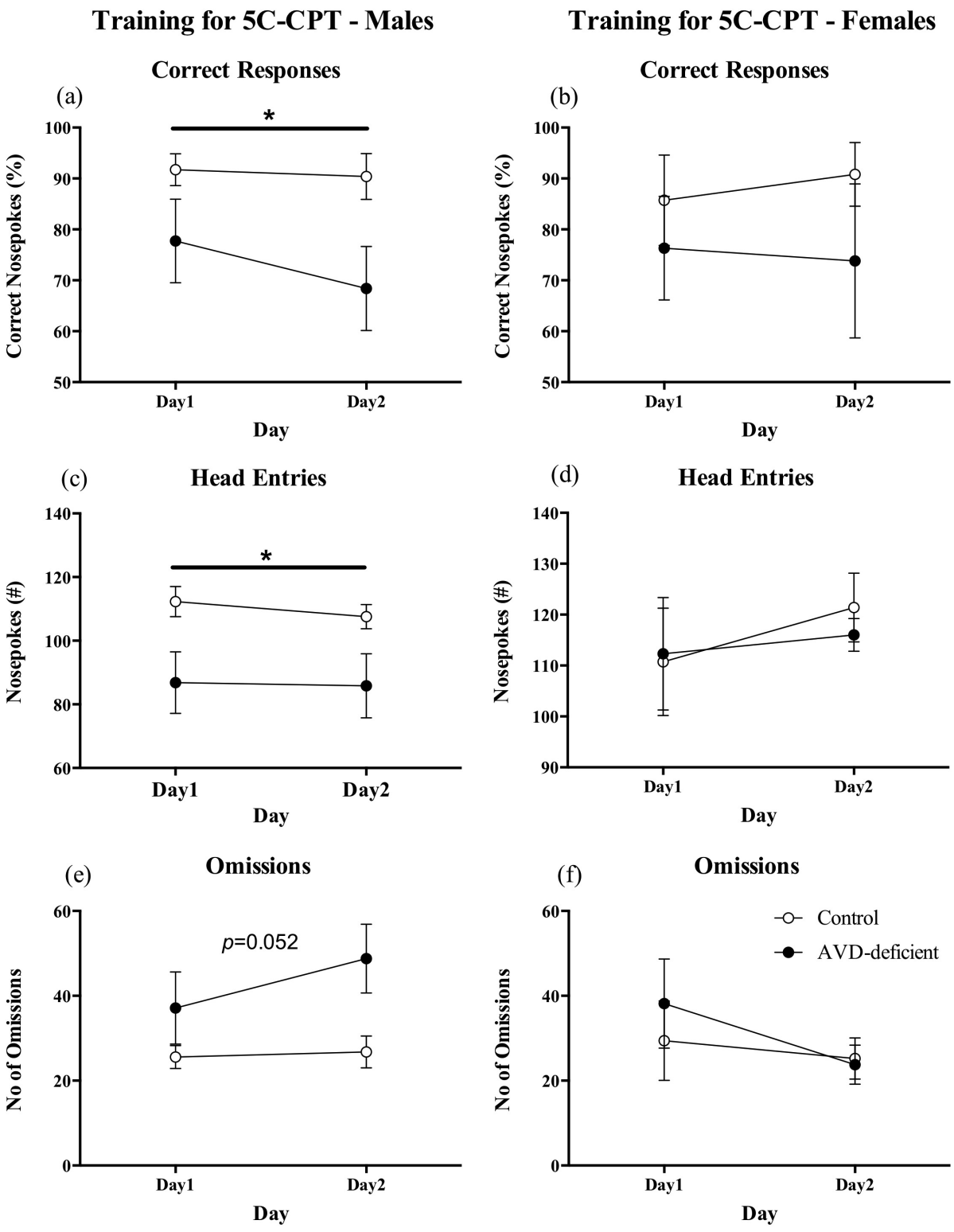

Figure 2.5 Results during training for 5C-CPT
Training involved increasing trials to 120 per session with variable inter-trial intervals (3 -7 s) (n = 5-7/group). Male results are shown in **a, c, and e**, with female results shown in **b, d, and f**. The percentage of correct responses are shown in **a and b**. There was a significant main effect of Diet on the percentage of correct responses given, with the AVD-deficient mice making fewer correct responses. This remained significant in males when separated for Sex, but not in females. The number of head entries into the reward magazine is shown in **c and d**, with AVD-deficient male mice making significantly fewer head entries compared to control males. The percentage of omissions is shown in **e and f**, with no significant effect of Diet. Mean ± SEM (* $p < 0.05$)

2.3.3.2 Testing on 5 choice – continuous performance task

Initially, all mice could not differentiate between target and non-target trials (d' = 0) but over the 20 sessions of 5C-CPT d' continually increased, demonstrating that all mice successfully learned to withhold a response during a non-target trial. AVD-deficient mice had similar levels of performance as controls on all measures analysed during the 5C-CPT, including p(Hit) ($F_{1,10} = 0.23$, $p = 0.639$, Figure 2.6a), p(FA) ($F_{1,10} = 0.08$, $p = 0.786$, Figure 2.6b), d' ($F_{1,10} = 0.38$, $p = 0.551$, Figure 2.6c) and B" ($F_{1,10} = 0.06$, $p = 0.820$, Figure 2.6d).

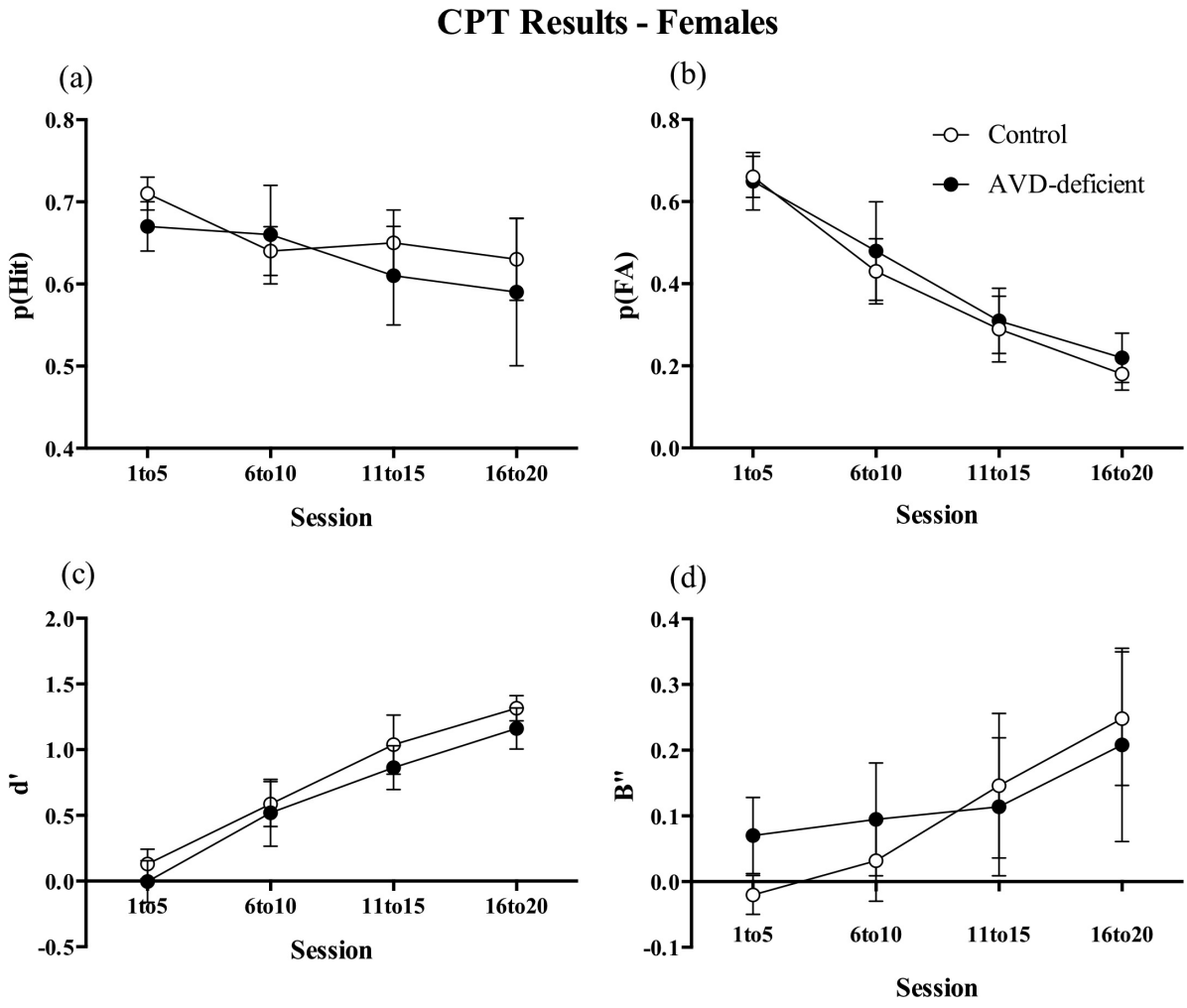

Figure 2.6 Results of the 5C-CPT in females
This figure shows the p(Hit) in **(a)**, p(FA) in **(b)**, d' in **(c)** and B" in **(d)** over the 20 testing sessions (four bins of five test sessions each). No significant effect of Diet was seen on any measure. Mean ± SEM (n = 6/group)

2.4 Discussion

The main finding from this study was that male AVD-deficient BALB/c mice showed deficits in attentional processes on the 5C-SRT. However, female AVD-deficient BALB/c mice were not different to controls on the attentional aspects of the task, although they did exhibit enhanced motivation (reduced latency to retrieve a reward) during the 5C-SRT task. To our knowledge this is the first study to show cognitive impairments with vitamin D deficiency in otherwise healthy adult rodents, in a model known to have normal serum calcium levels. Therefore, we can accept the hypothesis that AVD deficiency in male BALB/c mice can lead to cognitive deficits.

The male AVD-deficient mice took significantly longer to pass the initial training level for operant testing, compared to the control males. This may be due to difficulty in learning the rule initially or may be due to a reduction in exploratory behaviour. However, over all of the training levels there was no significant effect of the dietary manipulation. Furthermore, the AVD-deficient males were not significantly different to the control males in their number of correct responses during training (AVD-deficient males 82.56 ± 2.03 and Control males 85.01 ± 0.68, $p = 0.245$) and all mice reached the minimum criteria required during training and therefore could perform the task. This allowed different manipulations to be tested that would increase task difficulty and engage the prefrontal cortex to a greater degree (Robbins, 2002).

Male AVD-deficient mice were less accurate; took longer to respond when making a correct choice and were more likely to make an omission, without a change in the motivation to collect reward. Overall, these findings do not suggest a motor impairment in the AVD-deficient males or deficits in motivation. Motor impairments cannot explain a reduction in accuracy and longer response latencies without a change in reward latency are more likely to reflect deficits in decision processes (Robbins, 2002). Furthermore, according to Robbins (2002) increased omissions not accompanied by changes in reward latency may be due to gross impairments in attention.

The results seen here in the AVD-deficient males correspond to deficits seen following lesions to the medial PFC and the longer latency times suggest that the deficits may result in the animals trading speed for accuracy (Muir *et al.*, 1996). Lesion studies have also shown that the hippocampus is required for acquisition of the task (Bratt *et al.*, 1995) but not once the task has been acquired (Kirkby & Higgins, 1998). The 5C-SRT therefore requires both hippocampal-dependent learning and memory and PFC processes for different aspects of the tasks. These results suggest that AVD deficiency could have impacted on global cognitive function, rather than a specific cognitive domain.

It is well recognized that cognitive disturbances in schizophrenia are due at least in part to altered GABA and glutamate neurotransmission in the PFC (Lewis & Moghaddam, 2006). Furthermore, reductions in striatal glutamate levels have correlated selectively with performance on cognitive tests showing age-related decline (Zahr *et al.*, 2008). This is consistent with AVD deficiency in BALB/c male mice, in which we have shown an imbalance between excitatory and inhibitory neurotransmission with reductions in glutamate, glutamine and the enzymes GAD65 and 67, as well as increased levels of GABA across the whole brain (Groves *et al.*, 2013).

Post-mortem studies of the dorsolateral PFC of individuals with schizophrenia have shown reductions in mRNA levels of both GAD67 and a GABA membrane transporter 1 (GAT1), which is responsible for the reuptake of released GABA into nerve terminals, within subsets of parvalbumin GABA interneurons (Lewis *et al.*, 2005). Therefore, it is plausible that AVD deficient mice have altered activity of GABA interneurons, driven by changes in glutamate or GABA neurotransmission resulting in impaired attentional processes in male AVD-deficient BALB/c mice. However, we did not directly test this hypothesis in the current study and this would need to be investigated in future studies.

Despite seeing impairments in attention in male mice with AVD deficiency, there were no attentional impairments in AVD-deficient female mice in either the 5C-SRT task or the 5C-CPT. Neuropsychiatric and neurodegenerative disorders are often sexually dimorphic. For example, schizophrenia is more prevalent in young adult males compared to young adult females (Hafner, 2003); however, depression (Sagud *et al.*, 2002) and MS (Ahlgren *et al.*, 2011) are more prevalent in women compared to men.

Although MS is twice as prevalent in females as compared to males, the cognitive impairments that accompany MS are more common and are more severe in males (Beatty & Aupperle, 2002; Savettieri *et al.*, 2004). It is possible that sex hormones play a role in gender differences that are often seen in these types of disorders. There is evidence to suggest that females may be protected from deficits in cognition, but not from other aspects of these disorders, such as the autoimmune aspects of MS (Harbo *et al.*, 2013).

There is a large body of research on the protective effects of oestrogen, including in ischemic brain injury (Koh, 2014), for cognitive function, a reduction in AD risk and in schizophrenia (Pompili *et al.*, 2012). To determine if this is the mechanism behind a lack of cognitive impairment in female AVD-deficient mice, a future study would need to look at the effects of ovariectomising the mice and see if that leads to cognitive disturbances in AVD-deficient female mice. Future studies could also include testing the effects of AVD deficiency on other forms of cognitive tasks, such as a

hippocampal-dependent learning and memory task. This would determine if these deficits are specific to attention and PFC function or a more global deficit in performance. Furthermore, due to the necessity of using food-restriction with the 5C-SRT, a second test of cognition that doesn't involve food would determine if any additional metabolic stress resulting from food deprivation exacerbated the results seen in the AVD-deficient males.

Future studies should also determine if restoration of vitamin D levels would reverse the deficits seen with AVD deficiency, as this would have important health implications in humans. Vitamin D supplementation is a relatively easy and affordable treatment option if it could improve cognition in vitamin D deficient individuals. However, there is some evidence to suggest that treatment with vitamin D after the development of a disorder may be too late. A study looking at the effects of vitamin D deficiency on stroke outcomes in rats, indicated that vitamin D deficiency adversely affected outcomes of stroke and that treatment with vitamin D post-stroke was ineffective at improving outcomes (Balden *et al.*, 2012).

2.5 Conclusion

To our knowledge, this is the first study to show cognitive impairments with vitamin D deficiency in otherwise healthy adult male mice. Furthermore, we have shown sex differences in the susceptibility to vitamin D deficiency that should be explored further, with sex hormones as one possible explanation for this finding. This study provides the first clues to determine if preventing vitamin D deficiency in human populations may be able to prevent or delay cognitive impairments, particularly in at risk groups (McKenna, 1992).

Chapter 3 Structural and Diffusion Tensor Imaging in Adult Vitamin D-Deficient BALB/c Mice

3.1 Introduction

There is growing evidence to suggest that vitamin D deficiency can lead to cognitive impairments particularly later in life (Etgen *et al.*, 2012). For example, a study in community dwelling older woman showed that participants with deficient serum vitamin D levels had greater cognitive impairments on the Pfeiffer Short Portable Mental State Questionnaire, compared to participants with sufficient vitamin D levels (Annweiler *et al.*, 2010). Furthermore, a recent prospective study has shown that vitamin D deficiency was associated with accelerated decline in cognitive domains including episodic memory and executive function (Miller *et al.*, 2015).

Vitamin D deficiency has also been associated with increased risk of all-cause dementia and AD (Littlejohns *et al.*, 2014) as well as magnetic resonance imaging (MRI) indicators of cerebrovascular disease including white matter hyperintensities (WMHs) (Buell *et al.*, 2010). WMHs are areas of demyelination and axonal loss caused by chronic ischemia (Prins & Scheltens, 2015). Although, standard MRI can detect pathology such as WMHs, diffusion tensor imaging (DTI) may reveal more subtle impairments in white matter tract integrity before it becomes visible on standard MRI (Prins & Scheltens, 2015).

A recent study has shown that vitamin D deficiency in older individuals with memory complaints was associated with a disruption in neuronal integrity (Moon *et al.*, 2015). Researchers assessed DTI using tract-based spatial statistics (TBSS), a sensitive and objective method to assess changes in connectivity in the brain by measuring the anisotropic diffusion of water in white matter tracts (Smith *et al.*, 2006). Moon *et al.* (2015) found that vitamin D deficient patients have lower fractional anisotropy (FA) values primarily in frontal regions of the brain, compared to patients with sufficient vitamin D levels. FA is a measure of non-specific microstructural integrity (Pfefferbaum *et al.*, 2000). In addition, they found that the vitamin D deficient patients also had lower MMSE scores compared to the patients with sufficient vitamin D levels. In contrast to these findings, the researchers did not detect any differences in cortical thickness between vitamin D deficient and vitamin D sufficient patients (Moon *et al.*, 2015).

Reductions in hippocampal volume in subjects with AD are consistently reported in the literature (Jack *et al.*, 1999). However, there was no association between vitamin D and size of the hippocampus or amygdala, or total brain volumes after adjustment for intracranial volume, even though vitamin D deficiency was significantly associated with Alzheimer's disease (Buell *et al.*, 2010). Although there are limited studies addressing the effect of vitamin D deficiency on brain

volume and structure in adults, vitamin D does have a well-known role in cell proliferation, differentiation, survival and apoptosis (see Chapter 1 for review).

Both in vitro and in vivo studies have shown that the active form of vitamin D, $1,25(OH)_2D$, is antiproliferative and a pro-differentiator (Banerjee & Chatterjee, 2003). Moreover, studies have shown that vitamin D deficiency during development leads to increased proliferation and reduced apoptosis via altered gene regulation in rats (Ko *et al.*, 2004). Furthermore, rat pups that were exposed to vitamin D deficiency during development had a mild distortion in brain shape and increased lateral ventricle volumes, with reductions in differentiation and a lower expression of neurotrophic factors, such as nerve growth factor and glial cell line-derived neurotrophic factor (Eyles *et al.*, 2003). In contrast, DVD-deficient mice had reductions in lateral ventricular volumes, in C57BL/6J adult mice (de Abreu *et al.*, 2010; Harms *et al.*, 2012a) and in BALB/c mice in the fetal brain at E17.5 (Hawes *et al.*, 2015). DVD-deficient BALB/c mice also had elevated levels of brain derived neurotrophic factor (BDNF) and transforming growth factor β1 at E17.5 (Hawes *et al.*, 2015).

There have been no pre-clinical studies published to date that have looked at the effects of adult vitamin D deficiency on cognitive function and DTI. However, I have previously shown that adult vitamin D-deficient male BALB/c mice have mild cognitive impairments in attentional processing, assessed using the 5C-SRT task (see Chapter 2 for details).

Therefore, the first aim of this study was to assess control and AVD-deficient male BALB/c mice for volumetric differences in total brain volume and in a number of brain regions using automatic segmentation methods of 3D MRI images. In addition, the second aim of this study was to use ex-vivo DTI to measure connectivity in the brain using two well-known methods, voxel based morphometry (VBM) and TBSS (Smith *et al.*, 2006), in the control and AVD-deficient male mice. It was hypothesized that there would be no structural changes following 10 weeks of dietary manipulation in adult mice, however, that there would be disruptions to connectivity in the AVD-deficient mice.

3.2 Materials and methods

3.2.1 Animals and Housing

Fifteen ten-week old male BALB/c mice (Animal Resources Centre, Canning Vale, WA, Australia) were obtained and housed in groups of three-to-four in individually ventilated OptiMICE cages (Animal Care Systems, CO, USA), with bedding (Sanichips, Harlan Laboratories, USA) and

nesting material at the Queensland Brain Institute Animal House Facility, University of Queensland.

Mice (n = 7 control and 8 AVD-deficient) were assigned to either a control diet or a vitamin D-deficient diet as detailed in Chapter 2 until they were 20-weeks old. The mice were maintained on a 12-hour light-dark cycle (lights on at 07:00 h) with *ad libitum* access to food and water. They were housed under incandescent lighting free from UVB radiation. All experimental work was performed with approval from the University of Queensland Animal Ethics Committee, under the guidelines of the National Health and Medical Research Council of Australia.

3.2.2 Tissue collection

Behaviourally naïve mice were anaesthetized by an i.p injection of Lethabarb at 100 mg/kg body weight and transcardially perfused with 40 ml of phosphate buffered saline (PBS) and 40 ml of 4 % paraformaldehyde (PFA) in 0.1 M PBS. The brains were removed and post fixed in 4 % PFA for 24 h before being stored in sodium azide (0.05%) in 0.1M PBS (PBS azide) for future analysis.

3.2.3 Magnetic Resonance Imaging

Brains were incubated in 0.1% Magnevist® (gadopentetate dimeglumine, Bayer Healthcare Pharmaceuticals Inc., NJ, USA) in PBS for 4 days prior to imaging. MRI data were acquired with the samples immersed in Fomblin fluid (Solvay Solexis, Italy), using a 16.4 T (89 mm) Bruker micro-imaging system (Bruker Biospin, Karlsruhe, Germany) and a 15 mm SAW coil (M2M Imaging, USA). High-resolution anatomical images were acquired using three-dimensional (3D) gradient-echo FLASH (fast low angle shot) T_1/T_2*-weighted using repetition time (TR) 50 ms, echo time (TE) 12 ms, number of excitation averaging (NEX) 1 at 30 micron isotropic resolution. 3D Diffusion-weighted images (DWI) were acquired using Stjskal-Tanner DW spin-echo sequence using the following parameters: TR = 400 ms; TE = 22.8 ms; 80 micron isotropic resolution and a signal average of 1. DWI data were composed of two images acquired without diffusion weighting (b_0) and thirty direction DWI (b value of 5000 s/mm^2, δ/Δ = 2.5/14 ms). The samples were maintained at 22 ° C (Kurniawan *et al.*, 2014).

3.2.4 Brain volumetric measurements using segmented 3D MRI atlas

Anatomical MR images were cleaned of any non-brain tissue using a mask created in ITK-SNAP (www.itksnap.org). Inter-group differences in brain volumes were mapped using VBM (Ashburner & Friston, 2000). Firstly, a study-specific BALB/c template was created using Advanced Normalization Tools (ANTs) buildtemplateparallel script (http://picsl.upenn.edu/software/ants/),

where high-resolution gradient-echo images of all mice from the study (N = 15) were aligned to a common reference space via a 12 degrees-of-freedom affine alignment, followed by 5 iterative symmetric diffeomorphic registrations (Avants *et al.*, 2011). Secondly, the resulting BALB/c template was registered using ANTs diffeomorphic registration to an adult C57BL/6J MRI atlas that contains 18 segmentations of brain structures (Ma *et al.*, 2005). Finally, the warp information from the previous steps was used to register the segmented structures back to the individual subjects for analysis of inter-group differences in brain region volumes. Volumes for each brain segment were calculated using ITK-SNAP. Inter-group statistical analyses of volumes were carried out using a student's t-test. See Table 3.1 for brain regions analysed.

3.2.5 Voxel based morphometry of diffusion tensor imaging maps

Non-brain tissue was removed from diffusion magnetic resonance images by creating a mask of each brain in ITK-SNAP. DTI maps, consisting of fractional anisotropy (FA), mean diffusivity (MD), axial diffusivity (AxialD) and radial diffusivity (RadialD), were generated using MRTrix 0.2.9 (www.mrtrix.org). A study specific FA template was created using ANTs buildtemplateparallel script, and subsequently the other of DTI parameters were co-registered to the template space. VBM analyses were performed using FSL randomise (fsl.fmrib.ox.ac.uk) using 5000 permutations (Nichols & Holmes, 2002), threshold-free cluster enhancement and correction for multiple comparisons. The resulting t-map was thresholded > 0.95 to assess statistical significance.

3.2.6 Tract-based Spatial Statistics

The FA, MD, AxialD, and RadialD maps were assessed for inter-group differences using TBSS as previously described (Smith *et al.*, 2006). All FA images were aligned onto the ANTs FA template using a nonlinear registration algorithm within the TBSS package and the white matter tracts were skeletonized (using FA threshold of 0.3) to create a skeleton template. Subsequently, all DTI maps from each subject were skeletonized. The FA skeleton from each sample was registered to the template using TBSS nonlinear registration method. The other DTI maps (MD, AxialD, and RadialD skeletons) were co-registered to the template by applying the same transformation calculated for the FA. TBSS analyses were performed using 5000 permutations (Nichols & Holmes, 2002), threshold-free cluster enhancement and correction for multiple comparisons. The resulting t-map was thresholded > 0.95 to assess statistical significance.

3.3 Results

3.3.1 Anatomical measurements using automatic segmentation

There was no significant effect of Diet ($F_{1,13} = 0.16$, $p = 0.698$) on total brain volumes (Controls = 387.35 ± 3.90 mm^3 and AVD-deficient = 383.69 ± 7.90 mm^3). Total brain volumes excluded the olfactory bulbs, which are sometimes damaged during brain extraction. There was also no significant effect of Diet on any white matter structure volume ($F_{1,13} < 2.20$, $p > 0.160$) or brain region volume ($F_{1,13} < 1.49$, $p > 0.244$). See Table 3.1 for details.

Table 3.1 Volumetric brain analyses

Brain Volumes (mm^3)	Control (n = 7)	AVD-deficient (n = 8)
White Matter Structures[#]		
Anterior commissure	1.59 ± 0.03	1.60 ± 0.03
CC/Ext capsule	9.43 ± 0.11	9.29 ± 0.26
Fimbria	1.97 ± 0.02	1.91 ± 0.04
Internal capsule	1.67 ± 0.01	1.62 ± 0.05
Brain Regions[#]		
Amygdala	9.53 ± 0.08	9.46 ± 0.23
Basal forebrain	9.68 ± 0.11	9.76 ± 0.15
Rest of midbrain	10.49 ± 0.09	10.29 ± 0.18
Brain stem	48.11 ± 0.62	46.19 ± 1.36
Caudate putamen	20.63 ± 0.23	20.55 ± 0.36
Central grey	3.39 ± 0.05	3.39 ± 0.07
Cerebellum	41.76 ± 0.95	40.76 ± 1.14
Globus pallidus	2.12 ± 0.02	2.08 ± 0.05
Hippocampus	22.98 ± 0.12	22.92 ± 0.37
Hypothalamus	9.47 ± 0.12	9.47 ± 0.18
Inferior colliculi	4.19 ± 0.06	4.06 ± 0.10
Superior colliculi	6.58 ± 0.10	6.55 ± 0.14
Neocortex	109.02 ± 1.13	109.36 ± 1.98
Thalamus	20.85 ± 0.26	20.85 ± 0.46
Total Brain volume[$]	387.35 ± 3.90	383.69 ± 7.90

[#]The volumes of white matter structures and segmented brain regions were calculated using the registration of segmented 3D atlas

[$]Total brain volume was calculated excluding the olfactory bulbs.

There were no significant differences in brain volumes between control and AVD-deficient mice.

3.3.2 Diffusion tensor imaging

VBM and TBSS analyses of the FA, MD, AxialD, and RadialD maps showed no significant differences between control and AVD-deficient mice throughout the brain following threshold-free cluster enhancement and correction for multiple comparisons. Mean whole brain values were also analysed for FA ($F_{1,13} = 0.47$, $p = 0.505$) and MD ($F_{1,13} = 1.03$, $p = 0.330$) and there were no significant differences seen between control and AVD-deficient mice. See Table 3.2.

Table 3.2 Mean whole brain values for DTI parameters

Variable	Control (n = 7)	AVD-deficient (n = 8)
Whole brain FA	0.225 ± 0.005	0.216 ± 0.002
Whole brain MD, x10^{-3} mm^2/s	0.301 ± 0.003	0.297 ± 0.003

There were no significant differences between control and AVD-deficient mice for whole brain DTI parameters.

3.3.3 Post-hoc analysis

Overall, following correction for multiple comparisons across thousands of voxels there were no significant differences observed between control and AVD-deficient mice for any of the DTI parameters. Therefore, in the context of the current study, AVD deficiency did not alter connectivity or neuronal integrity following 10 weeks of dietary depletion in adult male BALB/c mice. However, there may be subtle differences in connectivity that cannot be picked up with a whole brain analysis. Therefore, future experiments should rely on a priori hypotheses regarding specific brain regions or white matter tracts that may be more susceptible to connectivity changes. For example, changes to white matter might be more visible in the larger white matter tracts; or in brain regions highly relevant to cognition such as the hippocampus or prefrontal cortex. Moon *et al.* (2015) found lower FA values in vitamin D deficient patients using TBSS primarily in frontal regions, including the largest white matter commissure, the corpus callosum.

3.3.3.1 Corpus callosum and hippocampal commissure

Therefore, as a hypothesis generating post-hoc analysis, the two largest commissural bundles, the corpus callosum and the hippocampal commissure were analysed using TBSS. Compared with controls, AVD-deficient mice had lower MD values and to a lessor extent, lower FA values ($p <$ 0.01, Figure 3.1) in regions of both commissures.

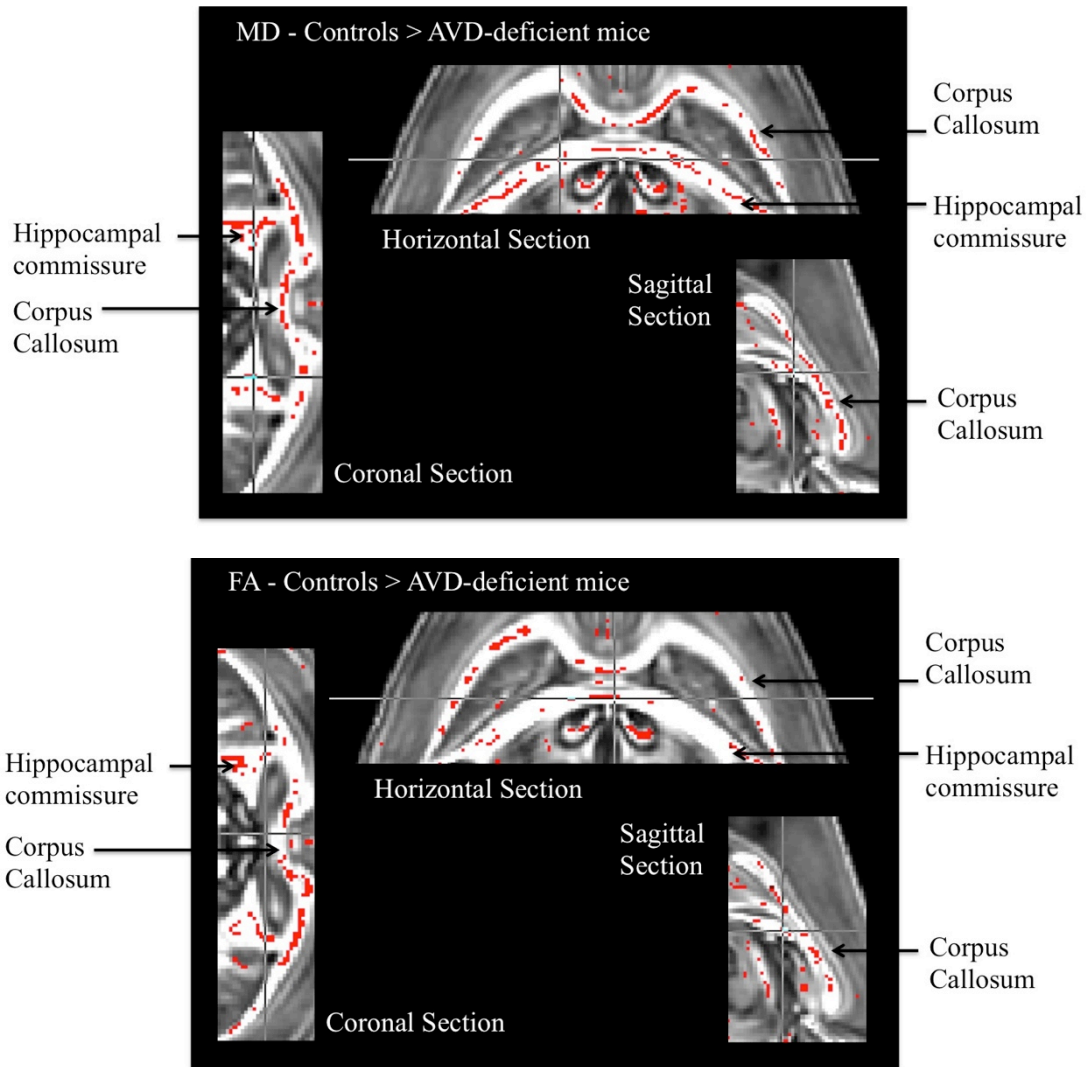

Figure 3.1 Post-hoc analysis of TBSS results for MD and FA values
Red = Controls > AVD-deficient mice ($p < 0.01$), overlaid on the MNI152 standard image. TBSS, Tract-based spatial statistics; FA, fractional anisotropy; MD, mean diffusivity.

3.4 Discussion

The results from the current study have indicated that AVD deficiency does not produce any gross structural abnormalities. Furthermore, this study has also shown that connectivity following whole brain analysis, measured by DTI was not altered with AVD deficiency. Post-hoc analysis suggests that there may be minor changes in connectivity within the larger white matter tracts, however this would need to be further explored in future studies designed to specifically address this.

At a gross structural level, the brains of AVD-deficient mice were not different to controls across four large white matter tracts and fourteen specific brain regions. Although, a developmental depletion of vitamin D deficiency can lead to changes in brain shape and size (Eyles *et al.*, 2009b), this did not occur following an adult exposure to vitamin D deficiency. This is consistent with the limited research performed to date, suggesting that vitamin D deficiency during adulthood does not produce changes in brain volumes.

Although, there is significant epidemiological evidence on the association between vitamin D deficiency and cognitive impairment, dementia, AD and cerebrovascular disease (Annweiler *et al.*, 2010; Buell *et al.*, 2010; Littlejohns *et al.*, 2014), very few studies have looked at the relationship between vitamin D deficiency and MRI pathologies in humans (Buell *et al.*, 2010; Moon *et al.*, 2015). Moreover, this is the first animal study assessing the effects of vitamin D deficiency on DTI.

Using both VBM and TBSS, there were no differences between control and AVD-deficient mice on a range of standard DTI parameters including FA, a measure of microstructural integrity (Pfefferbaum *et al.*, 2000). Whereas a single study performed in humans, which was the first of its kind, showed that vitamin D deficient patients with memory complaints had lower FA values in most areas of the brain (Moon *et al.*, 2015). However, we have not replicated this finding in our mouse model.

Recently, researchers using the same facility and a similar methodology found significant differences could be detected at a level of 10-15 % change. Other studies to measure the effect of learning and exercise using ex vivo samples showed that they could detect minimum changes as low as 3-5 % (Cahill *et al.*, 2015). However, the difference between one mouse brain and another may already be 5-10 % due to the fixation effect, plus additional distortion or damage that can occur to the brain when they are removed from the skull. Despite these limitations, the current study was effective as a preliminary study to determine that there were no large significant changes following AVD deficiency.

Previous studies have shown that reductions in FA values are consistently found in both AD patients, and those patients suffering from mild cognitive impairment (Medina *et al.*, 2006; Zhang *et al.*, 2007) and it's been proposed that changes in white matter microstructural integrity proceeds the development of dementia (Medina *et al.*, 2006). The model of AVD deficiency used in the current study has previously been shown to produce mild cognitive impairments in attention. Post-hoc analysis did suggest that there may have been subtle changes in connectivity, which may play a role in these impairments; however the changes were not significant enough to be identified using the formal analysis of DTI from whole brain.

The model of AVD deficiency used in the current study is relatively mild in comparison to other mouse models of vitamin D deficiency. These mice are only deficient for 10 weeks prior to testing and are young adults when first placed on the diet. One of the considerations in choosing this model of AVD deficiency was to limit confounding factors such as musculoskeletal deficits and hypocalcemia and we have successfully done so. However, it may be that a longer period of deficiency would be required before significant disruptions in neuronal integrity are visible using DTI. Future studies should examine the effects of an extended period on vitamin D deficiency prior to MRI or alternatively, since most of the studies linking vitamin D deficiency to cognitive impairment, Alzheimer's disease and MRI pathology have occurred in elderly populations, a future experiment should test the effects of AVD deficiency in older mice and assess both cognitive domains and connectivity in the brain using DTI.

Although, AVD deficiency did not alter gross structures within the brain, this does not preclude more subtle effects of AVD deficiency on cell proliferation or differentiation, such as an effect on adult hippocampal neurogenesis. Neurogenesis in the adult brain is limited to two main areas, the sub ventricular zone and the dentate gyrus of the hippocampus (Gage, 2002). Adult hippocampal neurogenesis is important in learning and memory (Yau *et al.*, 2015) and with significant associations observed between vitamin D deficiency and cognitive impairment; it is possible that AVD deficiency could be altering adult hippocampal neurogenesis, which would not be detected by a whole brain MRI analysis. This is explored in Chapter 4.

3.5 Conclusion

This is the first study in mice to assess the effects of AVD deficiency on brain structure and connectivity using MRI. We can conclude that using the current model of AVD deficiency there were no gross structural abnormalities or any significant changes in connectivity measured by DTI at the whole brain level. This does not rule out an effect of AVD deficiency on more subtle changes in specific areas (such as the corpus callosum or hippocampal commissure) or disturbances in other

aspects of brain function, such as neurotransmission or altered rates of proliferation and neurogenesis.

Chapter 4 Behavioural Effects of Adult Vitamin D Deficiency in BALB/c Mice are not Associated with Proliferation or Incorporation of Adult Born Hippocampal Neurons

4.1 Introduction

Epidemiological studies suggest that vitamin D deficiency during adulthood may be linked to adverse brain-related outcomes, such as cognitive impairment and depression in adults (Balion *et al.*, 2012; Anglin *et al.*, 2013); as well as in neuropsychiatric and neurodegenerative diseases (Cui *et al.*, 2013a; Eyles *et al.*, 2013). I have now shown that AVD deficiency in BALB/c male mice is associated with impaired cognition (Chapter 2), but had no impact on gross brain structure following 10 weeks of deficiency (Chapter 3).

A large population-based study showed that depression status and severity was associated with both decreased serum vitamin D and increased serum parathyroid hormone in older individuals (Hoogendijk *et al.*, 2008). A recent clinical trial was undertaken in adults suffering from depression who were also vitamin D deficient. Participants were given either a single dose of 150,000 IU or 300,000 IU vitamin D or no treatment and were tested again three months later. The single dose of 300,000 IU vitamin D not only proved safe but was also associated with a significant improvement in depression (Mozaffari-Khosravi *et al.*, 2013).

It has been hypothesized that hippocampal neurogenesis may play a role in the aetiology of depression, although overall studies have shown complex and inconsistent results, as reviewed by Miller and Hen (2015). It also remains unclear if this process mediates the link between vitamin D status and brain outcomes.

Vitamin D is known to be anti-proliferative and have pro-differentiation effects, as shown by the addition of $1,25(OH)_2D$ to cultures of normal and malignant cell lines (Ylikomi *et al.*, 2002; Banerjee & Chatterjee, 2003). Moreover, vitamin D has been shown to regulate a variety of neurotrophic factors, including NGF, providing further evidence for its ability to influence neuronal proliferation, differentiation, survival and growth (Korsching *et al.*, 1985; Maisonpierre *et al.*, 1990; Chao, 1994; Tomac *et al.*, 1995).

The addition of $1,25(OH)_2D$ to cultures of neonatal subventricular zone led to reduced neurosphere formation (Cui *et al.*, 2007), once again supporting the anti-proliferative nature of vitamin D in development. However, a recent study has shown that the addition of $1,25(OH)_2D$ to neural stem cells harvested from adult mice resulted in significantly enhanced proliferation and also drove them towards a more mature differentiated morphology (Shirazi *et al.*, 2015). The neural stem cells expressed significantly more NT-3, BDNF, ciliary neurotrophic factor and GDNF when cultured with $1,25(OH)_2D$ (Shirazi *et al.*, 2015). Although both studies confirm a role for vitamin D in neuronal proliferation, the results were mixed, perhaps due to the limitations involved with cell

culture studies. Therefore, in vivo studies are required to clarify the role of vitamin D in neuronal proliferation and differentiation.

Vitamin D deficiency during gestation in Sprague-Dawley rats led to increased cell proliferation in embryos via altered gene expression of many cell cycle genes (Ko *et al.*, 2004). Moreover, the absence of vitamin D during gestation led to greater proliferation of neuroprogenitor cells, as seen by increased neurosphere production from cells dissociated from neonatal rat subventricular zone (Cui *et al.*, 2007), providing further evidence from an animal model that the absence of vitamin D up-regulates proliferation during development. These studies were based on a developmental model of vitamin D deficiency (i.e. the rodents were exposed to low vitamin D only during gestation and early life). There is a paucity of information relating to neurogenesis and adult vitamin D deficiency.

A 1α-hydroxylase knockout mouse that lacks the ability to make $1,25(OH)_2D$, the active form of vitamin D, has been used to assess the effects of $1,25(OH)_2D$ deficiency on various aspects of adult hippocampal neurogenesis including proliferation and differentiation (Zhu *et al.*, 2012). In the 1α-hydroxylase knockout, hippocampal neurogenesis was no different to their wild type or heterozygote littermates at three weeks of age (Zhu *et al.*, 2012). However, the complete absence of $1,25(OH)_2D$ by eight weeks of age was associated with enhanced proliferation of progenitor cells in the dentate gyrus and a decrease in the survival of newborn cells without affecting differentiation (Zhu *et al.*, 2012). This study suggested that the absence of vitamin D may be linked to the regulation of adult hippocampal neurogenesis.

In the BALB/c strain, AVD deficiency produced changes in amino acid metabolism within the brain, including decreased levels of glutamine and glutamate and increased levels of GABA and glycine (Groves *et al.*, 2013). Studies have shown that one of the main molecules catabolized during cell proliferation is glutamine, as it is required in large quantities to support cell growth and division (Vander Heiden *et al.*, 2009). With AVD-deficient BALB/c mice exhibiting a reduction in brain glutamine levels, this lends further support to the hypothesis that adult depletion of vitamin D in the BALB/c strain may impair neurogenesis.

Adult neurogenesis is commonly investigated using markers for proliferation and incorporation of new neurons. Ki-67 protein, an endogenous protein only present during active phases of the cell cycle, allows the assessment of cell proliferation. A widely used molecule that marks newly dividing cells by integrating into DNA during mitosis, 5-Bromo-2'-Deoxyuridine (BrdU), is also a useful marker of cell proliferation (Gratzner, 1982). BrdU can be co-localised with a neuron-specific nuclear protein, neuronal nuclei (NeuN), which is specific for mature neurons and

expressed after terminal differentiation. Co-localisation of BrdU with NeuN marks newly generated neurons produced during BrdU administration (Mullen *et al.*, 1992) that have incorporated into the hippocampus.

Voluntary wheel running is robustly associated with increased hippocampal neurogenesis in adult mice (van Praag, 2008; Clark *et al.*, 2011) and therefore is widely used to stimulate this process above low baseline rates. For example, eight-week old BALB/cByJ mice with access to running wheels for 43 days had a 450% increase in $BrdU^+$ $NeuN^+$ neurons in the dentate gyrus compared to sedentary mice (Clark *et al.*, 2011). Although this protocol does not capture rates of proliferation, assessing the number of surviving newborn neurons is a functional readout of the integration of newborn neurons into the hippocampus. In combination, the assessment of cell proliferation and incorporation in an enhanced neurogenesis model should allow detection of any impact of AVD deficiency on these two aspects of adult hippocampal neurogenesis.

The forced swim test (FST) measures behavioural despair in rodents and is a well established behavioural assay related to the assessment of antidepressant agents (Porsolt *et al.*, 1977). We previously investigated the effects of AVD deficiency on the FST in group-housed male BALB/c mice and showed no impact of the dietary manipulation (Groves *et al.*, 2013). However, BALB/c mice are a highly anxious strain and all mice spent a significant time immobile. It was proposed that there may have been a ceiling effect that prevented an effect of AVD deficiency from being apparent (Groves *et al.*, 2013). In addition to stimulating neurogenesis, voluntary wheel running in mice has been shown to reduce immobility in both the FST and tail suspension tests (Duman *et al.*, 2008; Cunha *et al.*, 2013). To our knowledge, the effect of voluntary wheel running on immobility time in the FST has not been previously investigated in BALB/c mice. Impact of AVD deficiency on immobility time in the FST may indicate alterations in hippocampal neurogenesis.

The aims of this study were to test the effects of AVD deficiency on hippocampal neurogenesis. Our primary hypothesis was that AVD deficiency would alter proliferation and incorporation of adult born hippocampal neurons and that the increase in hippocampal neurogenesis stimulated by wheel running would be blunted by AVD deficiency. We aimed to test this hypothesis by assessing the number of $Ki67^+$ cells as a marker of cell proliferation following behavioural tests, and the number of $BrdU^+$ $NeuN^+$ cells, as a measure of the number of surviving newborn mature neurons integrated into the dentate gyrus, at baseline and following voluntary wheel running. Secondly, we hypothesised that AVD-deficient mice would demonstrate behavioural correlates of vitamin D deficiency, specifically, spending *more time immobile* compared to controls in the FST, and this would correlate with altered neurogenesis as measured by proliferation and incorporation. We

aimed to test this by exploring the effect of wheel running on immobility time the FST, and by assessing the correlation between neurogenesis and immobility time.

4.2 Materials and Methods

4.2.1 Animals and housing

A total of 87 BALB/c mice (57 male and 30 female) were used in this study. Ten-week old BALB/c mice (Animal Resources Centre, Canning Vale, WA, Australia) were obtained and housed in groups of four in individually ventilated OptiMICE cages, with bedding and nesting material at the Queensland Brain Institute Animal House Facility, The University of Queensland, Australia.

The mice were assigned to either a control diet or a vitamin D-deficient diet as detailed in Chapter 2 for 10 weeks prior to the start of behavioural testing and for the entire duration of the experimental procedures. The mice were maintained on a 12-hour light-dark cycle (lights on at 07:00 h) with *ad libitum* access to food and water. They were housed under incandescent lighting free from UVB radiation. All experimental work was performed with approval from The University of Queensland Animal Ethics Committee, under the guidelines of the National Health and Medical Research Council of Australia.

4.2.2 Experimental Design

After 10 weeks on the diet, the first wave of mice was separated into the following groups; twenty-eight mice (n = 5-8/sex/group) were individually housed with running wheels and 28 mice (n = 6-9/sex/group) were individually housed without running wheels, for the next six weeks. An additional four mice (2 male and 2 female) were individually housed without running wheels as non-BrdU injected controls. These mice were not included in the behavioural analysis but were used as a negative control to ensure that the only BrdU analysed was from the injections of BrdU. Mice were placed into the following five groups: Control Runner, Control Non-runner, AVD-deficient Runner, AVD-deficient Non-runner and No BrdU Control.

Wheel rotations for running groups were collected in 60 min time bins over the 6-week period using automated Wheel Manager software. During the first 10 days of individual housing all mice, excluding the non-BrdU controls, received a daily i.p injection of 50 mg/kg of BrdU at the same time each day, between 1000-1200 h, to assess hippocampal neurogenesis at the end of the behavioural testing. After 16 weeks on the diet, and at the end of running wheel access for the Running groups, mice were tested in a 30 min activity monitor test and in the FST (see section on behavioural testing below for full details of these methods). These tests were chosen as they are

altered by wheel running (Duman *et al.*, 2008) and the forced swim test was included as a measure of behavioural despair.

The second wave of mice included 27 male mice, 14 mice (n = 6-8/group) were individually housed with running wheels and 13 mice (n = 6-7/group) were individually housed without running wheels, for the next six weeks. These mice were not injected with BrdU, but were only analysed for serum 25(OH)D levels after 16 weeks on the diet.

4.2.1 Serum 25(OH)D levels

At the completion of experimental procedures, mice from the second wave were euthanized by i.p injection of an overdose of Lethabarb at 4 ml/kg body weight and a terminal blood sample was collected using a 25 gauge needle and a 3 ml syringe via cardiac puncture and blood was transferred to 1.5 ml eppindorf tubes. Blood was allowed to clot for 1h and then centrifuged for 5 min at 13,000 rpm. The sera was separated and then stored at -20 °C until testing. The levels of 25(OH)D was measured in serum samples using liquid chromatography-tandem mass spectrometry on a 4000 QTrap API AB mass spectrometer (Eyles *et al.*, 2009a).

4.2.2 Behavioural Testing

4.2.2.1 Activity monitor

Activity monitors were used to measure baseline locomotion (Walsh & Cummins, 1976). Eight open field activity monitors were used. The arenas were 27.5 x 27.5 x 30 cm high and made of clear Perspex with two arrays of 16 x 16 infrared beams. One array of beams was low (1 cm above floor) so that beams were broken when the animal was present or moving, and another high (8 cm above floor) so beams were broken only when the animal reared. Mice were placed in the centre of the arena and their activity was monitored for 30 min, in which time the Med Associates software used the beam break data to triangulate the position of the mouse and measure the distance travelled by the mouse over time.

4.2.2.2 Forced swim test

The apparatus used in the forced swim test was a clear round container (20 cm high x 14 cm diameter) with a column of water (16 cm deep) maintained at 25 °C. Each mouse was placed in the container for six min and recorded using a USB digital camera and recording software. Activity was scored using the mobility threshold settings within the Ethovision software by measuring the

percentage change in area of the tracked object from one sample to the next. Immobility was defined as less than 5% movement using these settings and was validated by a human observer.

4.2.3 Immunohistochemistry

To ensure our results were comparable with previous research on exercise induced hippocampal neurogenesis, immunohistochemistry was conducted following established protocols (Kempermann *et al.*, 1997; Clark *et al.*, 2011).

4.2.3.1 Perfusions

All mice were anaesthetized by an i.p injection of Lethabarb at 100 mg/kg body weight 24 h after FST and transcardially perfused with 40 ml of PBS and 40 ml of 4 % PFA in 0.1 M PBS. The brains were removed and post fixed in 4 % PFA for 24 h before being stored in PBS azide. BrdU incorporation was assessed in $n = 8$ per group. Brains included in the neurogenesis analysis were selected blind to behavioural results. Brains were transferred to a 30 % sucrose solution for 24 h and then rapidly frozen and 50 μm sections containing the hippocampus were cut coronally using a sledge microtome and stored in PBS azide at 4 °C until used.

4.2.3.2 Double-labelled immunofluorescence

A one-in-five series was stained to identify newly divided mature neurons. Sections were rinsed with PBS azide to remove all trace of sucrose. Sections were incubated in 1M HCl for 20 min at 40° C to denature DNA, then rinsed with 0.1M Boric acid for five min. The sections were then incubated in Antigen Recovery Solution for 30 min at 60° C, followed by 3x5 min rinses with PBS azide. Sections were blocked at RT with a solution of 3 % normal goat serum (NGS) and 0.05 % Saponin in PBS azide. Sections were incubated with the primary antibodies; rat anti-BrdU (1:500) (Accurate Chemical, NY, USA) and mouse anti-NeuN (1:500) (Merck, Millipore, MA, USA), or with the primary antibody, rabbit anti-Ki67 (1:5000) (Abcam, Cambridge, UK) for 56 h at 4° C. Sections were then washed for 3x15 min in PBS azide. Secondary antibodies raised in donkey or goat were conjugated with fluorescent markers (Alexa fluor-488 anti-rat (1:2000) and Alexa fluor-647 anti-mouse (1:500) (Jackson Lab. Inc, PA, USA), and Alexa fluor-568 anti-rabbit (1:1000) (Thermo Fisher, IL, USA) and the sections were incubated with the secondary antibodies overnight at 4° C. Sections were then washed with 1:1000 DAPI in PBS azide for 15 min, followed by 2x15 washes in PBS azide. Sections were mounted from a solution of chrome alum gelatin onto slides with DABCO.

4.2.3.3 Image analysis

Fluorescence images were captured on a Zeiss Axio Imager Z1 microscope using a 20x 0.8NA PlanApo objective and Zeiss AxioCam HRm camera. Optical sectioning was achieved using ApoTome structured illumination and the large area of the dentate gyrus was captured using the tiled imaging and stitching features of Zen 2009 software. Cells that exhibited complete incorporation of BrdU and those that exhibited partial accumulation of BrdU were all counted as $BrdU^+$ $NeuN^+$ cells. Cells with nuclei completely incorporated with BrdU and colabeled with NeuN, have not undergone any divisions. Partially immunolabelled cells, where nuclei were stained with NeuN but BrdU was only partially incorporated, have either undergone division after BrdU was injected into the animals (daughter cells) or were born late in the accumulation window (Figure 4.1). For Ki67 immunoreactivity, only $Ki67^+$ cells were included in the analysis.

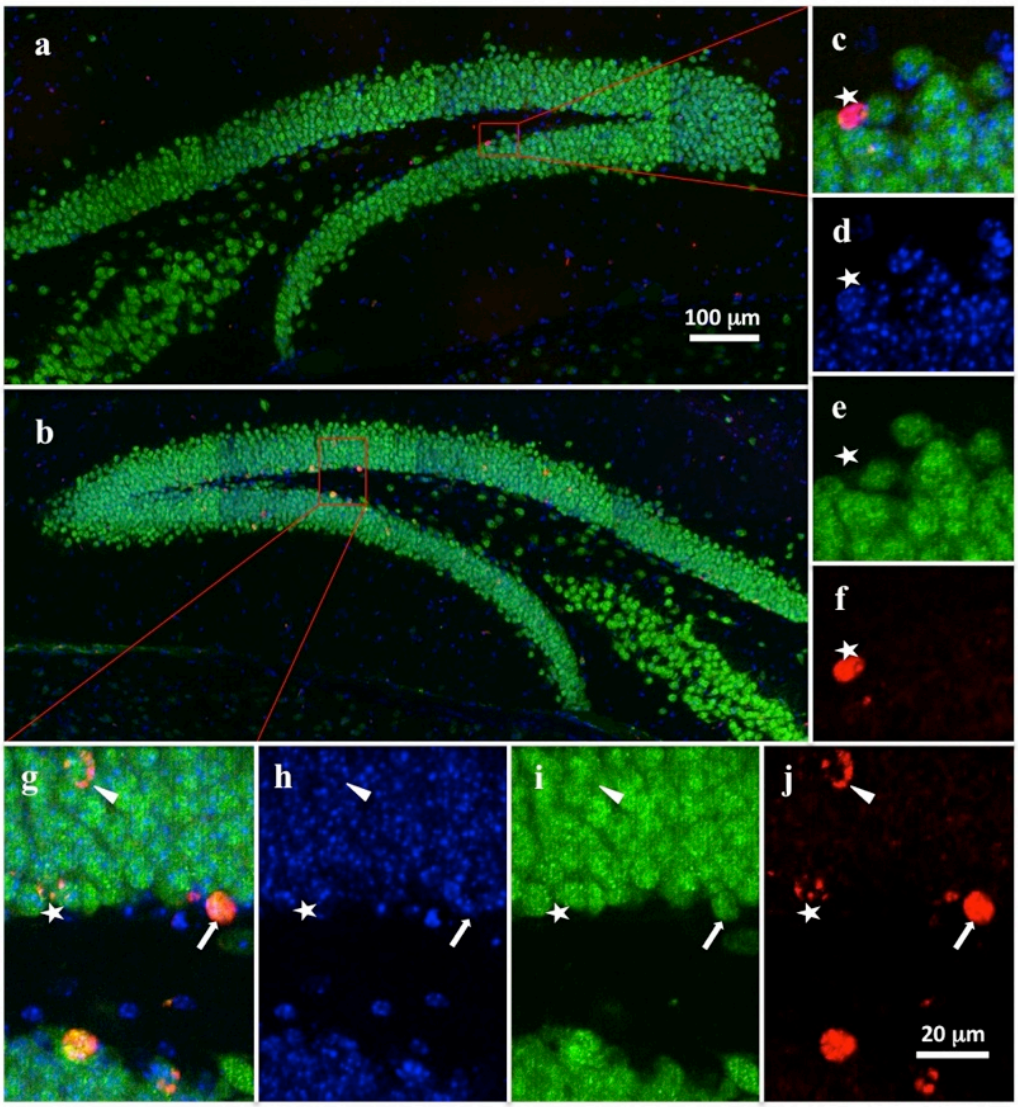

Figure 4.1 Representative images of BrdU incorporation within mature neurons in the dentate gyrus
A representative image of the dentate gyrus from a Non-Runner **(a)** and a Runner **(b).** A close up image from the section of the dentate gyrus from the Non-Runner **(c),** and the Runner **(g),** DAPI stain showing nuclei **(d and h)**, NeuN stain showing mature neurons **(e and i)** and BrdU staining showing newly born cells **(f and j)**. The line arrow points to a mature neuron (stained with NeuN) with complete BrdU incorporation; these cells have not undergone cell division after the uptake of BrdU. The arrow head points to a mature neuron (stained with NeuN) with only partial BrdU incorporation; these cells have divided following BrdU injection or were born late in the accumulation window. The star points to a cell with BrdU incorporation but no co-localisation with NeuN and is therefore not a neuron (not analysed). Blue – DAPI, Green – NeuN, Red – BrdU.

4.2.4 Statistical analysis

Data were analysed using SPSS version 20. All data were analysed for the main effects of Diet (control or AVD-deficient) and Sex using ANOVA. Repeated measures ANOVA was used for the wheel rotations, activity monitors and the forced swim test results. Significant differences ($p < 0.05$) were followed up with post-hoc t-tests. Correlations between neurogenesis and immobility time were assessed using Pearson's test for correlation. Adult hippocampal neurogenesis was measured as the total number of BrdU positive cells co-expressing NeuN averaged per section counted, and the number of Ki67 positive cells averaged per section counted, with the total area counted not different between groups.

4.3 Results

4.3.1 Serum 25(OH)D levels

There was a main effect of Diet ($F_{1,23} = 737.9$, $p < 0.001$) on serum 25(OH)D levels but no significant effect of Running ($F_{1,23} = 1.07$, $p = 0.313$), with no interaction ($F_{1,23} = 0.01$, $p = 0.940$). Levels of 25(OH)D were significantly reduced in both AVD-deficient Runners (2.23 ± 0.23 nM) and AVD-deficient Non-runners (3.44 ± 0.67 nM) compared to both Control Runners (31.99 ± 0.70 nM) and Control Non-runners 33.03 ± 2.21 nM. As no diet by sex interactions were found, data from male and female mice were pooled.

4.3.2 Running wheel activity

It has been well documented that volitional exercise stimulates adult neurogenesis in mice (Clark *et al.*, 2011). In order to ensure propensity for wheel running did not contribute to differences between AVD deficient and control mice, we compared mice individually housed with and without running wheels for six weeks (n = 5-9/group). There was no significant main effect of Diet ($F_{1,14} = 1.24$, $p = 0.285$) on the number of wheel rotations measured between Control (M = 15,388±1215 rotations per day) and AVD-deficient (M = 13,000±1907 rotations per day) mice. Hence it appears that AVD deficiency did not impact on voluntary wheel running in BALB/c mice, and that any differences between the groups are not due to variations in volitional exercise.

4.3.3 Spontaneous locomotion

In an earlier study (Groves *et al.*, 2013), we found significant differences in spontaneous locomotion between male AVD deficient and control BALB/c mice in both novel and familiar open field tests. In the novel environment, locomotion was increased for AVD deficient mice, however,

in the familiar environment, activity was significantly decreased compared to controls. To further explore differences in locomotion we compared the mice housed with and without running wheels in novel open field activity monitors. In this study, there was no significant main effect of Diet ($F_{1,51} = 1.03$, $p = 0.314$) on the distance travelled in the activity arena. It is possible that our previous results are restricted to males only, or that one of a number of possible environmental influences were at play in this study. There was, however, a main effect of Running ($F_{1,51} = 4.66$, $p = 0.036$), where Non-runners were more active than Runners with no interaction with diet ($F_{1,51} = 0.85$, $p = 0.772$) (n = 13-15/per group) (Figure 4.2a).

4.3.4 Immobility time in the forced swim test

Our final behavioural test aimed to determine any differences in immobility, a behavioural correlate of behavioural despair. There were significant main effects of Diet ($F_{1,27} = 4.67$, $p = 0.040$) and Running ($F_{1,27} = 17.71$, $p < 0.001$) on immobility time in the FST, with no interaction ($F_{1,27} = 0.23$, $p = 0.879$) (n=7-8/per group). Mice exposed to running wheels had a significant reduction in immobility time compared to those without access to running wheels. This result is not unexpected; running is associated with an anti-depressant effect reflected by decreased immobility in the FST (Cunha *et al.*, 2013). However, AVD-deficient mice spent less time immobile compared to controls (Figure 4.2b). These results are converse to those expected if vitamin D plays a role in depression, but are consistent with recent research demonstrating that immobility in the FST is independent of hippocampal neurogenesis (Holick *et al.*, 2008).

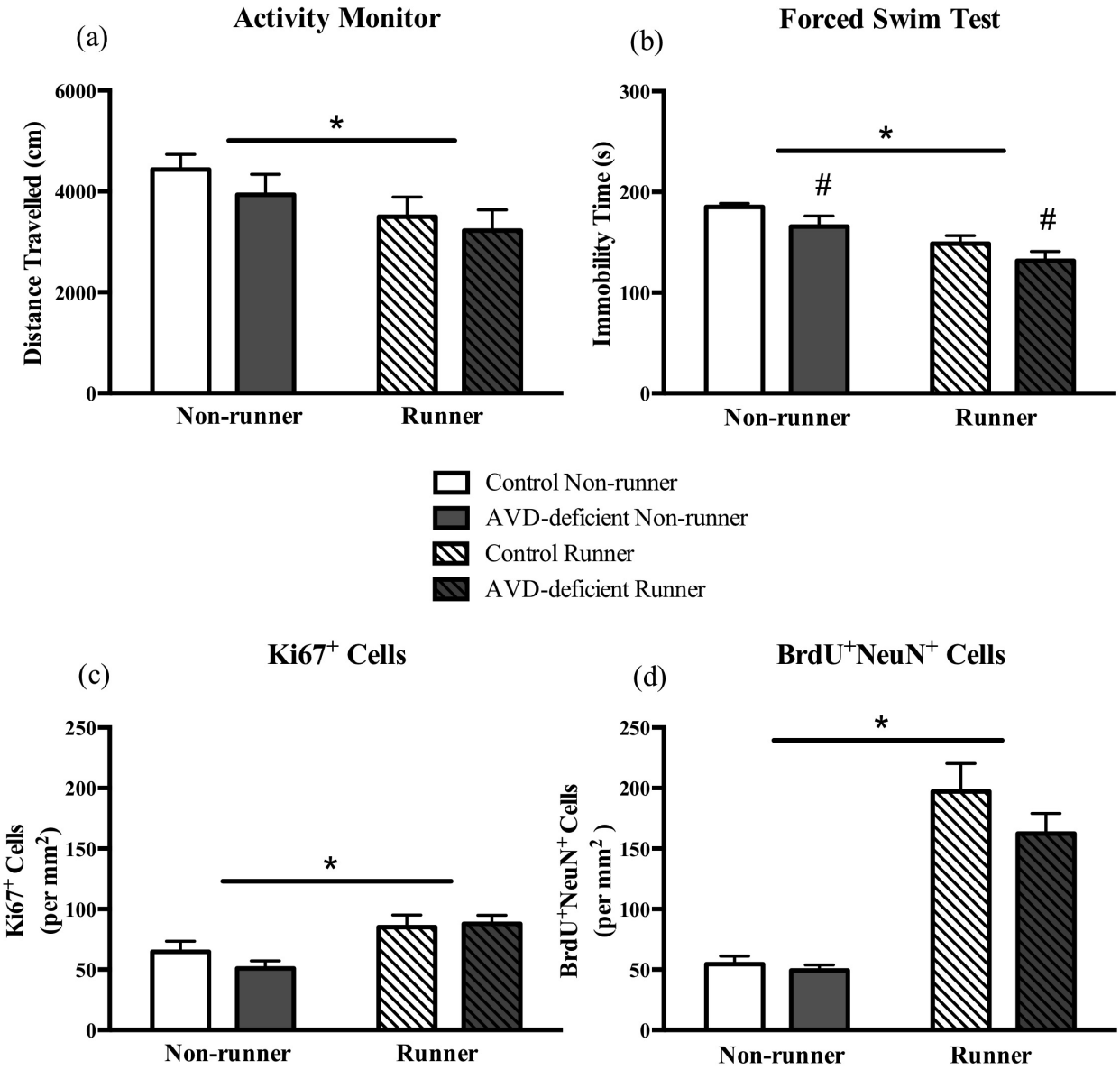

Figure 4.2 Results for the activity monitor, forced swim test, BrdU$^+$NeuN$^+$ cell counts and Ki67$^+$ cell counts

The distance travelled in the activity monitors shown in **(a)** (*n*=13-15/per group). There was no significant effect of Diet but there was a significant main effect of Running. Runners moved less than Non-runners. Immobility time during the FST is shown in **(b)** (*n*=7-8/per group). There was a main effect of both Diet and Running. Wheel running significantly reduced immobility time; moreover AVD deficiency also reduced immobility time. The number of Ki67$^+$ cells per mm^2 is shown in **(c)** (*n*=8/per group). There was no significant effect of diet but there was a significant effect of Running. Runners had higher numbers of Ki67$^+$ cells compared to Non-runners. BrdU$^+$ NeuN$^+$ cells per mm^2 are shown in **(d)** (*n*=8/per group). There was a significant increase in the number of BrdU$^+$ NeuN$^+$ cells following voluntary running. However, there was no significant effect of Diet on baseline cell numbers or cell numbers stimulated by voluntary running. The number of Ki67$^+$ cells per mm^2 is shown in **(d)** (*n*=8/per group). There was no significant effect of diet but there was a significant effect of Running. Runners had higher numbers of Ki67$^+$ cells compared to Non-runners. Mean ± SEM (* $p < 0.05$ # Main effect of Diet $p < 0.05$)

4.3.5 Cell proliferation and incorporation

While in utero vitamin D deficiency has been shown to lead to increased proliferation in rat neonatal neurospheres (Cui *et al.*, 2007), there is some evidence to suggest this effect is limited to development (Ko *et al.*, 2004). To determine if AVD deficiency regulated proliferation in BALB/c mice, we looked at proliferation of cells in the dentate gyrus following the behavioural tests. There was no significant difference between all groups in the area (mm^2) of the dentate gyrus in which $Ki67^+$ cells were counted ($F_{1,25} < 1.58$, $p > 0.220$). While there was no significant main effect of Diet ($F_{1,25} = 0.43$, $p = 0.517$) on the number of $Ki67^+$ cells per mm^2 (n = 6-8/per group), we did see a significant main effect of Running ($F_{1,25} = 11.29$, $p = 0.003$) where Runners had a greater number of $Ki67^+$ cells compared to Non-runners (Figure 4.2c). This suggests that, in BALB/c mice, AVD deficiency does not regulate proliferation, and nor does it impact processes that do regulate proliferation, such as volitional exercise.

To determine if AVD deficiency impacted neurogenesis in the adult hippocampus through mechanisms other than proliferation, we looked at the number of surviving newborn neurons integrated into the dentate gyrus. There was no significant difference between all groups in the area (mm^2) of the dentate gyrus in which $BrdU^+$ $NeuN^+$ cells were counted ($F_{1,28} < 0.55$, $p > 0.464$). In accord with previous research (van Praag, 2008; Clark *et al.*, 2011), there was a significant main effect of Running ($F_{1,28} = 74.85$, $p < 0.001$) on the number of BrdU-incorporated cells per mm^2, with Runners having a higher number of $BrdU^+$ $NeuN^+$ cells compared to Non-runners. There was, however, no significant main effect of Diet ($F_{1,28} = 1.82$, $p = 0.188$) on the number of BrdU-incorporated cells and there was no interaction ($F_{1,28} = 1.00$, $p = 0.326$) (n = 8/per group) (Figure 4.2d). These findings complement our proliferation results.

4.3.6 Correlations of cell proliferation and incorporation with immobility time in the FST

There was no significant correlation between $Ki67^+$ cells and time spent immobile ($R^2 = -0.085$, $p = 0.747$, N = 17). This finding held true when the groups were separated for Diet, with no significant correlation between $Ki67^+$ cells and time spent immobile in Controls ($R^2 = 0.098$, $p = 0.788$, $n = 10$) or AVD-deficient mice ($R^2 = -0.345$, $p = 0.449$, $n = 7$). This finding suggests that immobility time is unrelated to proliferation of adult born cells in the dentate gyrus.

Overall, there was no significant correlation between $BrdU^+NeuN^+$ cells and time spent immobile ($R^2 = -0.416$, $p = 0.077$, $n = 19$), however when the groups were separated, a significant correlation between $BrdU^+NeuN^+$ cells and time spent immobile was found in control mice ($R^2 = -0.810$, $p =$

0.005, $n = 10$), but not in AVD-deficient mice ($R^2 = -0.166$, $p = 0.670$, $n = 9$). This suggests that while AVD deficiency does not impact on the number of dividing cells, or their differentiation into mature neurons, it may impact the function of newly generated cells.

4.4 Discussion

The main finding from this study was that AVD deficiency did not affect proliferation or incorporation of adult hippocampal neurons within the dentate gyrus, at baseline or after voluntary wheel running in BALB/c mice. Therefore, we can reject the hypothesis that AVD deficiency impairs adult hippocampal neurogenesis through alterations in proliferation or integration into the dentate gyrus in BALB/c mice. As predicted, wheel running was associated with a significant increase in hippocampal neurogenesis in both male and female mice in this study, however this was not altered by AVD deficiency. Furthermore, there was no confounding factor from AVD deficiency altering the amount of voluntary wheel running and therefore confounding neurogenesis results, because voluntary wheel running was not significantly different between diets. Overall, this study provides convincing evidence that any putative link between AVD deficiency and adverse brain-related outcomes is not mediated via proliferation or incorporation of new hippocampal neurons in BALB/c mice as measured in this study.

Hippocampal neurogenesis was measured using well-known techniques, using a modified version of the protocol used in the Clark *et al.* (2011) study. We measured the number of $BrdU^+$ $NeuN^+$ cells and $Ki67^+$ cells within the dentate gyrus of the hippocampus, within a set number of sections from a one in five series. This allowed us to measure two aspects of hippocampal neurogenesis, proliferation and integration into the dentate gyrus. We have clearly shown that both these processes were not impaired by AVD deficiency. Furthermore, AVD deficiency had no effect on processes that enhance proliferation, such as wheel running.

One of the difficulties in unravelling the mechanisms by which AVD deficiency impacts rodent behaviour is in strain variation, not just in response to AVD deficiency but in neurogenesis itself. A comparison of neurogenesis in four strains found that while differences in proliferation rates did not reach significance, number of new neurons, neuronal differentiation and survival all differed (Kempermann *et al.*, 1997). Similar genetic variations are seen following exercise. The number of $BrdU^+$ cells varied from 6000 per mm^3 to 14000 per mm^3 across 12 strains of mice (Clark *et al.*, 2011).

The 1α-hydroxylase knockout mouse is most relevant to our study due to the role of 1α-hydroxylase in the conversion of 25(OH)D to $1,25(OH)_2D$ and the BALB/c background. These mice

have an increase in proliferation and a decrease in the survival of newborn neurons in the hippocampus at eight weeks of age (Zhu *et al.*, 2012), yet we have shown that after 16 weeks on a vitamin D deficient diet, with serum levels of 25(OH)D known to be deplete after six weeks, there was no impairment of hippocampal proliferation or integration of new neurons. This shows that either the AVD model used in this study may be subtler than a knockout animal model and therefore more reflective of vitamin D deficiency in human populations, or alternatively, it may be the developmental aspect of the 1α-hydroxylase knockout mouse that leads to hippocampal neurogenesis alterations in the adult animal.

Despite no change in cell proliferation or incorporation with AVD deficiency, there was a significant effect of diet on behaviour in the FST. We have previously shown that AVD deficiency did not impact immobility time on the FST in group-housed male BALB/c mice. However in this study, we chose to use individual housing to allow accurate measurement of individual wheel running in both male and female mice and we found that AVD deficiency resulted in a reduction in immobility time during the FST. A previous study in Swiss-Webster mice showed a significant difference in immobility time between individually housed mice and group-housed mice, showing that the FST was sensitive to housing conditions (Karolewicz & Paul, 2001). This study has showed that in individually housed BALB/c mice, wheel running for six weeks leads to a reduction in immobility time, and that this effect is enhanced for AVD deficient mice.

We hypothesized that AVD-deficiency would increase immobility compared to controls. Based on our results we can reject this hypothesis. AVD deficiency in BALB/c mice does not induce a depressive-like phenotype on the FST.

There are a number of epidemiological studies that show a significant association between vitamin D deficiency and depression (Hoogendijk *et al.*, 2008; Anglin *et al.*, 2013) and there have also been clinical trials showing reductions in depressive symptoms following vitamin D supplementation (Mozaffari-Khosravi *et al.*, 2013), yet this study produced no depressive-like symptoms in the AVD-deficient BALB/c mice as measured by immobility time in the FST. It is possible that the mice were not on the diet for a long enough period. While the AVD-deficient mice are deplete of serum 25(OH)D prior to the start of behavioural testing, it may require a longer period of depletion before some symptoms are detectable and this could be addressed in future studies.

Alternatively, it is possible that a secondary insult may be required to exacerbate the effects of vitamin D deficiency to induce depressive-like symptoms (Cui *et al.*, 2013a). For example, many of the epidemiological studies that show an association between vitamin D deficiency and depression are performed in older adults (Hoogendijk *et al.*, 2008). A recent study performed in rats showed no

effect of vitamin D supplementation in young (6 month old) rats, however in older rats (20 month old), vitamin D supplementation significantly improved learning and memory (Briones & Darwish, 2012). Another study showed that long-term vitamin D dietary manipulation in aging rats (11-13 months old at the start of the study and on diet for 5-6 months), had a significant impact on learning with only the rats on a high vitamin D diet able to perform a complex memory task, compared to those on low or mid levels (Latimer *et al.*, 2014). Therefore, perhaps combining vitamin D deficiency with ageing or another secondary insult such as social stress, could lead to a depressive-like phenotype in BALB/c mice.

Similar to the current study, a recent study in a transgenic Alzheimer's Disease mouse model showed a reduction in immobility time during the FST and it was proposed that this result may be due to a lack of cognitive flexibility and coping with stress strategies rather than from a change in behavioural despair (Torres-Lista & Gimenez-Llort, 2014). However, as determining the mechanism leading to the reduction in immobility time with AVD deficiency was beyond the scope of our experiments, future studies should include testing the cognitive abilities of AVD-deficient mice.

$BrdU^+$ $NeuN^+$ cells and $Ki67^+$ cells were counted in a subset of the population that also underwent the FST. Post-hoc analysis showed that immobility time was not significantly correlated with the level of proliferation. Interestingly, however, a significant negative correlation was found between BrdU+ NeuN+ cells and immobility time for control mice only, where a greater number of cells were correlated with reduced immobility. This effect was not seen for AVD deficient mice, potentially suggesting that the role of new neurons differs in the AVD-deficient model. Previous studies have shown that in C57BL/6J mice, hippocampal neurogenesis is required for the beneficial effects of antidepressants in the FST (reduction in immobility time) (Tyler *et al.*, 2014). However, in BALB/c mice ablation of neurogenesis does not prevent the reduction in immobility time seen with chronic antidepressant treatment (Holick *et al.*, 2008). Taken together, the results from Holick *et al.* (2008) and the results from this study may suggest that immobility time in the FST is independent of changes in neurogenesis in BALB/c mice and that there are other mechanisms involved.

While this study has shown that the number of proliferating cells and the number of newborn neurons integrated into the hippocampus were not affected by AVD deficiency, there are other measures of interest that may be impacted by AVD deficiency, such as the extent of branching of immature neurons rather than cell number, or on the level of apoptosis, or differentiation, which should be investigated in future studies.

AVD deficiency in the BALB/c strain was associated with a reduction in glutamine and glutamate levels in whole brain (Groves *et al.*, 2013). Glutamine is a constituent of proteins and is required in large quantities to support cell growth and division (Vander Heiden *et al.*, 2009), but is also very important for redox pathways (Mates *et al.*, 2002). Both glutamine and glutamate are used for the biosynthesis of the cellular antioxidant, glutathione. Furthermore, glutamine degradation via the citric acid cycle leads to the production of nicotinamide adenine dinucleotide phosphate, which is also essential for the maintenance of glutathione (Mates *et al.*, 2002).

Vitamin D has been shown to protect against oxidative stress in in vitro studies through regulation of glucose-6-phosphate dehydrogenase (Bao *et al.*, 2008) and γ-glutamyl transpeptidase (Garcion *et al.*, 1999), enzymes important in the glutathione pathway. Therefore, the absence of vitamin D during adulthood may lead to a depletion of brain glutathione and excessive oxidative stress, resulting in impaired brain function and this should be investigated in future studies.

4.5 Conclusion

This is the first study to show that AVD deficiency did not impair proliferation or incorporation of adult hippocampal neurons at baseline or after voluntary wheel running, despite a behavioural alteration on the FST. This suggests that the behavioural effects of AVD deficiency are not mediated via early hippocampal neurogenesis processes and that other mechanisms may be involved. In light of the fact that there is growing evidence to support the hypothesis that vitamin D deficiency in adulthood is related to adverse brain outcomes, it is now important to unravel the mechanisms by which vitamin D deficiency alters brain function.

Chapter 5 Preliminary Hippocampal Proteomics Provides Convergent Evidence of Adult Vitamin D Deficiency Impacting on Glutamate and GABA Neurotransmission

5.1 Introduction

Vitamin D is a neurosteroid that exerts its effects either as a transcription factor regulating gene expression (Ramagopalan *et al.*, 2010) or via rapid nongenomic actions through signal transduction systems (Falkenstein *et al.*, 2000). The genomic effects of vitamin D are pleiotropic with over 2,700 VDREs found genome wide (Ramagopalan *et al.*, 2010). There is considerable evidence to show that vitamin D has many functions in the brain, including calcium homeostasis and signaling, regulation of neurotrophic factors, neuroprotection, and modulating neurotransmission and synaptic plasticity. However, much of this research has been performed in cell cultures with the addition of vitamin D or in vivo following treatment with $1,25(OH)_2D$. For example, vitamin D added to primary rat hippocampal cultures, had a neuroprotective effect against excitotoxic insults and down-regulated L-VGCC expression (Brewer *et al.*, 2001).

The addition of vitamin D to cultured embryonic hippocampal cells not only increased NGF but also increased neurite outgrowth and decreased mitosis (Brown *et al.*, 2003). Furthermore, the injection of $1,25(OH)_2D$ into the rat hippocampus, was protective against brain inflammation via regulation of iNOS (Garcion *et al.*, 1998).

The effects of vitamin D deficiency during early periods of brain development has also been studied, for review see Eyles *et al.* (2013). Using a rat model, DVD deficiency has been extensively studied, including the analysis of gene and protein expression in different regions of the brain at different stages of development (Ko *et al.*, 2004; McGrath *et al.*, 2008). In the hippocampus of adult rats following DVD deficiency, analysis of protein expression levels revealed changes in mitochondrial function, calcium homeostasis, synaptic plasticity and neurotransmission (Almeras *et al.*, 2007).

While there is large number of epidemiological studies with significant associations between vitamin D deficiency in adulthood and a range of neuropsychiatric and neurodegenerative disorders (Balion *et al.*, 2012; Anglin *et al.*, 2013; Annweiler *et al.*, 2013), there is limited research on how the absence of vitamin D in the adult brain may be involved in the development of these disorders. In previous research using BALB/c mice, we have shown that amino acid metabolism is altered, with reduced levels of glutamate and glutamine and elevated levels of GABA and glycine, within whole brain tissue. Furthermore, we found that levels of GAD65 and GAD67, which are involved in the metabolism of glutamate and GABA, were down-regulated in AVD-deficient mice (Groves *et al.*, 2013). These results suggest that the balance between excitatory and inhibitory neurotransmission may be altered with AVD deficiency.

An imbalance in glutamate/GABA systems has been implicated in a range of neurological disorders including schizophrenia, autism and cognitive impairment (Lewis & Moghaddam, 2006; Nelson *et al.*, 2006; Kehrer *et al.*, 2008). Another study in adult rats showed that eight weeks of a vitamin D depleted diet led to decreased induction of LTP in the hippocampus of anesthetized animals, compared to a control diet or vitamin D supplemented diet (Salami *et al.*, 2012). It is well known that glutamate neurotransmission plays a central role in LTP (Bashir *et al.*, 1993). However, there is an important gap in the knowledge here in relation to what proteins are being affected by the absence of vitamin D to elicit these changes in excitatory and inhibitory neurotransmitter systems.

Therefore, the aim of this study was to use proteomics to screen hippocampal tissue for possible candidate proteins that are expressed differentially with AVD deficiency. With the changes in neurochemistry observed in this model, the hypothesis was that AVD deficient BALB/c mice would show changes in the expression of proteins specifically involved with glutamate and GABA signaling. Furthermore, we predicted that AVD deficiency would affect biological processes that were identified in vitamin D treatment studies, such as changes in synaptic plasticity, calcium regulation and neuroprotection.

5.2 Materials and methods

5.2.1 Animals and housing

Sixteen ten-week old male BALB/c mice (Animal Resources Centre, Canning Vale, WA, Australia) were obtained and housed in groups of 4 in individually ventilated OptiMICE cages (Animal Care Systems, CO, USA), with bedding (Sanichips, Harlan Laboratories, USA) and nesting material at the Queensland Brain Institute Animal House Facility, University of Queensland.

Mice (n = 8 control and 8 AVD-deficient) were assigned to either a control diet or a vitamin D-deficient diet as detailed in Chapter 2 until they were 20-weeks old. The mice were maintained on a 12-hour light-dark cycle (lights on at 07:00 h) with *ad libitum* access to food and water. They were housed under incandescent lighting free from UVB radiation. All experimental work was performed with approval from the University of Queensland Animal Ethics Committee, under the guidelines of the National Health and Medical Research Council of Australia.

5.2.2 Tissue collection and serum 25(OH)D levels

The mice were euthanized by CO_2, a terminal blood sample was collected via cardiac puncture, followed by decapitation. Serum was collected as per the details in Chapter 4. The level of 25(OH)D was measured in the serum samples using liquid chromatography-tandem mass

spectrometry (Sciex Instruments, ON, Canada) on a 4000 QTrap API AB mass spectrometer (Eyles *et al.*, 2009a).

The whole brain was removed and the whole hippocampus was collected using a mouse 1mm brain block (David Kopf Instruments, California, USA) and free-hand dissection using The Mouse Brain in Stereotaxic Coordinates as a reference (Franklin, 2012). Tissue was frozen on dry ice and stored at -80° C until further processing.

5.2.3 Tissue preparation

Hippocampal tissue from two mice from each cage, totaling 8 mice (n = 4 control and 4 AVD-deficient) was used in the proteomics analysis. The hippocampal tissue was homogenized and then sonicated in 8M urea, 20mM Tris, 4% Chaps and 1% DTT lysis buffer with 1X protease and phosphatase inhibitors (Roche Diagnostics, Castle Hill, AUS). The samples were placed on a rotator and spun slowly for 4 h, followed by centrifugation at 15° C for 30 min at 13,000 g. The supernatant was removed and placed into a new tube. Protein concentration of the supernatant was determined using a Bradford assay following the standard protocol.

5.2.4 Isobaric tag for relative and absolute quantification labeling

For each sample, 100 μg of protein was reduced with 2.5 mM tris(2-carboxyethyl)phospshine at 60 ºC for 1 h and cysteine residues alkylated with 10 mM methyl methanethiosulfonate (room temperature for 15 min). Protein was precipitated with 4 volumes of ice-cold acetone overnight at -20° C. Protein was collected by centrifugation and the pellet resuspended in 0.5 M tetraethylammonium bromide containing 0.1 % sodium dodecyl sulfate. Protein was digested with sequencing grade trypsin (Promega, WI, USA) overnight. For each sample, the isobaric tag for relative and absolute quantification (iTRAQ) reagent (AB Sciex, CA, USA) was added to the digest and incubated for 1 h at room temperature. After labeling the eight-labeled peptide samples were pooled and evaporated to dryness in a speed vac.

5.2.5 Strong cation exchange peptide fractionation

The labeled peptide mix was resuspended in 0.1 % acetic acid containing 2% acetonitrile. Chromatography was performed on an 1100 series high performance liquid chromatography (HPLC) (Agilent, CA, USA) equipped with a fraction collector. Samples were fractionated using a Zorbax 300-strong cation exchange (SCX) column (5 μm, 4.6 x 50 mm, Agilent, CA, USA) using a linear gradient from 0 – 250 mM ammonium acetate containing 2 % acetonitrile at 0.5 mL per minute with 30 s fractions. Fractions were combined accordingly to give a total of 15 fractions.

Combined fractions were desalted using Empore C-18 7mm/ 3 mL solid phase extraction cartridges (Agilent, CA, USA).

5.2.6 Mass spectrometry

Peptides were dissolved in 1% formic acid and injected onto a Shimadzu Prominence nanoLC system (Shimadzu, Kyoto, Japan) at a flow rate of 1 μL/min onto a Vydac Everest C18 300 A, 5 μm, 150 mm x 150 μm column (Grace, Colombia, MD, USA). Chromatographic separation was performed using a linear gradient 10 – 60% solvent B (0.1% formic acid, 90% acetonitrile) over 48 min. Mobile phases consisted of solvent A (1% acetonitrile, 0.1% formic acid) and solvent B. The HPLC eluent was interfaced to a TripleTOF 5600 LC/MS/MS system using a Nanospray III ionisation source (Applied Biosystems, Forster City, CA). Source conditions included an ion spray voltage of 2700 V, nebulizer gas flow of 10, curtain gas flow of 30, interface heater temperature at 150° C and collision-induced dissociation settings included CAD gas of 10 and a declustering potential of 80 V. MS TOF spectra were acquired across the range *m/z* 350 – 1800 for 0.5 s followed by 20 data-dependent MS/MS measurements on pre-cursor ions (100 counts/s threshold, +2 to +5 charge state, collision energy of 40 with collision energy spread of 15). Data was acquired and processed using Analyst TF 1.5**™** software (AB Sciex, CA, USA).

5.2.7 Protein identification and quantification

ProteinPilot**™** v4.5 software (Applied Biosystems, Forster City CA) utilising the Paragon Algorithm was used for the identification of proteins. MS/MS data was searched against the Uniprot FASTA formatted database. Search parameters included trypsin as the digestion enzyme, MMTS as cys-modification, biological modifications allowed (using the set provided with the software) and 'thorough' search setting. The criterion for a positive identification was a confidence score of ≥ 95% and a minimum of 2 peptides per protein.

5.2.8 Statistical analysis

Data were analysed using SPSS version 20. All data were analysed for the effects of Diet (control or AVD-deficient) using ANOVA. Proteins were considered significantly different between control and AVD-deficient diets when a *p*-value of less than 0.05 was given. Without correcting for multiple comparisons, a dataset of these proteins was generated with fold change (FC) from control and the UniProtKB protein codes for analysis using Ingenuity Pathway Analysis (IPA) (Ingenuity System Inc, Redwood City, CA).

5.2.9 Ingenuity pathway analysis

Data was analysed using the 'Core Analysis' function in IPA. Data was analysed in the context of canonical pathways, biological processes and networks. The proteins included in the analysis were not corrected for multiple comparisons, so that a larger list of candidate proteins (the dataset) could be analysed using IPA. However, results from the pathway analysis were only considered significant with *p*-values less than 0.01. Significance of the canonical pathways was tested by the Fisher Exact test *p*-value. The significance calculated was a measurement of the likelihood that the pathway is associated with the dysregulated proteins by chance. Canonical pathways were ordered by significance and the ratio value (number of significant proteins in a given pathway, divided by total number of molecules that make up that pathway).

Bio functions were grouped in: Diseases and Disorders, Molecular and Cellular Functions and Physiological System Development and Function. Proteins from the dataset that were associated with bio functions in the Ingenuity Pathways Knowledge Base were considered for analysis. Fischer's exact test was used to calculate a *p*-value determining the probability that each bio function assigned to the dataset was due to chance alone.

Networks were generated when differentially regulated genes could be related according to previously known associations, but independently of established canonical pathways. Networks were associated to bio functions according to the molecules involved based on Ingenuity Pathways Knowledge Base.

5.3 Results

5.3.1 Serum 25(OH)D levels

There was a main effect of Diet ($F_{1,14} = 319.05$, $p < 0.001$) on serum 25(OH)D levels. Levels of 25(OH)D were significantly reduced in AVD-deficient mice (5.72 ± 0.35 nM) compared to Control mice (38.78 ± 1.82 nM). As expected, the AVD-deficient mice had levels of 25(OH)D at the lower level of detection.

5.3.2 Differentiated proteins

This study identified 2,648 proteins, 1,373 of these proteins had 2 or more peptides identified with > 95 % confidence. All other proteins had less peptides (0 or 1) identified with 95 % confidence. Levels of 45 proteins were significantly different between control and AVD-deficient mice ($p <$

0.05) and 32 of these had a FC >1.5 (Figure 5.1). Of the significantly different proteins, 15 proteins were up-regulated following AVD deficiency and 30 proteins were down-regulated (Table 5.1).

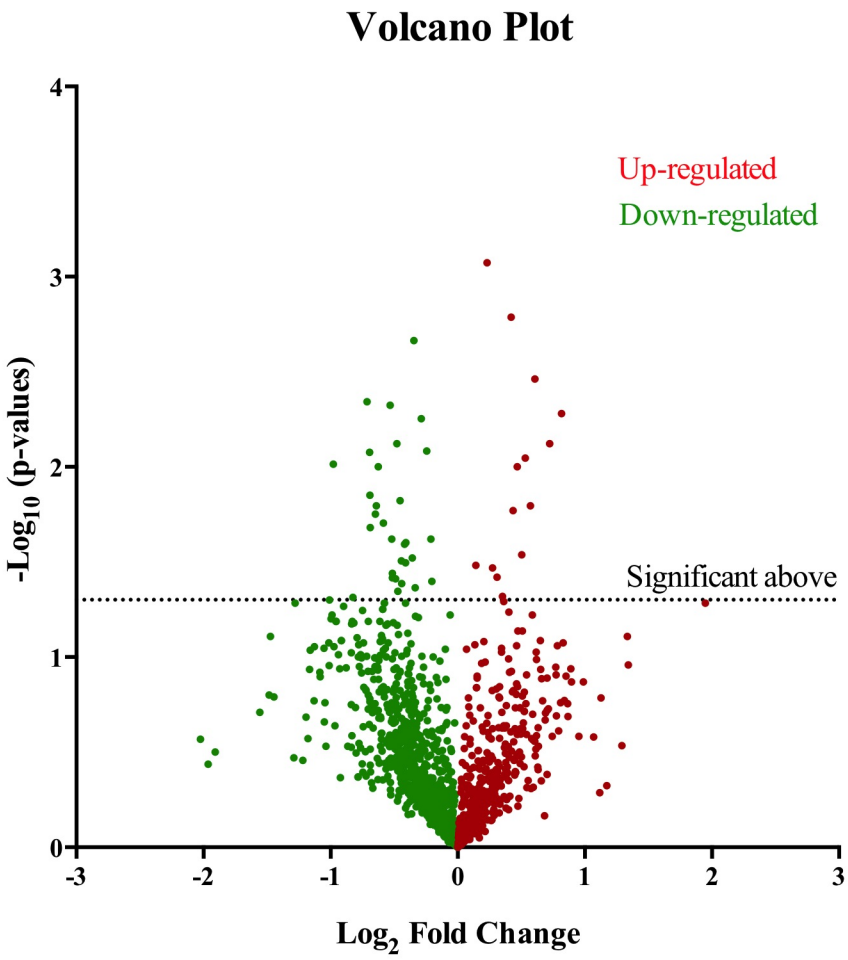

Figure 5.1 A volcano plot of all the proteins identified with more than two peptides
The green dots show proteins that were down-regulated with AVD deficiency and the red dots are proteins that were up-regulated with AVD deficiency. Dots shown above the dotted line are significantly different between controls and AVD-deficient mice ($p < 0.05$).

Table 5.1 Proteins significantly different between control and AVD-deficient BALB/c mice

Proteins significantly different (p <0.05) in order from lowest p-value to highest.

Protein No.	Accession No.	Gene Name	Protein Name	Peptides	% Cov	Mean Cont	Mean AVD	FC	P-value
422	Q9WV80	Snx1	Sorting nexin-1	3	5.75	1.0001	1.2605	1.2603	0.001
1007	Q99LI8	Hgs	Hepatocyte growth factor-regulated tyrosine kinase substrate	4	3.23	1.0047	0.7119	-1.4112	0.002
1374	Q56A07	Scn2b	Sodium channel subunit beta-2	2	5.58	0.8280	1.2618	1.5238	0.002
1063	P63325	Rps10	40S ribosomal protein S10	2	13.94	1.2953	2.3771	1.8351	0.003
673	O54774	Ap3d1	AP-3 complex subunit delta-1	6	3.67	1.2388	0.6064	-2.0427	0.005
984	Q9R0P5	Dstn	Destrin	3	15.15	0.7890	1.7844	2.2615	0.005
1189	Q9Z2D6	Mecp2	Methyl-CpG-binding protein 2	2	3.31	1.0342	0.6078	-1.7015	0.005
163	Q8BGQ7	Aars	Alanine--tRNA ligase, cytoplasmic	8	6.71	1.0410	0.7817	-1.3316	0.006
747	Q61595	Ktn1	Kinectin	3	1.58	1.0268	0.5136	-1.9992	0.008
1241	Q9CYR6	Pgm3	Phosphoacetylglucosamine mutase	3	4.98	0.9864	0.7734	-1.2754	0.008
329	Q9D051	Pdhb	Pyruvate dehydrogenase E1 component subunit beta	5	10.03	0.7227	1.4883	2.0593	0.008
1420	Q3UBX0	Tmem109	Transmembrane protein 109	3	7.41	1.0633	0.6594	-1.6125	0.008
1745	Q8VDP6	Cdipt	CDP-diacylglycerol--inositol 3-phosphatidyltransferase	2	4.7	0.9876	1.6796	1.7006	0.009
1593	Q9WV54	Asah1	Acid ceramidase	2	3.05	0.8656	1.3823	1.5969	0.010
732	Q60605	Myl6	Myosin light polypeptide 6	4	21.85	0.9320	0.3505	-2.6588	0.010
1069	Q9D883	U2af1	Splicing factor U2AF 35 kDa subunit	2	8.79	0.7820	0.4190	-1.8663	0.010
553	Q68EF6	Begain	Brain-enriched guanylate kinase-associated protein	3	5.50	0.9216	0.4622	-1.9936	0.014
1104	P27671	Rasgrf1	Ras-specific guanine nucleotide-releasing factor 1	3	2.46	0.9780	0.6214	-1.5738	0.015
631	P15105	Glul	Glutamine synthetase	4	8.58	0.8174	1.4467	1.7698	0.016
667	Q9CXZ1	Ndufs4	NADH dehydrogenase [ubiquinone] iron-sulfur protein 4	2	12.00	1.0614	0.5591	-1.8983	0.016
558	Q8C8N2	Scai	Protein SCAI	3	7.10	0.8066	1.2477	1.5468	0.017
1272	Q9ES28	Arhgef7	Rho guanine nucleotide exchange factor 7	2	2.90	0.9912	0.5186	-1.9114	0.018
287	P63024	Vamp3	Vesicle-associated membrane protein 3	6	38.83	0.9981	0.5565	-1.7936	0.020
538	Q9CYZ2	Tpd52l2	Tumor protein D54	3	18.18	0.9264	0.4654	-1.9906	0.021
45	P60469	Ppfia3	Liprin-alpha-3	11	9.88	1.0726	0.8688	-1.2345	0.024
1608	O54724	Ptrf	Polymerase I and transcript release factor	2	4.59	1.1661	0.6941	-1.6800	0.024
1030	P51855	Gss	Glutathione synthetase	4	7.38	0.8774	0.5838	-1.5029	0.025
263	P12023	App	Amyloid beta A4 protein	6	6.75	1.0800	0.7106	-1.5198	0.026

601	Q8C854	Myef2	Myelin expression factor 2	2	3.89	1.0670	1.7657	1.6548	0.029
529	O35874	Slc1a4	Neutral amino acid transporter A	3	7.52	0.7254	1.0378	1.4306	0.030
95	P02468	Lamc1	Laminin subunit gamma-1	9	5.41	0.9576	0.6145	-1.5582	0.031
50	Q9QYX7	Pclo	Protein piccolo	21	3.37	1.2671	0.8413	-1.5061	0.032
536	B1AXV0	Frrs1l	DOMON domain-containing protein FRRS1L	3	11.6	1.0615	1.2256	1.1545	0.033
1032	P31648	Slc6a1	Sodium- and chloride-dependent GABA transporter 1	3	3.84	1.3463	1.7726	1.3166	0.034
103	P09405	Ncl	Nucleolin	10	9.19	1.1258	0.6735	-1.6715	0.036
858	Q80VP1	Epn1	Epsin-1	2	2.44	0.9593	1.3059	1.3613	0.038
293	Q8CHU3	Epn2	Epsin-2	4	6.72	1.0502	0.6278	-1.6727	0.038
2185	Q9D8B7	Jam3	Junctional adhesion molecule C	2	5.16	0.9084	0.5570	-1.6309	0.039
703	Q922D8	Mthfd1	C-1-tetrahydrofolate synthase	2	2.67	0.8567	0.6992	-1.2252	0.040
199	Q80U49	Kiaa0284	Protein KIAA0284	7	4.70	1.0813	0.6964	-1.5528	0.041
543	P61089	Ube2n	Ubiquitin-conjugating enzyme E2 N	5	17.76	1.1432	0.8175	-1.3984	0.043
2209	Q61290	Cacna1e	Voltage-dependent R-type calcium channel subunit alpha-1E	2	0.75	1.3296	0.8312	-1.5996	0.045
1157	Q8K010	Oplah	5-oxoprolinase	2	1.63	0.9406	1.3386	1.4231	0.048
630	P48320	Gad2	Glutamate decarboxylase 2	2	4.44	0.8542	0.4905	-1.7416	0.048
1444	Q924M7	Mpi	Mannose-6-phosphate isomerase	3	4.96	1.1433	0.5007	-2.2834	0.049

Abbreviations = Accession No. is the UniProtKB protein code, % Cov = % Coverage (95 CI), FC = Fold Change, Cont = Control AVD = AVD-deficient

5.3.3 Pathway and functional analysis

5.3.3.1 Canonical Pathways

Analysis using IPA showed the top canonical pathway affected by AVD deficiency as the γ-glutamyl cycle ($p = 0.0004$) (Table 5.2), which is involved with the synthesis of glutathione, biosynthesis of reductants and may be involved with amino acid transport (Vina *et al.*, 1989). Other identified significant canonical pathways were Glutamine Biosynthesis I ($p = 0.002$), Glutamate Receptor Signaling ($p = 0.006$), Glutathione Biosynthesis ($p = 0.006$) and GABA Receptor Signaling ($p = 0.008$). See Table 5.2 for the full list of significant ($p < 0.01$) canonical pathways.

Table 5.2 Significant canonical pathways identified using Ingenuity Pathway Analysis
Significant ($p < 0.01$) canonical pathways in order of significance. Ratio values are the number of proteins dysregulated out of the number of proteins in the pathway.

Canonical Pathways	*p*-value	Ratio values
γ-glutamyl Cycle	0.0004	0.13
Glutamine Biosynthesis I	0.002	1.00
D-mannose Degradation	0.002	1.00
Epithelial Adherens Junction Signaling	0.003	0.02
Glutamate Dependent Acid Resistance	0.004	0.50
Tight Junction Signaling	0.005	0.02
Sertoli Cell-Sertoli Cell Junction Signaling	0.005	0.02
Glutamate Receptor Signaling	0.006	0.04
Glutathione Biosynthesis	0.006	0.33
GABA Receptor Signaling	0.008	0.03
Agrin Interactions at Neuromuscular Junction	0.008	0.03

5.3.3.2 Bio Functions

The top six most significant bio functions associated with the dataset were 'Small Molecule Biochemistry' (*p*-value 0.000001 – 0.009, 19 molecules), 'Cell-To-Cell Signaling and Interaction' (*p*-value 0.000001 – 0.009, 17 molecules), 'Molecular Transport' (*p*-value 0.000001 – 0.009, 17 molecules), 'Cellular Assembly and Organisation' (*p*-value 0.000002 – 0.009, 15 molecules), 'Amino Acid Metabolism (*p*-value 0.000002 – 0.008, 10 molecules) and 'Behaviour' (*p*-value 0.000002 – 0.009, 10 molecules). These bio functions are listed in Table 5.3.

Within these significant bio functions, IPA identified a number of biological processes associated with the dysregulated proteins including release of amino acids and neurotransmitters, uptake and metabolism of amino acids, plasticity of synapse, quantity of vesicles and synapses, retraction of dendrites and remodeling of actin filaments; as well as behaviours involved in emotion, contextual conditioning, fear, and learning and memory. For a list of the most significant ($p < 0.001$) functions associated with the dysregulated proteins refer to Table 5.3.

5.3.3.3 Networks

The most significant biologically relevant network (Figure 5.2**,** score 69 and 26 proteins) involved functions corresponding to 'Behaviour', 'Cellular Assembly and Organisation' and 'Cell Morphology'.

Table 5.3 The top six most significant bio functions identified using Ingenuity Pathway Analysis
Including detailed lists of biological processes that may be impacted by the dysregulated proteins within each bio function to $p < 0.001$. If a biological process was identified in two bio functions, it is still only listed once.

Bio Functions	***p*-value**	**No. of Molecules**
Small Molecule Biochemistry	**0.000001 – 0.009**	**19**
- release of neurotransmitter	- 0.000001	6
- release of amino acids	- 0.000002	5
- metabolism of amino acids	- 0.00003	5
- modification of L-glutamic acid	- 0.00009	2
- transport of amino acids	- 0.0002	4
- uptake of amino acids	- 0.0004	3
- synthesis of amino acids	- 0.005	3
- metabolism of glutamine	- 0.005	2
- release of L-glutamic acid	- 0.005	3
- accumulation of ceramide	- 0.005	2
- secretion of catecholamine	- 0.006	3
Cell-To-Cell Signaling and Interaction	**0.000001 – 0.009**	**17**
- binding of lymphoma cell lines	- 0.00007	3
- plasticity of synapse	- 0.0001	4
- quantity of synapse	- 0.0004	3
- binding of carcinoma cell lines	- 0.0005	2
- neurotransmission	- 0.0006	6
Molecular Transport	**0.000001 – 0.009**	**17**
- secretion of molecule	- 0.00001	9
- transport of molecule	- 0.0003	13
Cellular Assembly and Organisation	**0.000002 – 0.009**	**15**
- quantity of vesicles	- 0.000002	5
- quantity of synaptic vesicles	- 0.0001	3
- retraction of dendrites	- 0.0002	2
- translocation of vesicles	- 0.0003	2
- retraction of neuritis	- 0.0003	3
- remodeling of actin filaments	- 0.0003	2
- quantity of synapse	- 0.0003	3
- enlargement of endosomes	- 0.0004	2
- secretion of vesicles	- 0.0008	2
Amino Acid Metabolism	**0.000002 – 0.008**	**10**
Behaviour	**0.000002 – 0.009**	**10**
- emotional behaviour	- 0.000002	7
- contextual learning	- 0.00001	2
- contextual conditioning	- 0.00004	4
- conditioning	- 0.0001	5
- cued conditioning	- 0.0002	3
- fear	- 0.0003	3
- long-term memory	- 0.0003	3
- active avoidance response	- 0.0003	2
- discrimination	- 0.0005	2
- startle response	- 0.0007	3
- spatial learning	- 0.0009	4

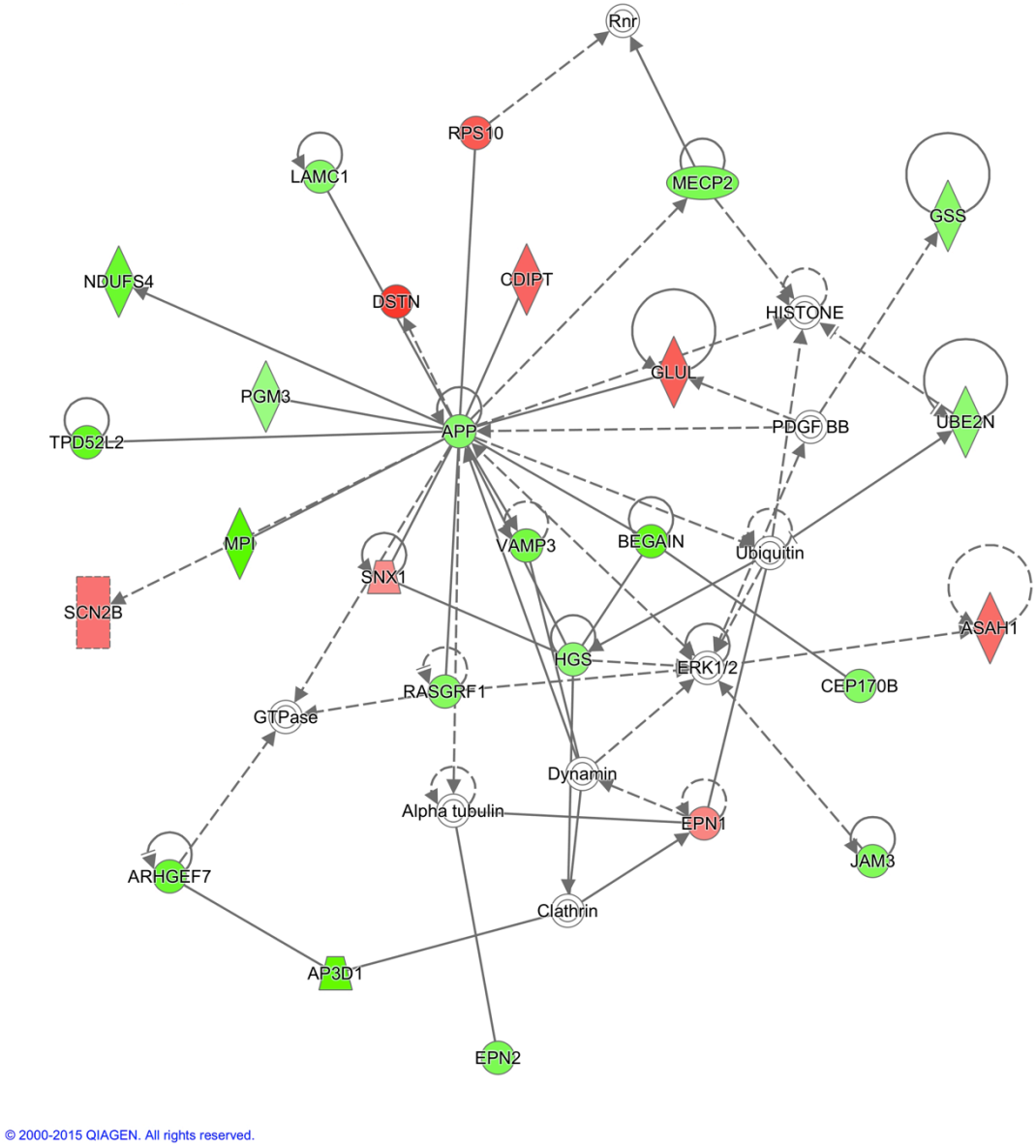

Figure 5.2 The top biologically relevant network identified by Ingenuity Pathway Analysis
The network impacts on functions corresponding to 'Behaviour', 'Cellular Assembly and Organisation' and 'Cell Morphology'. Red denotes proteins up-regulated and green denotes proteins that are down-regulated in the dataset.

5.4 Discussion

This study has shown that the expression levels of 45 proteins have been altered in the hippocampus with AVD deficiency. IPA has implicated a number of canonical pathways affected by AVD deficiency including the γ-glutamyl cycle, important for the production of glutathione, an important antioxidant; and glutamate and GABA receptor signaling and glutamine biosynthesis. Furthermore, IPA has identified some key biological functions that may be affected by AVD deficiency including small molecule biochemistry, cell-to-cell signaling and interaction, molecular transport, cellular assembly and organisation, amino acid metabolism and behavioural outcomes.

5.4.1 γ-glutamyl cycle

The top recognized pathway altered by AVD-deficiency was the γ-glutamyl cycle with significant changes to the level of two enzymes; glutathione synthetase was down-regulated and 5-oxoprolinase was up-regulated as shown in Figure 5.3. A secondary significant canonical pathway was also identified involving glutathione, which was glutathione biosynthesis. We have previously published a reduction in glutamate levels and an increase in glycine levels with AVD deficiency in whole brain (Groves *et al.*, 2013) and these changes have also been added to Figure 5.3. Furthermore, vitamin D treatment has previously been shown to up-regulate γ-glutamyl transpeptidase and increase glutathione levels in vitro (Garcion *et al.*, 1999) (also noted on Figure 5.3).

5-oxoprolinase may be up-regulated in an attempt to compensate for lower glutamate levels and with a reduction in glutamate plus a reduction in the level of glutathione synthetase; this would theoretically lead to lower levels of glutathione. Glutathione has many biological functions including detoxification of free radicals, organic peroxides and reactive non-radicals. It is also important in the maintenance of the cellular thiol pool and for regeneration of other antioxidants, such as vitamin C (Dringen, 2000). Importantly, a reduction in glutathione would lead to increased levels of oxidative stress.

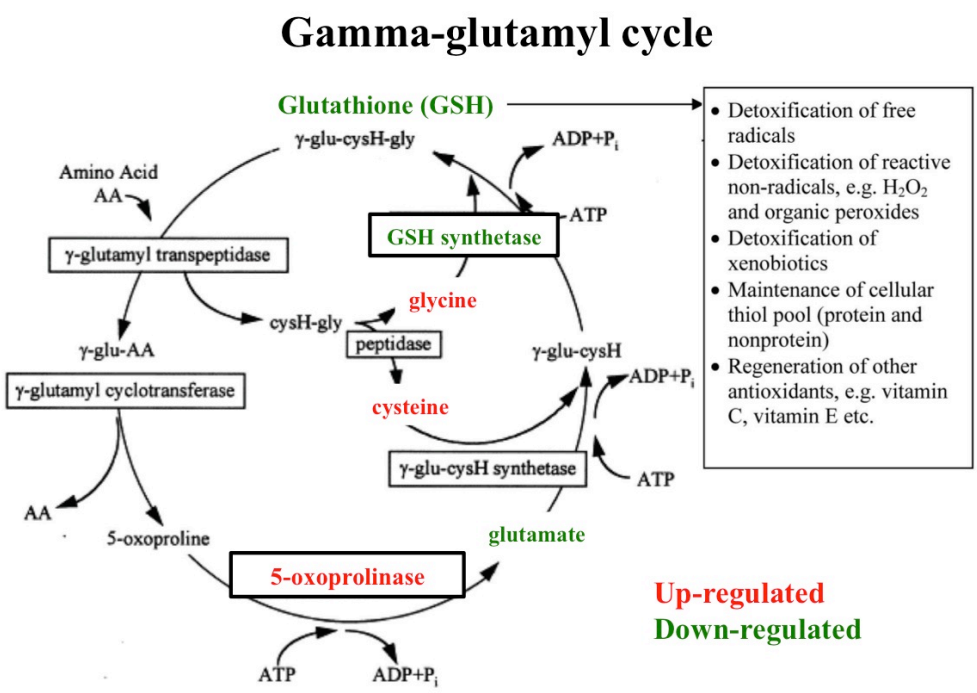

Figure 5.3 The impact of AVD deficiency on the γ-glutamyl cycle
Elevated levels of glycine and reduced levels of glutamate were observed in AVD-deficient BALB/c mice in a previous study (Groves *et al.*, 2013). In vitro treatment of vitamin D has previously been shown to stimulate γ-glutamyl transpeptidase, which resulted in increased levels of glutathione (Garcion *et al.*, 1999). Finally, in this study we observed a decrease in glutathione synthetase and an increase in 5-oxoprolinase. Figure modified from Singh *et al.* (2013).

5.4.2 Glutamate, glutamine and GABA

Four out of the eleven significant canonical pathways identified by IPA, involved glutamate, glutamine and GABA. Previous research has shown a reduction in levels of glutamine and glutamate with AVD deficiency in BALB/c mice in whole brain, with increased levels of GABA (Groves *et al.*, 2013). The results of the proteomics in this study have shown that AVD-deficiency has up-regulated glutamate-ammonia ligase. As glutamine is essential for many cellular and metabolic processes (Smith & Wilmore, 1990; Vander Heiden *et al.*, 2009), low levels of glutamine would stimulate the enzyme, glutamate-ammonia ligase to be up-regulated in an attempt to increase glutamine levels.

The proteomic results also show an up-regulation in the transporter responsible for the re-uptake of glutamate from the synaptic cleft and in the transporter responsible for re-uptake of GABA. In addition, the proteomics has shown convergent evidence that GAD65 is down-regulated, as we have seen previously in AVD-deficient mice (Groves *et al.*, 2013). Within the significant bio function 'Small Molecule Biochemistry' the dysregulated proteins could have significant downstream effects on the release of neurotransmitters. For example, reductions in VAMP3 and CACNA1E, both

proteins down-regulated in this study, have previously been shown to decrease release of glutamate from astrocytes and glutamatergic neurons respectively (Long *et al.*, 2009; Liu *et al.*, 2011).

5.4.3 Synaptic Plasticity

Dysregulated proteins in the proteomics dataset may also have significant effects on other biological processes including quantity of synaptic vesicles, plasticity and quantity of synapses, retraction of dendrites and remodeling of actin filaments. For example, reduced MECP2, down-regulated in this study, has been previously shown to decrease the number of synapses formed, lead to a reduction in synaptic response and an increased retraction of dendrites (Chao *et al.*, 2007; Nguyen *et al.*, 2012). Furthermore, reductions in APP, MECP2, PPFIA3 and RASGRF1, all proteins down-regulated in the proteomics, have previously been reported to reduce synaptic plasticity (Schoch *et al.*, 2002; Moretti *et al.*, 2006; Tomiyama *et al.*, 2010; Lee *et al.*, 2011).

RASGRF1 normally facilitates neurite outgrowth in response to NGF and BDNF (Talebian *et al.*, 2013). NGF has previously been shown to be upregulated in vitro in response to $1,25(OH)_2D$ (Brown *et al.*, 2003) and reduced in neonatal mice exposed to developmental vitamin D deficiency (Feron *et al.*, 2005). Many of the same proteins involved in 'Cellular Assembly and Organisation' were also involved with the 'Behaviour' biological processes. For example, reductions in MECP2 and RASGRF1 have been shown to impair spatial learning and memory (Giese *et al.*, 2001; Moretti *et al.*, 2006).

5.4.4 Integrated Network

IPA has indicated that there is a significant network of associated dysregulated proteins that impact on 'Behaviour', 'Cellular Assembly and Organisation', and 'Cell Morphology'. These results suggest that AVD deficiency may lead to altered behaviour, including impaired learning and memory, through molecular processes affecting cellular assembly and organisation, such as synapse formation and plasticity and changes in dendritic arborisation.

5.4.5 Developmental versus adult vitamin D deficiency

Hippocampal proteomic analysis has previously been performed in a DVD deficiency rat model using 2D gel electrophoresis (Almeras *et al.*, 2007). In their study 23 differentially expressed proteins were identified in the hippocampus; however none of these proteins were identified as significantly altered in the current study of AVD deficiency. This may be due to the differences in the models, for example the use of Sprague Dawley rats versus BALB/c mice, or due to the difference in the experimental procedures and sample preparation. A comparison study of 2D

difference gel electrophoresis, cleavable isotope-coded affinity tags and iTRAQ proteomics showed limited overlap of differentially expressed proteins between the three methods suggesting that they are complementary in nature and cannot be used interchangeably (Wu *et al.*, 2006).

Despite no overlap in differentially expressed proteins between DVD deficiency and AVD deficiency, there were common dysregulated biological functions between the two models, which included redox balance, neurotransmission, synapse plasticity, and cytoskeleton maintenance (Almeras *et al.*, 2007). This may suggest that the role vitamin D plays in development and adulthood may be via similar biological mechanisms.

5.4.6 Limitations and Strengths

The purpose of this study was to generate hypotheses by finding links between vitamin D deficiency and specific pathways or biological processes that can be followed up in future studies. For this reason only a small number of male mice were used and we targeted a very specific area of the brain, the hippocampus. For a more complete analysis of protein changes following AVD deficiency, other brain areas should also be analysed. Furthermore, increasing the sample size may provide a greater number of dysregulated proteins to follow up.

One of the drawbacks to using iTRAQ proteomics is the limited availability of isobaric tags. There are eight different isobaric tags available and therefore this limits the number of samples in any run to eight. Performing separate runs would introduce variability to the experiment and therefore for the purpose of hypothesis generation it was decided to perform one run with eight samples. Despite this drawback, iTRAQ has been shown to be much more sensitive than both 2D difference gel electrophoresis and cleavable isotope-coded affinity tags, identifying significantly more peptides from each protein and a greater number of differentiated proteins without affecting accuracy (Wu *et al.*, 2006).

The results from this preliminary proteomic study have provided a number of exciting pathways that might be affected by AVD deficiency to follow up on. However, the protein expression changes have not been validated, either by replication of the iTRAQ experiment or by an alternative method such as through western blot confirmation or by performing ELISA's on a few key proteins. Therefore, the results must be considered as preliminary results and in need of technical, proteomic or biological validation of the candidate proteins. For example, a key pathway identified following analysis was that of the γ-glutamyl cycle, suggesting that glutathione levels would be reduced by AVD deficiency, and this is explored in Chapter 6.

The proteomic results have provided a list of up and down-regulated proteins. However, this study cannot tell you if these proteins are altered by vitamin D directly, altered through down-stream effects of vitamin D-targeted gene changes, or as a result of compensatory effects. Following up the individual pathways identified through IPA will provide a more complete picture on how these protein changes evolved.

5.4.7 Future Directions

This study has shown that multiple pathways that involve glutathione, glutamate and GABA have been dysregulated by AVD deficiency, including glutathione biosynthesis, glutamate and GABA receptor signaling and glutamine biosynthesis. Furthermore, IPA has implicated AVD deficiency in changes to neurotransmission and amino acid metabolism. Changes to amino acid metabolism that alter the availability of glutamate and glutamine could then have downstream effects on neurotransmission and glutathione maintenance. Alternatively, elevated oxidative stress could deplete glutathione stores, leading to reductions in glutathione components, such as glutamate, which would then lead to impaired neurotransmission. Future experiments should address these issues. For example, if oxidative stress is driving all these changes, treating AVD-deficient mice with n-acetylcysteine, a molecule known to increase glutathione levels (De Rosa *et al.*, 2000), should correct reductions in glutamate levels and changes in neurotransmission.

Future studies should also test markers of oxidative stress. For example, 8-oxo-deoxyguanosine is a marker of DNA oxidation and is commonly assessed using immunohistological techniques (Steullet *et al.*, 2010). Furthermore, if changes seen here, to the γ-glutamyl cycle and glutathione biosynthesis, lead to increased oxidative stress, this could leave AVD-deficient mice more vulnerable to secondary insults or exacerbate underlying conditions (Cui *et al.*, 2013a).

One important consequence of both oxidative stress and deficits in glutathione levels that has been repeatedly reported in the literature is impairments to parvalbumin (PV) interneurons (Steullet *et al.*, 2010; Cabungcal *et al.*, 2013a; Hardingham & Do, 2016). PV interneurons are fast-spiking interneurons with high metabolic demand and therefore may be more susceptible to oxidative stress compared to other neurons (Cabungcal *et al.*, 2013b). Future studies should test if AVD deficiency leads to changes in PV interneurons in the hippocampus and PFC using immunohistochemistry. Furthermore, if alterations in PV interneurons are observed, impairments to gamma oscillations may be responsible for the cognitive deficits observed with AVD deficiency (Hardingham & Do, 2016).

The effects of AVD-deficiency on hippocampal-dependent learning and memory are explored in Chapter 6 and future experiments should also be conducted to assess changes in synaptic plasticity

and dendritic arborisation in the animals to pursue the findings in this study. This could elucidate one possible mechanism behind the effects of AVD deficiency.

5.5 Conclusion

AVD deficiency in male BALB/c mice has impacted on a number of canonical pathways involving glutathione, glutamate, glutamine and GABA. AVD-deficient mice have a number of proteins dysregulated involved in amino acid metabolism, transport, uptake, and neurotransmitter release. This study has provided convergent evidence on the effects of AVD deficiency on glutamatergic and GABAergic neurotransmission. Finally, the proteomics results has also elucidated other biological pathways, processes and functions that may be impacted by AVD deficiency and therefore generated a number of hypotheses that need to be followed up in future studies.

Chapter 6 Reductions in Glutathione and Impairments in a Hippocampal-Dependent Learning and Memory Task in Male AVD-Deficient BALB/c Mice

6.1 Introduction

There is increasing evidence from epidemiological studies that vitamin D deficiency is associated with cognitive impairments, particularly in older individuals (Balion *et al.*, 2012). Furthermore, epidemiological studies have shown that a decrease in cognitive performance and decline is associated with increased oxidative stress in the elderly (Berr *et al.*, 2000; Pratico *et al.*, 2002; Baierle *et al.*, 2015). For example, a study in elderly patients from Brazil reported a significant association between markers of oxidative stress and cognitive parameters, as measured by the MMSE, Verbal fluency and the Boston Naming Test (Baierle *et al.*, 2015). Another study showed that cognitive decline over a four-year period was associated with increased markers of oxidative stress and a lower antioxidant status (Berr *et al.*, 2000). Therefore, it is possible that increased oxidative stress may lead to cognitive impairments.

Vitamin D has been shown to have a role in antioxidant metabolism via downregulation of the expression of iNOS (Garcion *et al.*, 1998) and regulation of gamma glutamyl transpeptidase (Garcion *et al.*, 1999), an enzyme important in the glutathione pathway. More recently, an *in vivo* study has shown that treatment with $1,25(OH)_2D$ leads to upregulation of glutathione reductase and glutamate cysteine ligase, enzymes involved in glutathione synthesis, and to an increase in glutathione levels (Jain & Micinski, 2013). In addition, two proteins involved in the production of glutathione were altered by AVD deficiency, identified in the proteomics analysis of the hippocampus in Chapter 5, suggesting that the biosynthesis of glutathione would be impaired. Therefore, it is likely that AVD-deficient BALB/c mice have reductions in glutathione levels and that this could contribute to cognitive impairment.

Furthermore, proteomic analysis identified a number of dysregulated proteins within the hippocampus in AVD-deficient BALB/c mice that have functions in cellular assembly and organisation, including synapse formation and plasticity, and changes in dendritic arborisation (Chapter 5). Dysregulation of these proteins, as seen with AVD deficiency, are predicted to impair spatial learning and memory, among other behaviours.

The active place avoidance (APA) task is a spatial learning and memory task for rodents that is dependent on avoidance of an aversive event. This is different to the 5C-SRT task that was used in Chapter 2, which was reward based and required food-restriction. The APA has greater complexity than the more widely used Morris water maze, and yet only requires a single trial per day (Cimadevilla *et al.*, 2000; Vukovic *et al.*, 2013). The APA test has been shown to be hippocampal-dependent during both acquisition and for performance once the procedure is learnt (Cimadevilla *et*

al., 2001). This is due to the continuous rotation of the apparatus and the animal having to continually update their place information in relation to the shock zone (Cimadevilla *et al.*, 2000; Vukovic *et al.*, 2013). Furthermore, the role of hippocampal neurogenesis has been more clearly established for the acquisition of the APA, as seen in a study in which ablation of immature neurons produced deficits in acquiring the task (Vukovic *et al.*, 2013).

There is currently no published data on using the APA in BALB/c mice and therefore one aim of this study was to establish the APA in the BALB/c strain for the first time, to ensure that they are capable of learning this task. We have shown that AVD deficiency is not associated with altered adult hippocampal neurogenesis, in the form of altered proliferation or the survival of newborn neurons within the dentate gyrus of the hippocampus (Chapter 4), however, this does not preclude an effect of AVD deficiency on spatial learning and memory via oxidative stress, or by other mechanisms highlighted by the analysis of the proteomics data reported in Chapter 5.

The primary aim of this study was to assess the effects of AVD deficiency in male BALB/c mice on their performance in a hippocampal-dependent learning and memory task, the APA. The hypothesis was that male AVD-deficient BALB/c mice would show impairments on the APA. A secondary aim of this study was to assess glutathione levels using HPLC, with the hypothesis that there would be a reduction in glutathione levels in AVD-deficient mice. We have previously showed that males, but not females, were impaired on the 5C-SRT task (Chapter 2). Therefore, the current experiments focused on the effects of AVD deficiency in male mice.

6.2 Materials and methods

6.2.1 Animals and housing

Thirty-eight male BALB/c mice (Animal Resources Centre, Canning Vale, WA, Australia) were included in this study in two cohorts. The first cohort included 23 (n = 11 control and 12 AVD-deficient mice) nine-week-old male BALB/c mice and the second cohort included 15 (n = 8 control and 7 AVD-deficient mice) ten-week-old mice. Mice were obtained and housed in groups of 4 as detailed previously in Chapter 2. Cohort 1 mice underwent testing on the APA for five days, at nine-weeks old, prior to being placed on diet at 10-weeks old. After the APA testing, the mice were placed into dietary groups, ensuring that there was no significant difference between the two groups and at 20-weeks old underwent re-testing on the APA to test for the effects of AVD deficiency.

At 10-weeks of age, all mice (including the mice from Cohort 2) were placed on either a control diet or a vitamin D-deficient diet as detailed in Chapter 2, until they were 20-weeks old. The mice

were maintained on a 12-hour light-dark cycle (lights on at 07:00 h) with *ad libitum* access to food and water. They were housed under incandescent lighting free from UVB radiation. All experimental work was performed with approval from the University of Queensland Animal Ethics Committee, under the guidelines of the National Health and Medical Research Council of Australia.

6.2.2 Active place avoidance

The apparatus for the APA (Bio-Signal Group, NY, USA) consisted of an elevated arena with a grid floor surrounded by a 30 cm high transparent circular barrier, producing an enclosed arena 77 cm in diameter. The arena was located in a room with visual cues placed on the walls. The arena, including the grid floor, rotated clockwise at a speed of 1 rpm and an electric shock could be delivered through the grid floor. The position of the animal in the arena was tracked using an overhead camera linked to Tracker software (Bio-Signal Group, NY, USA). During trials, a mouse was placed in the arena and trained to avoid a 60° shock zone, the positioning of which was kept constant in relation to the room and to the external visual cues; the mouse's "start" position was always opposite the shock zone, near the barrier of the arena (Figure 6.1).

On Day 1 (habituation day), the mice were free to explore the arena with no shock given, for 5 min. On the next 4 consecutive days, Days 2 to 5 (test days), entrance into the shock zone led to the delivery of a brief foot shock (500 ms, 60 Hz, 0.5 mA). If, after the initial shock, the animal remained in the shock zone, further shocks were delivered at 1.5 s intervals until the animal moved out of the zone. Each test session lasted 10 min and recorded tracks were analysed offline using Track Analysis software (Bio-Signal Group, NY, USA). To assess the extent of learning, the following parameters were measured: number of foot shocks delivered during the 10 min trial, latency to first entry into the shock zone, and the distance travelled during the trial.

The second APA test that was performed at 20-weeks old, had the location of the shock zone and start zones moved, compared to the initial APA at 9-weeks old, to increase the difficulty of the task. The extra-maze cues were also changed for the second APA test (Figure 6.1).

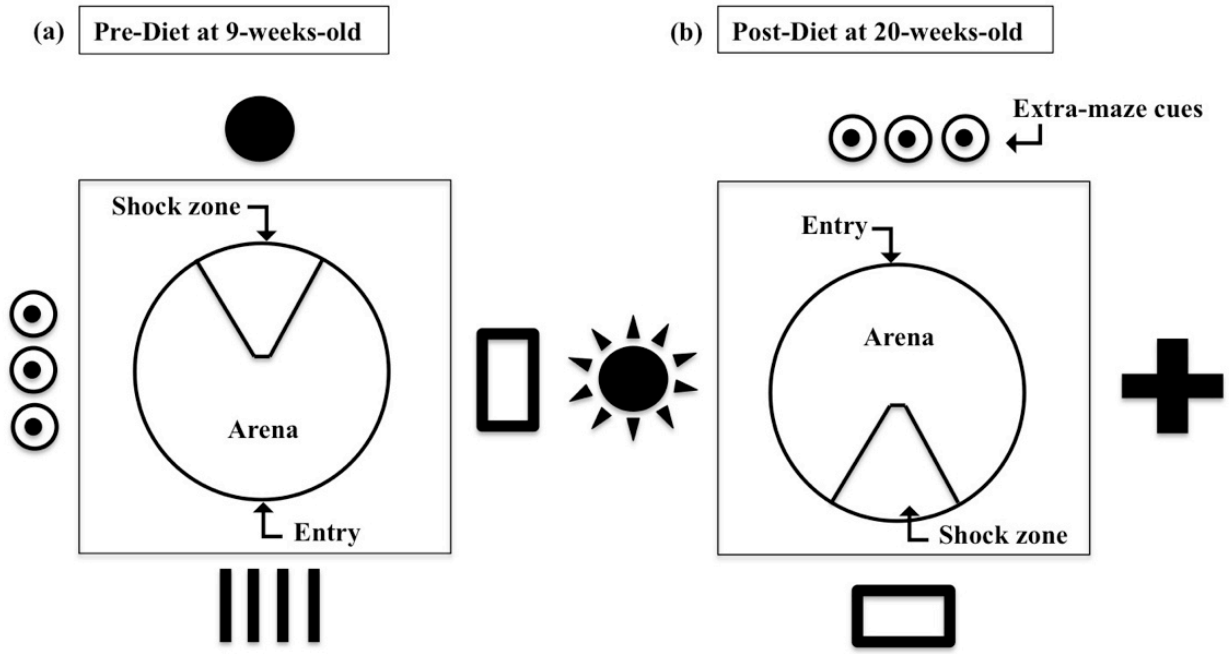

Figure 6.1 The active place avoidance arena layout
The layout in (a) was used pre-dietary manipulation. The location of the shock zone and entry position was moved as per layout in (b) for the second APA test. The extra-maze cues were also changed for the second APA test.

6.2.3 Tissue collection

Mice from Cohort 1 were anaesthetized by an i.p injection of Lethabarb at 100 mg/kg body weight 24 h after the APA and transcardially perfused with 40 ml of PBS and 40 ml of 4 % PFA in PBS. The brains were removed and post-fixed in 4 % PFA for 24 h before being stored in PBS azide for future analysis not included in this thesis.

Mice from Cohort 2 were euthanized by i.p injection of an overdose of Lethabarb at 4 ml/kg body weight, followed by decapitation. The whole brain was removed and the left hand cerebrum was collected using free-hand dissection. Tissue was frozen on dry ice and stored at -80° C until further processing for HPLC analysis.

6.2.4 High performance liquid chromatography analysis

Tissue extracts were analysed for total glutathione based on the method described by (Khan *et al.*, 2011). Basically, analyses were performed on an Agilent 1100 series HPLC system equipped with a Coulochem III electrochemical detector with a glassy carbon electrode (ESA Inc., MA, USA). Analyte separation was performed on a Jupiter C18 5 µm 300A column (4.6 x 250 mm, Phenomenex, NSW, Australia) using an isocratic mobile phase consisting of 3 % methanol and 0.1

% trifluoroacetic acid in water. The column flow rate was 0.7 ml/min and analytes were detected using an electrochemical detector in DC mode, using a detector potential of 900 mV and 1000 mV potential for the guard cell. The data was acquired and processed using Chemstation software (Agilent, CA, USA).

6.2.5 Statistical Analysis

Results were analysed for statistical significance using SPSS (version 20.0) software. All data were analysed for the main effects of Diet (control or AVD-deficient) using ANOVA or, where appropriate, repeated measures ANOVA. Significant differences ($p < 0.05$) were followed up with post-hoc t-tests.

6.3 Results

6.3.1 Active place avoidance – pre Dietary manipulation

All mice learnt to avoid the shock zone over the four test days ($F_{1,22} = 34.40$, $p < 0.001$, Figure 6.2c) and their latency to enter the shock zone was significantly increased over the four days of testing ($F_{1,22} = 13.08$, $p < 0.001$, Figure 6.2a). The distance travelled during the 10 min test was significantly reduced over the course of the four test days for all mice ($F_{1,22} = 29.29$, $p < 0.001$, Figure 6.2e).

6.3.2 Active place avoidance – post Dietary manipulation

There was a significant effect of Diet ($F_{1,21} = 15.52$, $p = 0.001$, Figure 6.2b) on the latency to enter the shock zone over the test days, with controls showing an increased latency compared to AVD-deficient mice. There was no significant effect of Diet ($F_{1,21} = 0.60$, $p = 0.446$) on the maximum time to avoid shock over the course of the test days but there was a significant effect of Diet ($F_{1,21} = 5.30$, $p = 0.032$) on Day 2, with a shorter maximum time to avoid in the AVD-deficient mice (209.18 s ± 31.93 in Controls and 124.67 s ± 19.52 in AVD-deficient mice).

The number of shocks received by all mice was significantly reduced over the test days ($F_{1,21} = 47.49$, $p < 0.001$). Furthermore, there was no significant effect of Diet ($F_{1,21} = 2.62$, $p = 0.121$, Figure 6.2d) on the number of shocks over the four test days. On the final test day, Day 5, the number of shocks failed to reach significance ($F_{1,21} = 4.10$, $p = 0.056$). However, when analysing the first 5 min of Day 5, AVD-deficient mice received significantly more shocks compared to controls ($F_{1,21} = 6.27$, $p = 0.021$) (1.09 shocks ± 0.28 in controls and 3.83 shocks ± 1.01 in AVD-deficient mice), but not in the second 5 min ($F_{1,21} = 0.01$, $p = 0.947$) of Day 5 (1.55 shocks ± 0.41 in

controls and 1.58 shocks ± 0.38 in AVD-deficient mice). Furthermore, there was no significant difference in the number of shocks between the first 5 min and the second 5 min by control mice ($F_{1,10} = 1.10$, $p = 0.320$). However, there was a significant reduction in shock number for AVD-deficient mice in the second 5 min ($F_{1,11} = 5.75$, $p = 0.035$), suggesting that they were still learning how to avoid the shock zone.

There was no significant main effect of Diet ($F_{1,21} = 2.95$, $p = 0.180$) on the distance travelled over the five days of testing. There was however, a significant Diet x Day interaction ($F_{1,21} = 3.85$, $p = 0.015$, Figure 6.2f), with AVD-deficient mice exhibiting hyperlocomotion compared to controls towards the end of the testing. On Day 1, there was no significant effect of Diet ($F_{1,21} = 1.89$, $p = 0.184$) on the distance travelled during habituation to the arena. On test days, when shock was applied to the shock zone, there was still no effect of Diet ($F_{1,21} = 0.19$, $p = 0.667$) on the distance travelled on Day 2. However, there was a significant effect of Diet ($F_{1,21} = 5.55$, $p = 0.028$) over the four test days, with AVD-deficient mice travelling more than the controls.

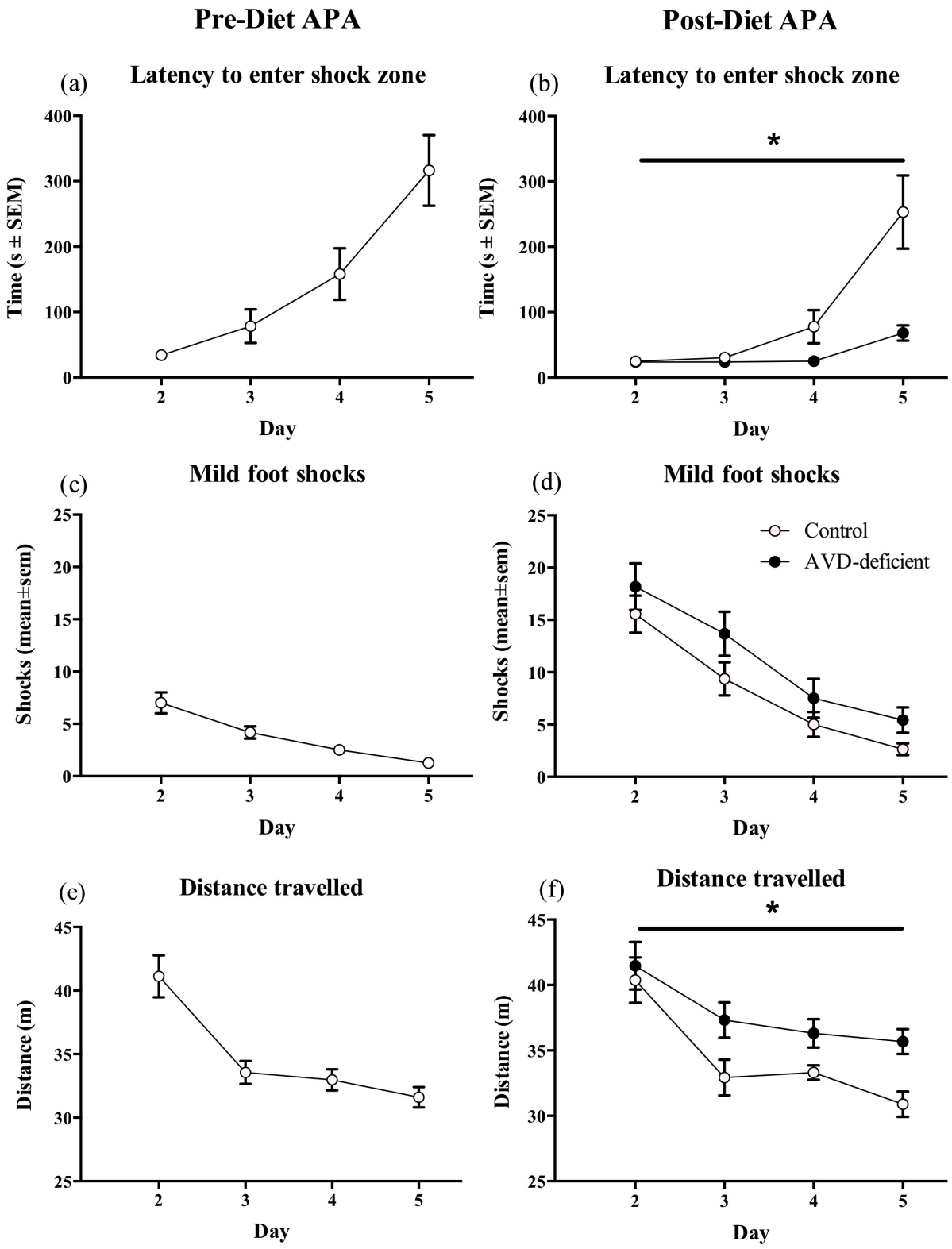

Figure 6.2 Results for the active place avoidance
The results of the APA prior to dietary manipulation are shown in **a**, **c** and **e** with the results of the second APA post-dietary manipulation shown in **b**, **d** and **f**. During the first APA, all mice increased their latency to enter the shock zone over the four test days (**a**) and in the second APA, there was a significant difference between controls and AVD-deficient mice in their latency to enter the shock zone (**b**), AVD-deficient mice had a reduced latency compared to controls. All mice significantly reduced the number of shocks they received over the four test days (during both APA tests) and overall there was no significant effect of Diet (**c** and **d**). Finally, the distance travelled in the arena is shown in **e** and **f**. During the second APA post-dietary manipulation, AVD-deficient travelled further than controls. Mean ± SEM (n = 11-12, * $p < 0.05$)

Assessing the difference between the number of shocks received on the final day of testing before being placed on diet, compared to the number of shocks on the final day of testing after dietary manipulation, there was a significant Diet x Day ($F_{1,21} = 5.14$, $p = 0.034$, Figure 6.3) effect. AVD-deficient mice received significantly more shocks during the second APA test compared to control mice.

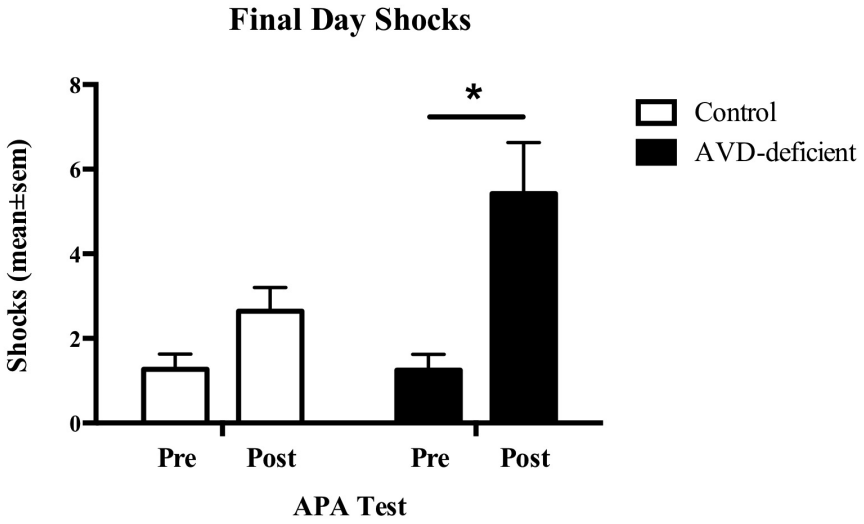

Figure 6.3 Final day of the active place avoidane
The difference in the number of shocks received on the last day of testing pre-diet compared to post-diet. There was a significant increase in shocks received in AVD-deficient mice, but no significant difference in controls. Mean ± SEM (n = 11-12, * $p < 0.05$)

6.3.3 Glutathione levels

There was a significant effect of Diet ($F_{1,13} = 26.43$, $p < 0.001$, Figure 6.4) on total glutathione levels, with a significant decrease in the levels of glutathione in AVD-deficient mice (513.27 μm ± 53.72 for controls and 210.91 μm ± 12.22 for AVD-deficient mice).

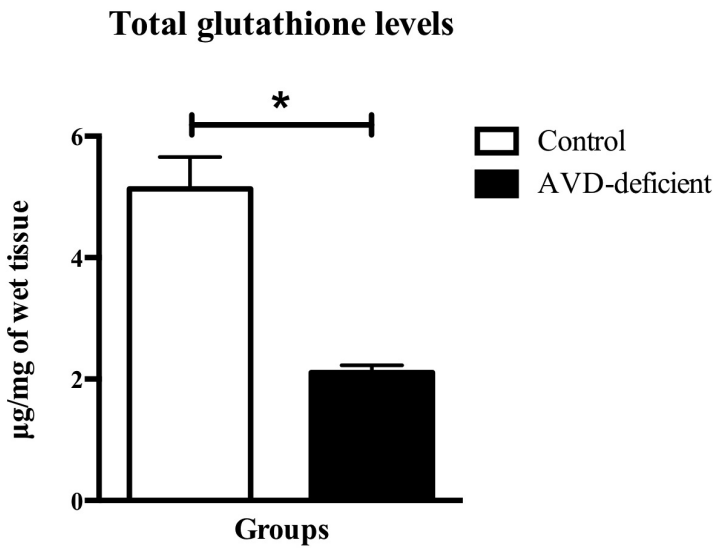

Figure 6.4 Total glutathione levels
Total glutathione levels in brain tissue from the left cerebrum. AVD-deficient mice had a significant reduction in total glutathione levels. Mean ± SEM (n = 7-8 * $p < 0.05$)

6.4 Discussion

I have established for the first time that BALB/c mice are capable of learning the APA task. Furthermore, this study has shown that AVD-deficient male mice have impaired performance on this task. As a secondary aim, total brain glutathione was measured in a separate cohort of male mice, showing that AVD-deficient mice have a significant reduction of glutathione. The mice underwent testing on the APA prior to starting the dietary manipulation, this was to ensure that BALB/c mice could learn to avoid the shock zone and show a significant improvement over the four days. All mice successfully learned to avoid the shock zone and increased their latency to enter the shock zone. When the mice were retested following the dietary manipulation, the extra-maze cues and the location of the shock zone were changed. This was to ensure the task involved both memory and learning, as they were still required to learn the new cues and locations.

After 10 weeks on the diet, in the second round of APA testing, during both habituation and on the first test day, there was no difference in the distance travelled in the arena. Therefore, baseline locomotion was not altered in the AVD-deficient mice. However, over the remaining test days, AVD-deficient mice exhibited hyperlocomotion compared to controls.

Previous studies in BALB/c mice have shown that AVD deficiency enhanced their response to heat and produced quicker responses to shock, in an active avoidance paradigm that was without spatial cues (Groves *et al.*, 2013), therefore the increased locomotion could be a response to shock.

However, this does not explain their decreased latency to avoid the shock zone at the start of each day.

The difference in latencies to first enter the shock zone each day, between AVD-deficient mice and controls, could show that the consolidation of memories from one day to the next is impaired with AVD deficiency. Furthermore, the differences seen overall on this task between AVD-deficient mice and controls, suggests that while the AVD-deficient mice were able to learn the requirements of this task over time, their learning appears to be delayed compared to controls.

AVD-deficiency led to a reduction in whole brain glutathione levels compared to controls. Glutathione is an endogenous antioxidant important for neutralising reactive oxygen species, in detoxification, in immune responses, and also has a wide range of cellular reactions including regulation of protein and gene expression (Townsend *et al.*, 2003). Although total glutathione levels were measured as an indication of oxidative stress, a more complete analysis of the glutathione antioxidant system is now warranted. For example, measuring the ratio of oxidised to reduced glutathione, as well as measuring the levels of enzymes important in the antioxidant function of glutathione, such as glutathione peroxidase and glutathione reductase, would better indicate the oxidation status of the AVD-deficient mice (Urso & Clarkson, 2003).

Alterations in glutathione homeostasis that lead to greater oxidative stress have been implicated in a range of neuropsychiatric and neurodegenerative diseases, including cognitive impairment (Zhao & Zhao, 2013; Baierle *et al.*, 2015; Black *et al.*, 2015). Furthermore, increased oxidative stress can lead to an elevated stress response, and stimulation of apoptosis pathways, resulting in ‘sick’ cells and ultimately cell death (Townsend *et al.*, 2003). Increased oxidative stress can also lead to impairments in PV interneurons and alterations to neuronal synchronization resulting in cognitive deficits (Hardingham & Do, 2016).

Following the induction of oxidative stress, synaptic membranes have been shown to have elevated markers of oxidative stress including lipid peroxides and protein carbonyls, and a change in membrane surface potential. These changes could result in reduced membrane fusion between nerve terminal membranes and synaptic vesicles, produce a subsequent decline in neurotransmission and result in impaired learning and memory (Urano *et al.*, 1997; Fukui *et al.*, 2002).

Acquisition of the APA task requires adult neurogenesis as shown in the study in which ablation of immature neurons within the hippocampus, produced deficits in acquiring the task (Vukovic *et al.*, 2013). Although, we have not measured levels of immature neurons following AVD deficiency, we

have previously shown that levels of both proliferating cells and the survival of newborn neurons in the dentate gyrus of the hippocampus were not altered by AVD deficiency.

In addition to altered glutathione biosynthesis, the results from the proteomic analysis of the hippocampus suggested that AVD deficiency may be impacting on branching, synapse formation and synapse plasticity, rather than cell numbers. Previous research has shown that increased dendritic arborisation and density of dendritic spines was associated with higher performance in spatial learning and memory tasks (Leggio *et al.*, 2005).

Using the Ts65Dn genetic mouse model of Down Syndrome it was shown that hippocampal-dependent cognitive deficits were associated with lower synapse density and dendritic arbors that were less branched and less spinous (Kurt *et al.*, 2004). Furthermore, there has been extensive research on the necessity of synaptic plasticity, long-term depression (LTD), and LTP for learning and memory (Elgersma & Silva, 1999), therefore, it is possible that AVD-deficient mice may have impairments in LTP or LTD and this should be investigated in future experiments.

Future experiments will be needed to discover the mechanism behind AVD deficiency's impact on hippocampal-dependent learning and memory. Experiments should include analysing the degree of dendritic arborisation of immature neurons within the hippocampus of AVD-deficient and control mice. Furthermore, future studies should examine changes in synapse formation and strength; as well as investigate how proteins shown to be dysregulated following AVD deficiency (Chapter 5) could lead to these changes. Finally, markers of oxidative damage should be assessed in the brain of AVD-deficient mice, as this may be a key mechanism in the effects of AVD deficiency.

This study has shown that the APA can be retested in the same animals and still be sensitive to show an effect of AVD-deficiency, therefore this test could be used to determine if repletion of vitamin D could reverse the deficits seen and also be used to test possible treatments, such as antioxidant treatments.

6.5 Conclusion

With epidemiological studies showing an association between AVD deficiency and cognitive impairment and decline, it is important to assess and unravel this link in a rodent model. This study has now provided further evidence that AVD deficiency leads to cognitive impairments in male BALB/c mice and that AVD deficiency leads to reduced glutathione levels. The direct implication of oxidative stress as the mechanism for cognitive impairment should now be explored further.

Chapter 7 AVD Deficiency Exacerbated Impairments Caused by Social Stress in BALB/c and C57BL/6J Mice

7.1 Introduction

Neuropsychiatric disorders such as schizophrenia have complex aetiology involving both genetic factors and environmental influences (McDonald & Murray, 2000). The idea that multiple factors may contribute to the development of a disorder, led to the creation of the 'two-hit hypothesis' (Maynard *et al.*, 2001). However, recent genome wide association studies show that within the genetics of schizophrenia alone, prior to any environmental exposures, there are thousands of genetic variants of small effect sizes that lead to an increased risk of developing schizophrenia (Wray & Visscher, 2010). Moreover, there are multiple environmental risk factors that can influence the development of disorders. For example, epidemiological studies have identified a number of non-genetic risk factors for schizophrenia including pregnancy and birth complications, maternal infection, immigration, adverse life events, and substance abuse (McDonald & Murray, 2000).

Therefore, it is likely that the accumulation of multiple risk factors leads to the development of complex neuropsychiatric disorders. However, using animal models it is not feasible to look at many 'hits' and therefore a 'two-hit hypothesis' may be a more reasonable approach. For example, a recent study investigated the consequences of combining prenatal immune activation with stress during puberty in mice. They found that the prenatal insult markedly increased the vulnerability of offspring to subsequent stress. Furthermore, the interaction between the two 'hits' led to behavioural alterations relevant to neuropsychiatric disorders, including deficits in sensorimotor gating as assessed by prepulse inhibition (PPI) of the acoustic startle response (ASR) (Giovanoli *et al.*, 2013).

Epidemiological studies show that first generation, dark-skinned migrants to cold climates have increased risk of developing psychosis (Cantor-Graae & Selten, 2005). It was proposed that social stress related to being a member of a minority ethnic group in a foreign country may contribute to this increased risk (Dealberto, 2010). Another hypothesis given was that it may be vitamin D deficiency, after moving from a country with high sun exposure to one of limited exposure, that increased their risk of developing psychosis (Dealberto, 2010). Developmental vitamin D deficiency has been shown to be a risk factor for the development of schizophrenia later in life (McGrath *et al.*, 2010a), however recent animal experiments have suggested that vitamin D deficiency during adulthood also compromises brain function.

7.1.1 Vitamin D's neuroprotective properties

Vitamin D has been shown to be neuroprotective against a range of neuronal insults including excitoxicity and ischemia, both directly and via regulation of NGF and GDNF (Kume *et al.*, 2000; Wang *et al.*, 2001; Wang *et al.*, 2002b). $1,25(OH)_2D$ treatment upregulates antioxidant molecules, such as glutathione and iNOS, protecting against free radicals (Garcion *et al.*, 2002; Jain & Micinski, 2013). Furthermore, a recent study in PD has shown that vitamin D supplements prevented a decline in PD-related outcomes compared to those on placebo, suggesting a protective effect of vitamin D against PD.

Vitamin D deficiency has now been shown to reduce glutathione levels in BALB/c mice, potentially leaving the mice at risk for increased oxidative stress (Chapter 6). Therefore, the absence of vitamin D could leave an individual more vulnerable to a second 'hit'. For example, it was recently shown in a stroke model, that vitamin D deficient rats had greater infarct volumes compared to controls and this corresponded with greater impairments, post-stroke, in sensorimotor behavioural testing (Balden *et al.*, 2012). Furthermore, the vitamin D deficient animals had significantly lower levels of IGF-1, a neuroprotectant molecule usually elevated after injury, compared to controls (Balden *et al.*, 2012).

7.1.2 Modelling a two-hit hypothesis

Social stress can be assessed in rodents using a number of different approaches, but chronic social defeat has been shown to have long lasting effects on behaviour and brain function (Golden *et al.*, 2011). Using this procedure, mice are exposed to repeated bouts of social defeat by a larger aggressive mouse and then further subjected to continuous psychological stress from sensory interaction through a clear perforated divider in their home cage (Golden *et al.*, 2011). This protocol has been repeatedly shown to produce social avoidance in a subset of 'susceptible' mice. Susceptible mice show behavioural and physiological changes suggestive of depressive and anxiety symptoms, while 'resilient' mice do not (Golden *et al.*, 2011). For example, a study in rats showed that socially defeated animals displayed weight loss and an enhanced and prolonged response to acoustic startle (Pulliam *et al.*, 2010).

A recent study reported that BALB/c and C57BL/6J mice show a different susceptibility to social defeat stress, with only the BALB/c mice exhibiting long-term social withdrawal (Razzoli *et al.*, 2011). Furthermore, BALB/c mice displayed greater effects of AVD deficiency when compared directly with C57BL/6J mice (Groves *et al.*, 2013). AVD-deficient BALB/c mice also displayed enhanced acoustic startle, suggesting an altered response to stressful stimuli (Groves *et al.*, 2013).

Therefore, the aim of the current study was to examine the effects of a two-hit model combining AVD deficiency and chronic social defeat in both BALB/c and C57BL/6J mice on behaviour, using a social interaction paradigm and PPI of the ASR, two tests relevant to neuropsychiatric disorders and psychosocial stress. It was proposed that AVD deficiency would leave the mice more vulnerable to the second hit of social stress, and that these effects would be greater in the 'susceptible' BALB/c strain

7.2 Materials and methods

7.2.1 Animals and housing

Thirty male BALB/c mice, 23 male C57BL/6J mice and 16 CD-1 male mice were used in this study. Ten-week-old BALB/c and C57BL/6J mice (Animal Resources Centre, Canning Vale, WA, Australia) were obtained and housed in groups of 4 as detailed in Chapter 2. The mice were assigned to either a control diet or a vitamin D-deficient diet for 10 weeks prior to the start of behavioural testing; and for the entire duration of the experimental procedures. Dietary manipulation and housing has been detailed previously in Chapter 2. The 16 CD-1 male mice (Animal Resources Centre, Canning Vale, WA, Australia) were used as the aggressor mice in a social defeat paradigm, and were obtained at 9-10 months old and housed individually in OptiMICE cages (Animal Care Systems, CO, USA). The CD-1 mice were maintained on standard mouse chow (Specialty Feeds, WA, Australia, **Appendix 2**). All experimental work was performed with approval from the University of Queensland Animal Ethics Committee (QBI/202/13/NHMRC), under the guidelines of the National Health and Medical Research Council of Australia.

7.2.2 Experimental design

BALB/c and C57BL/6J mice were tested separately, and after a minimum of 10 weeks on diet the mice were further separated into one of two groups for social treatment, either Separated (SEP) or Social Defeat (DEF). SEP mice were placed two per cage with a perforated Plexiglas divider between them (n=7-8 BALB/c, n=5-6 C57BL/6J). These mice were moved to a new cage daily for 10 days, so that each day the mice were in a new cage with a new cage mate. At the end of the 10 days, the mice remained separated until the end of behavioural testing, 2 weeks later. The DEF mice underwent 10 days of social defeat using the protocol described by (Golden *et al.*, 2011) (detailed in Section 7.2.5) and then were housed two per cage with a perforated Plexiglas divider between them to the end of behavioural testing, 2 weeks later.

7.2.3 Body weight

Body weight was measured on Day 1 prior to the start of the 10-day social stress paradigm and on Day 11 to assess the effects of the stress paradigm on food intake and body weight maintenance.

7.2.4 Serum biochemistry

At the completion of experimental procedures, mice were euthanized by i.p injection of an overdose of Lethabarb at 4 ml/kg body weight and a terminal blood sample was collected via cardiac puncture. Serum was collected as per the details in Chapter 4. The level of 25(OH)D was measured in the serum samples using liquid chromatography-tandem mass spectrometry (Sciex Instruments, ON, Canada) on a 4000 QTrap API AB mass spectrometer (Eyles *et al.*, 2009a). To measure a range of other biochemical parameters, serum samples from 32 mice (n = 4/diet/social treatment/strain) were sent to IDEXX Laboratories (Brisbane, Australia). For the full list of biochemical parameters measured, see Table 7.2.

7.2.5 Chronic social defeat

Sixteen ex-breeder 9-10 month old CD-1 male mice (Animal Resources Centre, Canning Vale, WA, Australia) were used as aggressive residents. DEF mice from both diets were exposed to a different unfamiliar CD-1 resident mouse each day for 10 days, for up to 10 min of interaction. During this exposure all subject mice showed signs of subordination (i.e submissive postures, withdrawal, fleeing, lying on its back, or freezing), assessed visually by the experimenter. After the interaction period, subject mice were separated from the aggressive resident by placing a Plexiglas perforated divider (to allow sensory contact) between them in the resident's home-cage. The mice were housed in this way for the next 24 h, with food and water provided *ab libitum*.

7.2.6 Behavioural testing

7.2.6.1 Social interaction test

A modified version of the social interaction test described by (Golden *et al.*, 2011) was used to measure approach/avoidance behaviour toward an unfamiliar target, 24 hours after the final defeat. Social interaction was measured in a 30 x 30 cm arena with a metal wire cage (12 x 5 cm x 8 cm high) at one end (Figure 7.1). In the first phase of the test, subjects were individually placed in the arena opposite the metal wire cage while it was empty and their movement was recorded for 150 s (habituation phase) using a USB digital camera and recording software. They were returned to their home-cage for 30 s. In the second phase of the test, an unfamiliar aggressive CD-1 mouse was

confined within the metal wire cage and the subject was placed back in the arena for a further 150 s and their movement was again recorded (interaction phase). The test was performed under red light conditions.

The duration of the subject's presence within the interaction zone (defined as a 5 cm wide area surrounding the wire cage) and in the avoidance zone (defined as the far corners opposite the metal cage, 7.5 cm square) (Figure 7.1) as well as the average distance of the test mouse to the centre point of the metal cage and the total distance travelled (cm) were obtained using Ethovision v9 software (Noldus Information Technology, The Netherlands).

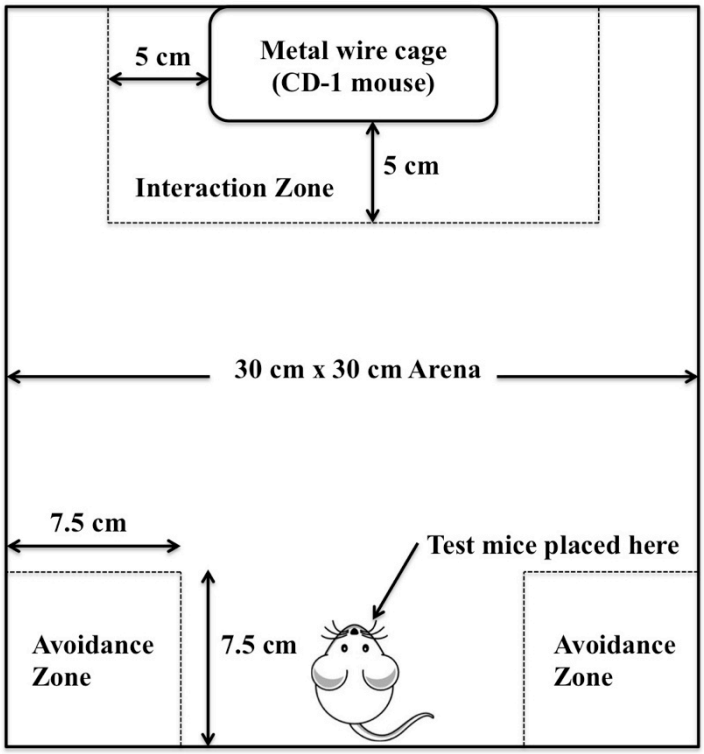

Figure 7.1 Social interaction arena layout

7.2.6.2 Prepulse inhibition of the acoustic startle response

PPI of the ASR was used to measure sensorimotor gating (Harms *et al.*, 2008) using startle chambers (SR-Lab, San Diego Instruments, CA, USA), which consisted of a Plexiglass cylinder (5 cm diameter x 12 cm long) mounted on an elevated Plexiglass base within a dark chamber. A speaker situated 24 cm above the cylinder was used to provide background noise within the chamber set to 70 dB as well as the acoustic pulses of white noise throughout the testing.

Testing began with an acclimatisation period of 300 s of 70 dB background noise. The mice then underwent a total of 130 trials (26 different blocks of 5 trials). To assess within-session habituation

(WSH), startling pulses of 110 dB were presented at the start (post acclimatisation) and end of the testing. The mice were exposed to a range of pulse intensities (80, 90, 100, 110 and 120 dB) to measure ASR and a range of prepulse to pulse intervals (8, 16, 32, 64, 128 and 256 ms) before a 120 dB pulse to measure PPI. The median values for each block of 5 trials were used for analysis, with PPI being calculated with the formula: %PPI = [(startle amplitude of ASR trial - startle amplitude on prepulse trial)/startle amplitude of ASR trial] x 100 (Harms *et al.*, 2008).

7.2.7 Statistical analysis

Results were analysed for statistical significance using SPSS (version 20.0) software. All data were analysed for the main effects of Diet (control or AVD-deficient), Social Treatment (SEP or DEF), and Strain (C57BL/6J or BALB/c) using ANOVA or, where appropriate, repeated measures ANOVA. Significant differences ($p < 0.05$) were followed up with post-hoc student t-tests.

7.3 Results

7.3.1 Body Weight

Starting body weights were not different for Social Treatment ($F_{1,43} = 0.34$, $p = 0.562$) and Diet ($F_{1,43} = 0.40$, $p = 0.530$) groups, but were significantly different between Strains ($F_{1,43} = 42.91$, $p < 0.001$) with C57BL/6J mice weighing more than BALB/c mice. At the end of the Social Treatment, the change in bodyweight was significantly different for Social Treatment ($F_{1,43} = 5.99$, $p = 0.019$) but not for Strain ($F_{1,43} = 0.93$, $p = 0.339$) or Diet ($F_{1,43} = 2.38$, $p = 0.130$) groups, with DEF mice losing weight compared to SEP mice (Table 7.1), providing conformation that the social defeat protocol worked.

Table 7.1 Summary of results for social defeat and social interaction not included in Figure 7.2
Mean ± SEM

Social Defeat and Interaction	BALB/c Control SEP	BALB/c AVD SEP	BALB/c Control DEF	BALB/c AVD DEF	C57BL/6J Control SEP	C57BL/6J AVD SEP	C57BL/6J Control DEF	C57BL/6J AVD DEF
Social Defeat - Ave Duration (s)	—	—	417.5 ± 13.2	382.5 ± 24.9	—	—	600.0 ± 0	600.0 ± 0
Habituation - Ave Dist to CD-1 (cm)	13.4 ± 1.9	14.0 ± 1.1	13.6 ± 1.9	15.1 ± 2.4	9.0 ± 1.5	10.5 ± 0.4	9.3 ± 0.8	10.5 ± 0.2
Habituation - Dist travelled (cm)	599.9 ± 63.6	722.2 ± 71.5	636.6 ± 69.5	805.3 ± 98.5	774.0 ± 165.0	822.7 ± 44.0	713.9 ± 67.3	725.0 ± 43.5
Habituation - Interaction Zone (s)	80.58 ± 14.03	64.16 ± 4.78	44.93 ± 10.97	50.43 ± 12.92	93.45 ± 18.14	87.02 ± 19.40	91.02 ±10.47	105.87 ± 7.20
Habituation - Avoidance Zone (s)	16.88 ± 11.99	19.10 ± 8.16	6.69 ± 2.11	27.35 ± 13.77	6.06 ± 1.99	6.91 ± 1.00	5.98 ±1.85	6.96 ±1.25
Interaction - Dist travelled (cm)	475.6 ± 63.0	474.8 ± 89.3	284.9 ± 34.0	175.9 ± 31.7	599.8 ± 62.1	582.4 ± 56.3	380.9 ± 75.7	493.7 ± 41.7
Starting Body Weight (g)	27.4 ± 0.6	26.9 ± 0.8	25.6 ± 1.0	26.8 ± 0.4	32.3 ± 1.9	32.2 ± 0.8	33.0 ± 1.7	30.9 ± 1.4
Finishing Body Weight (g)	26.9 ± 0.6	27.1 ± 0.8	23.5 ± 0.8	25.3 ± 0.8	31.5 ± 1.4	31.7 ± 0.9	27.9 ± 1.1	29.4 ± 0.7

7.3.2 Serum biochemistry

There was a significant main effect of Strain ($F_{1,43} = 152.41$, $p < 0.001$) and Diet ($F_{1,43} = 937.07$, $p < 0.001$) on 25(OH)D levels, with higher levels seen in C57BL/6J mice compared to BALB/c mice and higher levels in Controls compared to AVD-deficient mice, see Table 7.2 for details. As expected, the AVD-deficient mice had levels of 25(OH)D at the lower level of detection.

There was a significant main effect of Strain ($F_{1,17} > 4.38$, $p < 0.047$) on the level of a number of other biochemical parameters including sodium, potassium, glucose, calcium, total protein, albumin, bilirubin, aspartate aminotransferase, and creatine kinase.

There was a significant main effect of Social Treatment ($F_{1,17} = 5.49$, $p = 0.032$) on the level of total serum protein, with a Strain x Diet interaction ($F_{1,17} = 4.51$, $p = 0.049$). When analysing the strains separately, there was a significant effect of Social Treatment ($F_{1,11} = 6.27$, $p = 0.029$) on total serum protein levels in C57BL/6J mice, but no effect of Diet ($F_{1,11} = 4.26$, $p = 0.063$), with reduced levels in the Social Defeat mice. There were no significant effects in BALB/c mice.

Analysing the strains separately, there was only a significant effect of Social Treatment ($F_{1,12} = 4.86$, $p = 0.048$) on sodium levels in BALB/c mice, with lower levels in DEF mice compared to SEP mice. There were no significant effects of Diet on any measure when strains were analysed separately.

Table 7.2 Summary of results for biochemical parameters

Mean ± SEM

Biochemistry (*n* = 4/group)	BALB/c Control SEP	BALB/c AVD SEP	BALB/c Control DEF	BALB/c AVD DEF	C57BL/6J Control SEP	C57BL/6J AVD SEP	C57BL/6J Control DEF	C57BL/6J AVD DEF
25-OHD$_3$ (*n* = 5-8)	26.85 ± 0.56	2.21 ± 1.11	28.57 ± 2.06	2.99 ± 1.23	62.15 ± 4.11	1.90 ± 0.36	66.54 ± 2.89	1.02 ± 0.30
Sodium	161 ± 1	161 ± 1	159 ± 1	159 ± 1	156 ± 1	156 ± 1	158 ± 4	157 ± 1
Potassium	6 ± 0	6 ± 0	6 ± 0	6 ± 0	10 ± 2	8 ± 1	8 ± 0	7 ± 1
Chloride	113 ± 1	111 ± 0	110 ± 1	112 ± 1	108 ± 1	108 ± 1	114 ± 3	110 ± 1
Glucose	8 ± 1	9 ± 1	7 ± 2	15 ± 5	28 ± 8	27 ± 2	18 ± 7	20 ± 4
Urea	8 ± 0	8 ± 0	9 ± 1	9 ± 0	8 ± 1	8 ± 1	8 ± 1	10 ± 3
Calcium	2.1 ± 0.1	2.1 ± 0.0	1.7 ± 0.4	2.0 ± 0.0	2.4 ± 0.1	2.5 ± 0.1	2.4 ± 0.1	2.3 ± 0.3
Phosphate	3 ± 0	3 ± 0	3 ± 1	3 ± 0	4 ± 1	4 ± 0	4 ± 1	4 ± 0
Protein Total	50 ± 1	50 ± 1	40 ± 8	50 ± 1	56 ± 2	54 ± 2	53 ± 0	43 ± 5
Albumin	24 ± 1	24 ± 0	20 ± 4	25 ± 0	27 ± 1	29 ± 1	28 ± 1	29 ± 5
Globulin	25 ± 0	25 ± 1	20 ± 4	26 ± 1	28 ± 3	25 ± 1	25 ± 0	15 ± 9
A:G Ration	1 ± 0	1 ± 0	1 ± 0	1 ± 0	1 ± 0	1 ± 0	1 ± 0	0 ± 1
Bilirubin Total	1 ± 0	1 ± 0	0 ± 0	1 ± 0	3 ± 1	2 ± 0	3 ± 1	1 ± 1
Alkaline Phosphatase	78 ± 4	93 ± 8	69 ± 15	81 ± 6	87 ± 18	69 ± 9	63 ± 14	51 ± 9
Aspartate Aminotransferase	273 ± 57	178 ± 11	138 ± 5	172 ± 44	476 ± 143	374 ± 129	315 ± 84	247 ± 121
Alanine Aminotransferase	46 ± 7	30 ± 2	25 ± 2	28 ± 7	410 ± 244	217 ± 96	92 ± 31	78 ± 48
Creatine Kinase	42 ± 11	58 ± 8	39 ± 11	41 ± 10	39 ± 8	33 ± 8	25 ± 2	31 ± 8
Cholesterol	3 ± 0	3 ± 0	2 ± 0	3 ± 0	4 ± 0	4 ± 0	3 ± 1	3 ± 0
Triglyceride	1 ± 0	1 ± 0	1 ± 0	1 ± 0	1 ± 0	1 ± 0	1 ± 0	1 ± 0

7.3.3 Social defeat duration

In the socially defeated mice, there was no significant effect of Diet ($F_{1,22} = 1.52$, $p = 0.230$) on the duration of the daily defeats. There was a significant effect of Strain ($F_{1,22} = 198.83$, $p < 0.001$) on the duration of the daily defeats. Defeats were terminated by the experimenter early in BALB/c mice due to the intensity of the defeats (multiple attacks within a 60 sec time period, despite defensive behaviour shown by the BALB/c mice), however all C57BL/6J mice completed the full ten min defeat each day for the 10 days (Table 7.1).

7.3.4 Social interaction test

7.3.4.1 Habituation phase

During the habituation phase of the social interaction test there was no significant main effect of Diet or Social Treatment on any measure, there was however a significant main effect of Strain on the average distance to the metal cage ($F_{1,44} = 13.88$, $p = 0.001$), with the C57BL/6J mice having a shorter average distance compared to the BALB/c mice (Table 7.1).

7.3.4.2 Interaction phase

During the interaction phase there were four main measures analysed; the average distance to the CD-1, distance travelled, time in the interaction zone and time in the avoidance zone.

7.3.4.2.1 Average distance to CD-1

There were significant main effects of Diet ($F_{1,44} = 4.64$, $p = 0.037$), Strain ($F_{1,44} = 19.19$, $p < 0.001$) and Social Treatment ($F_{1,44} = 30.48$, $p < 0.001$) on the average distance to CD-1 with a Strain x Social Treatment interaction ($F_{1,44} = 15.31$, $p < 0.001$). Analysing the strains separately, there was a significant effect of Social Treatment in C57BL/6J ($F_{1,19} = 4.69$, $p = 0.043$) and in BALB/c mice ($F_{1,25} = 31.88$, $p < 0.001$), with DEF mice having a greater average distance to CD-1 compared to SEP mice. There was also a significant effect of Diet ($F_{1,25} = 4.70$, $p = 0.040$) in the BALB/c strain only, with AVD-deficient mice having a greater distance to CD-1 compared to control mice (Figure 7.2a and b).

7.3.4.2.2 Distance travelled during interaction

There was a significant main effect of Social Treatment ($F_{1,44} = 21.05$, $p < 0.001$) and Strain ($F_{1,44} = 13.80$, $p = 0.001$) but not of Diet ($F_{1,44} = 0.01$, $p = 0.935$) and no interaction. C57BL/6J mice travelled further than BALB/c mice and SEP mice travelled further than DEF mice (Table 7.1).

7.3.4.2.3 Time spent in the interaction zone

There was a significant main effect of Strain ($F_{1,44} = 33.48$, $p < 0.001$) and Social Treatment ($F_{1,44} = 15.49$, $p < 0.001$), but not of Diet ($F_{1,44} = 0.11$, $p = 741$) on the time spent in the interaction zone. The DEF mice spent less time in the interaction zone compared to SEP mice and BALB/c mice spent less time in this zone compared to C57BL/6J mice (Figure 7.2c and d).

7.3.4.2.4 Time spent in the avoidance zone

There were significant main effects of Strain ($F_{1,44} = 10.11$, $p = 0.003$), Social Treatment ($F_{1,44} = 6.34$, $p = 0.016$) and Diet ($F_{1,44} = 4.86$, $p = 0.033$) on the time spent in the avoidance zone. When analysing the strains separately, there was no significant effect of Social Treatment or Diet in the C57BL/6J mice. In the BALB/c mice, there was a significant effect of Social Treatment ($F_{1,25} = 6.32$, $p = 0.019$) and Diet ($F_{1,25} = 4.75$, $p = 0.039$) on the time spent in the avoidance zone. DEF mice spent more time in the avoidance zone compared to SEP mice and AVD-deficient mice spent more time in the avoidance zone compared to control mice (Figure 7.2e and f).

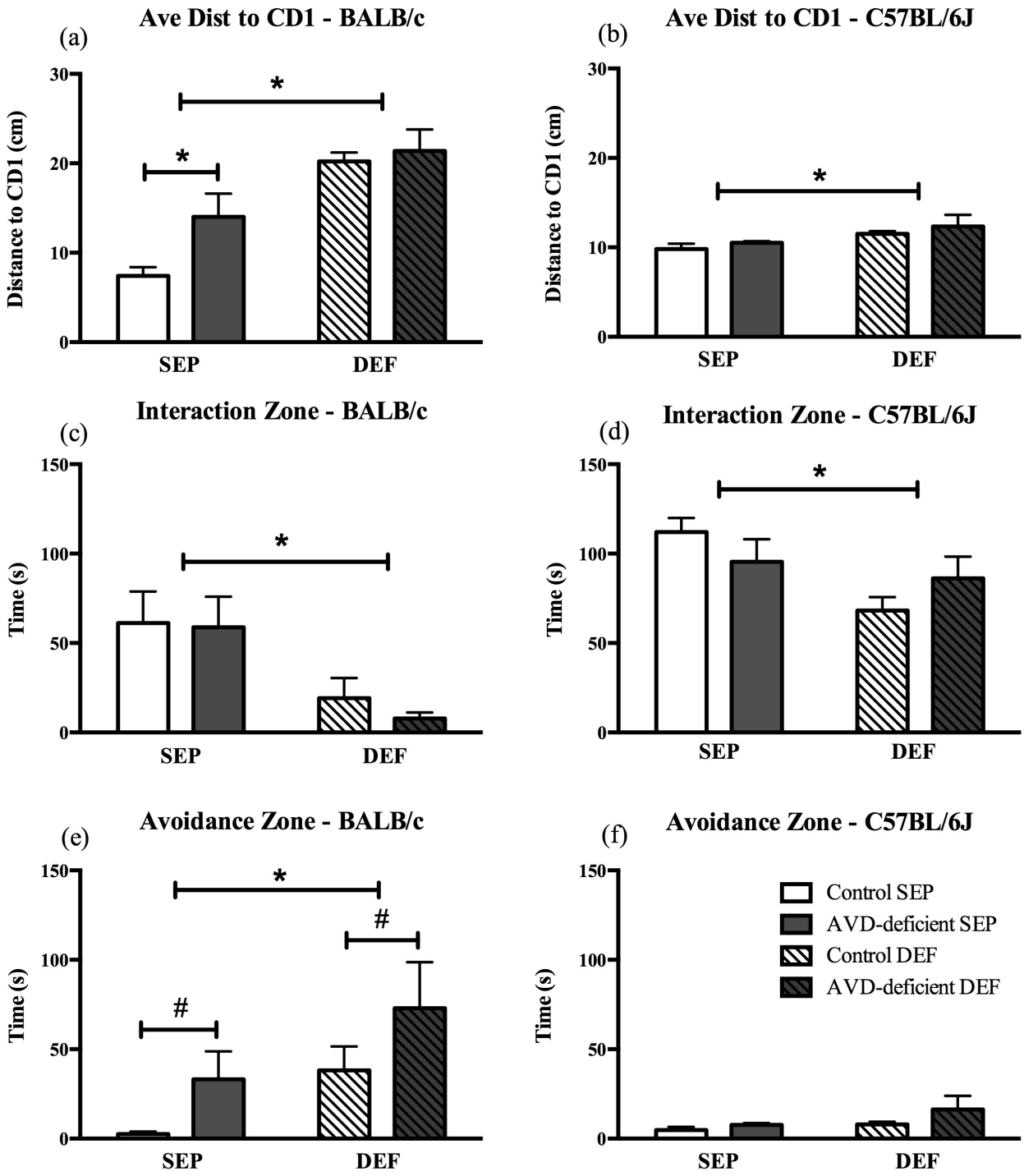

Figure 7.2 Results of the social interaction test
The results of the social interaction test in BALB/c mice **(a, c and e)** and in C57BL/6J mice **(b, d and f)**. The average distance to the CD-1 mouse was significantly further in DEF mice compared to SEP mice in both strains **(a and c)**, however in the BALB/c strain **(a)**, there was also a significant increase in the average distance to the CD-1 mouse in AVD-deficient SEP mice compared to control SEP mice. The time spent in the interaction zone was significantly less for DEF mice compared to SEP mice in both strains **(b and d)**, with no significant differences between AVD-deficient and control mice in either strain. The time spent in the avoidance zone was significantly greater in DEF mice compared to SEP mice in BALB/c mice only **(e)**, furthermore AVD-deficient mice spent significantly (# main effect of Diet, $p < 0.05$) more time in the avoidance zone compared to controls **(e)**. Mean ± SEM (*$p < 0.05$)

7.3.5 Prepulse inhibition of the acoustic startle response

7.3.5.1 Acoustic startle response

There was a significant Strain x Diet x Social Treatment interaction ($F_{1,43} = 12.54$, $p = 0.001$) with significance found in the main effects of Strain, Social Treatment and Diet in the ASR. In the BALB/c strain (Figure 7.3a and c), there was a Diet x Social Treatment interaction ($F_{1,24} = 4.38$, $p = 0.047$). Post-hoc t-tests showed a significant difference between DEF controls and SEP control mice, with DEF leading to a blunted ASR but no significant difference between Social Treatments in AVD-deficient mice. In C57BL/6J mice (Figure 7.3b and d), there was a main effect of Diet ($F_{1,19} = 15.79$, $p = 0.001$) but not of Social Treatment ($F_{1,19} = 1.67$, $p = 0.212$), with a Diet x Social Treatment interaction ($F_{1,19} = 7.52$, $p = 0.013$). In the DEF mice, AVD deficiency led to a blunted ASR compared to controls and compared to SEP mice.

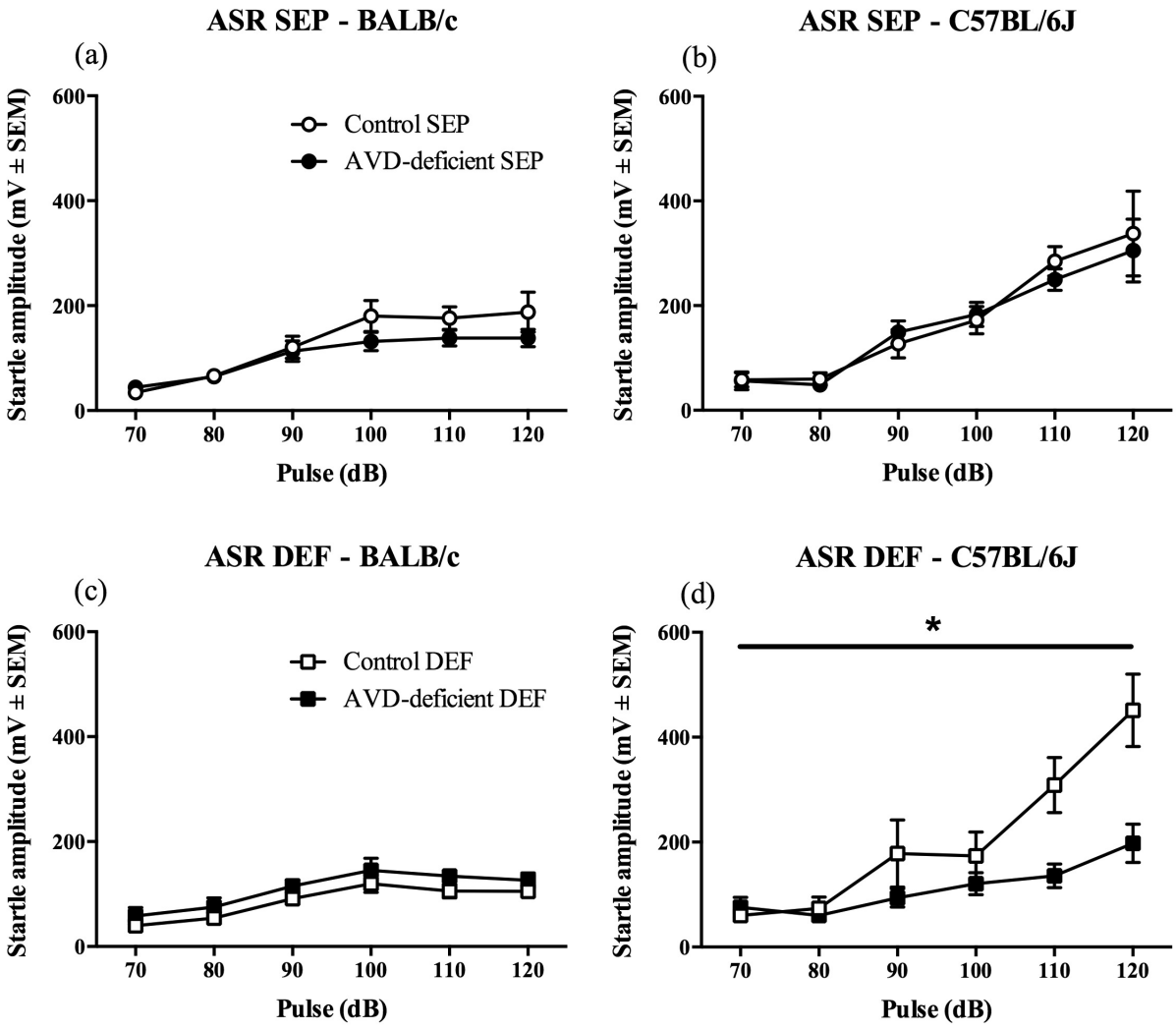

Figure 7.3 Results for the acoustic startle response
ASR in BALB/c **(a and c)** and C57BL/6J **(b and d)** mice. There was a significant Diet x Social Treatment interaction in BALB/c mice. Post-hoc t-tests showed a significant difference between DEF controls and SEP control mice, with DEF leading to a blunted ASR but no significant difference between Social Treatments in AVD-deficient mice. In C57BL/6J mice, AVD deficiency led to a blunted acoustic startle response in DEF mice **(d)**. Mean ± SEM (*$p < 0.05$)

7.3.5.2 Within-session habituation

There was a significant main effect of Strain ($F_{1,43} = 6.16$, $p = 0.017$) and Diet ($F_{1,43} = 9.31$, $p = 0.004$) on the response to the initial 110 dB pulse given at the start of the session, with AVD-deficient mice reacting less than controls and BALB/c mice reacting less than C57BL/6J mice. Due to the effect of Diet on the response to the initial pulse, AVD-deficient mice showed no WSH ($F_{1,19} = 0.01$, $p = 0.933$), however, control mice did show a significant WSH ($F_{1,24} = 6.58$, $p = 0.017$) (Figure 7.4). There was no effect of Social Treatment on the initial pulse ($F_{1,43} = 1.61$, $p = 0.212$) or on WSH ($F_{1,43} = 0.03$, $p = 0.876$).

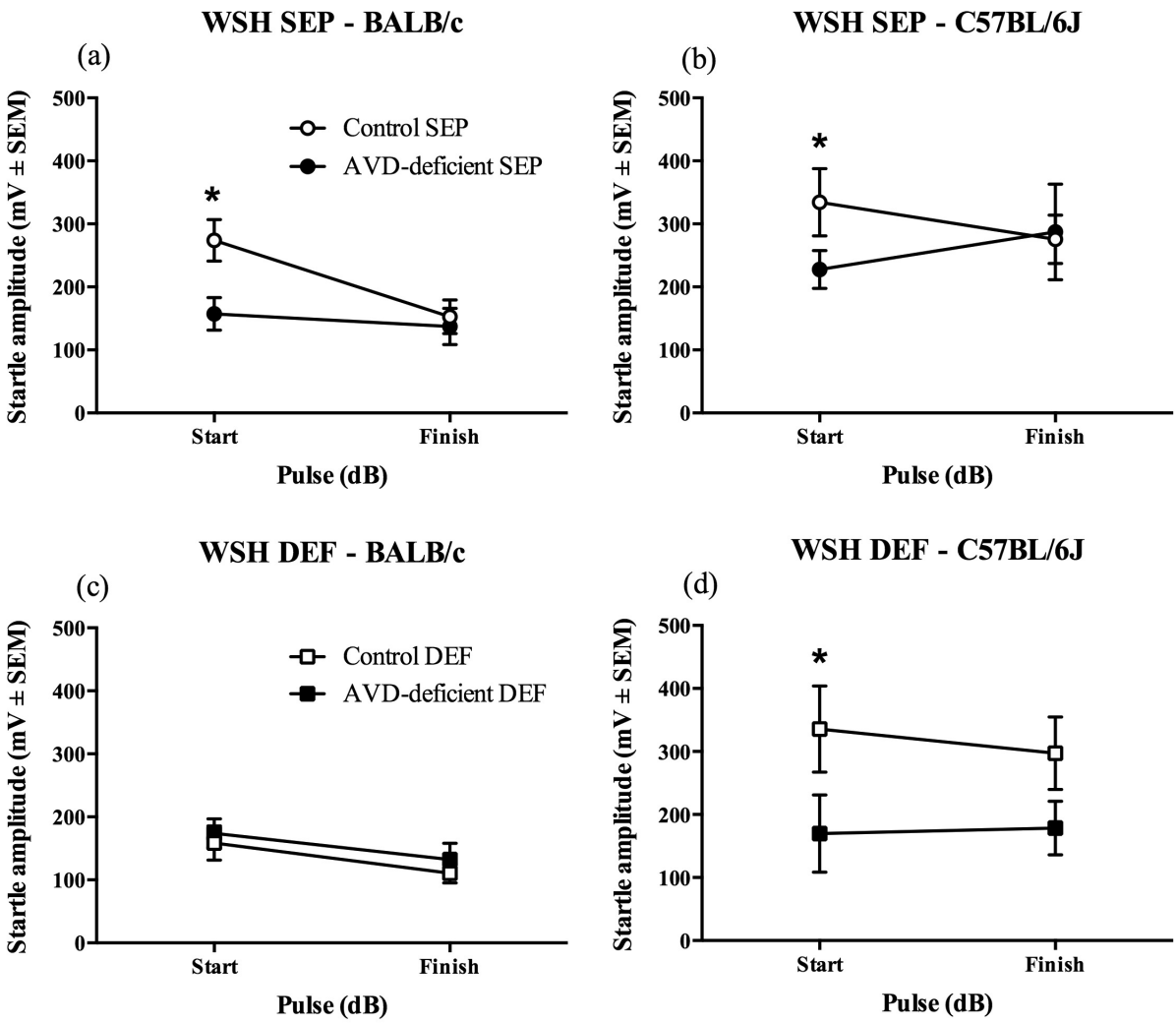

Figure 7.4 Results for within-session habituation
WSH in BALB/c strain **(a and c)** and in C57BL/6J strain **(b and d),** SEP mice shown in **a** and **b**, with DEF mice shown in **c** and **d**. Overall, AVD deficiency led to a significantly reduced response to the initial 110 dB pulse. Due to this reduced initial response, AVD-deficient mice had no WSH, although controls did show WSH. Mean ± SEM (*$p < 0.05$)

7.3.5.3 Prepulse inhibition

There were no significant differences in PPI scores for Strain ($F_{1,43} = 0.70$, $p = 0.407$), Diet ($F_{1,43} = 0.23$, $p = 0.633$) or Social Treatment ($F_{1,43} = 0.18$, $p = 0.671$) (Figure 7.5). When strains were analysed separately, there were still no significant differences seen.

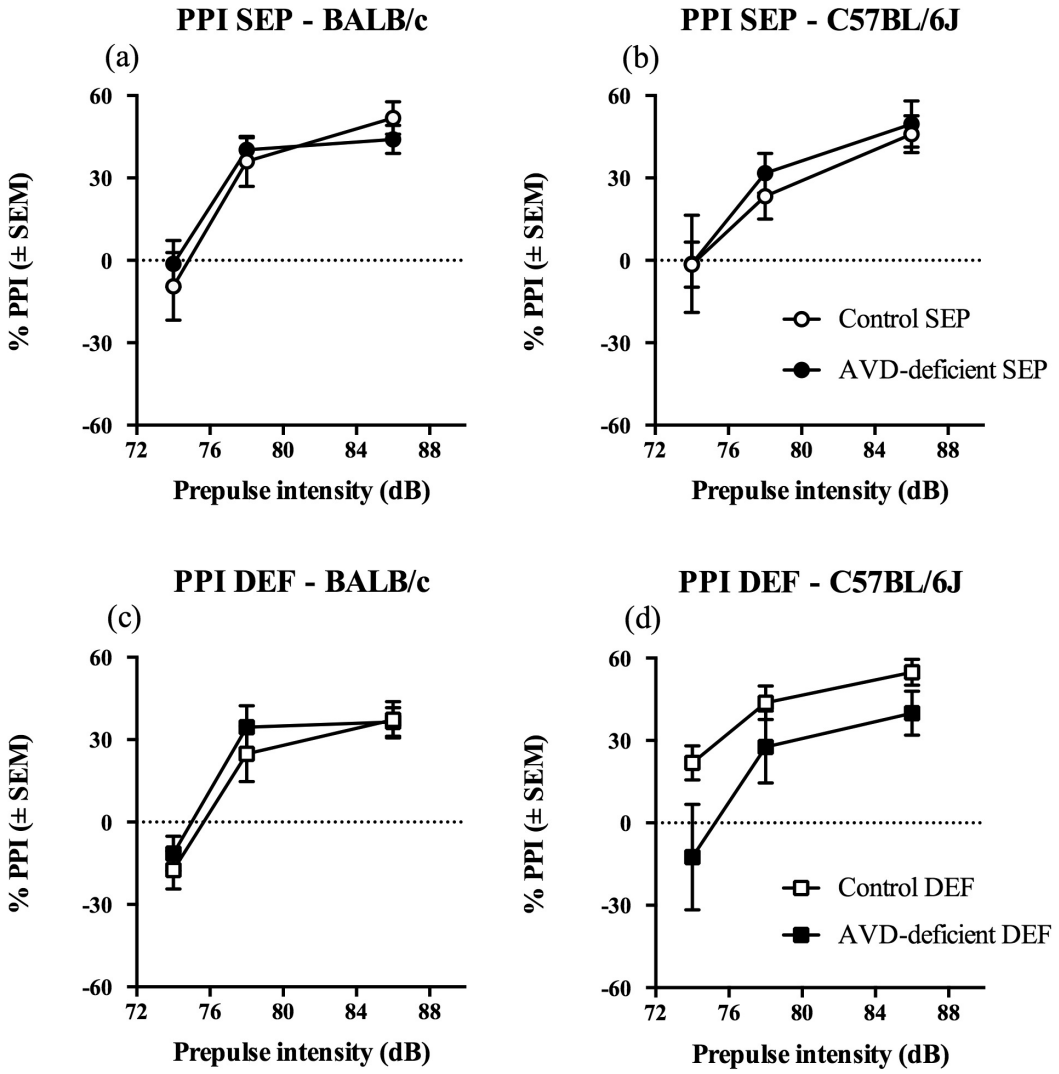

Figure 7.5 Results for prepulse inhibition of the acoustic startle response
There were no significant differences on PPI scores. Mean ± SEM

7.4 Discussion

The main finding from this study was that AVD deficiency exacerbated impairments caused by social stress and the degree of impairment was dependent on strain. In BALB/c mice but not C57BL/6J mice, AVD deficiency exacerbated the social deficit seen following social stress compared to controls. In the ASR, the main difference was seen in the C57BL/6J mice; when AVD deficiency was combined with social defeat, it led to a blunted ASR, compared to either treatment on their own. In addition, AVD deficiency altered WSH across both strains due to a blunted initial response. Therefore we can accept the hypothesis that combining two-hits leads to greater detrimental outcomes compared to either treatment alone.

Biochemical parameters were measured in a subset of mice from each group and although there were strain differences for a number of parameters, there was no significant difference between AVD-deficient mice and control mice on any measure other than vitamin D levels. Importantly, calcium and phosphate levels, which are known to be regulated by vitamin D (Holick, 2007) were not altered in the AVD-deficient mice. Therefore, we can conclude that the model of AVD deficiency used in the current study and in the previous chapters has limited confounding factors and we can be more confident that alterations reported using this model have occurred due to the effects of vitamin D deficiency.

7.4.1 Relevance to neuropsychiatric disorders

Social avoidance is a negative symptom of schizophrenia (Hansen *et al.*, 2009), a symptom of autism (Richer, 1976) and it is also a symptom of depression and anxiety (Gorman, 1996). A previous study in group-housed BALB/c and C57BL/6J mice showed no social interaction impairments with AVD deficiency (Groves *et al.*, 2013). Although the current study showed that chronic social defeat led to a mild social deficit in C57BL/6J mice, AVD deficiency did not exacerbate the mild social deficit caused by the chronic social defeat. However in BALB/c mice, AVD deficiency did exacerbate the social deficits following chronic social defeat and also increased the deficit following the milder separated housing condition.

Impairments in PPI of the ASR are relevant to schizophrenia (Moriwaki *et al.*, 2009), however ASR on its own is generally not considered important as a behavioural readout for schizophrenia. It has been suggested that PPI is difficult to assess when the ASR itself has been modulated by a specific treatment, as we have seen in these experiments. However, we did not see any significant difference in PPI. The % PPI values in the C57BL/6J mice are comparable to previously published data using this strain; with the same protocol and equipment that was used in the current study (Harms *et al.*,

2008). Although, results using the BALB/c strain have not previously been published using this protocol, there was no significant difference in % PPI between the two strains in this study. PPI values do vary depending on the particular protocol used, as well as the facility and batch of mice. For example, a study by (van den Buuse, 2013) showed higher rates of % PPI and ASR in the BALB/c strain compared to the C57BL/6J strain and compared to the BALB/c mice in this study.

The relevance of altered ASR is not as easily defined, although changes in fear and anxiety can alter ASR (Davis *et al.*, 1997), as can changes in stress response pathways (Wang *et al.*, 2002a). Furthermore, enhanced response to acoustic startle has been previously proposed to reflect aspects of traumatic psychosocial stress (Pulliam *et al.*, 2010).

AVD deficiency in group-housed BALB/c mice has previously been shown to increase the ASR compared to controls (Groves *et al.*, 2013). In this experiment there was no significant main effect of diet on the ASR when analysing BALB/c mice separately. It is possible that the combination of social stress and AVD deficiency has blunted the ASR back to levels comparable to controls. However, there were differences in housing between the current study and the previous study (Groves *et al.*, 2013) and the different housing conditions may have impacted on the ASR response. The level of ASR in this experiment was comparable to the controls in the previous study (Groves *et al.*, 2013). In C57BL/6J mice, there was a significant effect on ASR when AVD deficiency was combined with chronic social defeat leading to a blunted ASR. Studies in humans have shown blunted startle in unipolar and bipolar depression (Forbes *et al.*, 2005; Dichter & Tomarken, 2008), and in Parkinson's disease patients (Bowers *et al.*, 2006), suggesting that it may be indicative of a depressive-like phenotype.

7.4.2 Limitations

The duration of the social defeats were often terminated early by the experimenter (average of six and a half min, in lieu of the standard 10 min duration), due to the aggressive nature of the social defeats for the BALB/c strain. Due to the intensity of the protocol in the BALB/c mice, there may have been ceiling effects that would prevent all differential effects of diet from being seen. Interestingly, we noted effects of AVD deficiency in the separated mice in the BALB/c strain and propose that being separated from cage mates during this period was enough to produce a mild social stress in this strain. Therefore, a better control such as group-housed mice should be used in any future experiments. Furthermore, a milder form of social stress such as social isolation or restraint stress may be enough of a second hit in this strain.

By contrast, the C57BL/6J strain was a more robust, larger sized, aggressive strain compared to the BALB/c strain and was more resilient to the social defeat paradigm. All bouts of defeat lasted the full 10 min, however all mice were submissive towards the CD-1 mice and there was a significant difference in behaviour between the separated C57BL/6J mice and the social defeat C57BL/6J mice. However, overall the C57BL/6J mice were more resilient to social defeat compared to the BALB/c mice.

7.4.3 Future Directions

This experiment is the first to analyse the effects of combining the two adult environmental exposures of vitamin D deficiency and social stress. Future studies should extend the behavioural tests to address a more comprehensive screen of domains relevant to neuropsychiatric disorders, such as tests of anxiety, psychomimetic-induced locomotion and cognitive impairments. Other important experiments would be to look at changes in brain neurochemistry. Perhaps in future experiments, alternatives to social stress as the 'second hit' could be used, for example cannabis use is a risk factor for the development of schizophrenia and could be combined with AVD deficiency.

There is also growing evidence of an association between vitamin D deficiency and a substantially increased risk of all-cause dementia and AD (Littlejohns *et al.*, 2014). Future experiments could test the additional effect of vitamin D deficiency on already well-characterized animal models of Alzheimer's disease. It is possible that a lack of neuroprotection caused by vitamin D deficiency may exacerbate the symptom severity of diseases such as Alzheimer's disease.

7.5 Conclusion

We found that AVD deficiency exacerbated impairments caused by social stress and the degree of impairment was strain dependent. These results extend our previous work related to developmental vitamin D exposures, and indicate that AVD deficiency may leave the brain more vulnerable to stress-related second hits. Furthermore, these findings could provide a mechanism of action underpinning the increased risk of psychosis in dark skinned migrants to cold climates; or the increased risk of AD, or PD severity in vitamin D deficient individuals.

Chapter 8 General Discussion

8.1 Introduction

There is increasing evidence from epidemiological studies to suggest that vitamin D deficiency during adulthood is associated with adverse brain outcomes (Eyles *et al.*, 2013; Groves *et al.*, 2014), however a causal relationship has not yet been established. We have previously shown in a mouse model that vitamin D deficiency during adulthood impacts on a range of brain functions (Groves *et al.*, 2013) and should therefore be considered as a biologically plausible risk factor for the development of neuropsychiatric and neurodegenerative disorders. The purpose of this thesis was to extend our understanding of how vitamin D deficiency is impacting on brain function and provide evidence towards deciphering the mechanisms by which this occurs.

Based on a preliminary proteomic study, I found convergent evidence of an effect of AVD deficiency on glutamate and GABA signaling, enabling a greater understanding of how AVD deficiency may be altering neurotransmission. Furthermore, we have established that AVD deficiency impacts on glutathione synthesis leading to a reduction in glutathione levels within the brain, which would potentially increase exposure to oxidative stress. Oxidative stress has been identified as a risk factor for a number of neuropsychiatric and neurodegenerative disorders (Zhao & Zhao, 2013; Baierle *et al.*, 2015; Black *et al.*, 2015) and therefore, this may be one key mechanism for the effects of AVD deficiency.

This study has also shown for the first time that AVD deficiency was associated with cognitive impairments in otherwise healthy male mice on two separate tasks; (a) an operant-based task to measure attentional processes, and (b) a hippocampal-dependent spatial learning and memory task. Additionally, pathway analysis of the proteomic results has highlighted a number of dysregulated proteins known to impact on synaptic plasticity, which may be responsible for the alterations in cognitive performance. Mindful that the proteomics data requires confirmation and/or replication, we note with interest the convergent findings with other findings from the AVD deficiency models.

Despite significant evidence towards an effect of vitamin D on proliferation and differentiation during development (see Chapter 1 for review), there were no cellular level changes in adult hippocampal neurogenesis in AVD-deficient mice. Furthermore, I found that brain volume and structure were not different between controls and AVD-deficient mice using MRI, nor were there any changes to connectivity in the brain as measured by diffusion tensor MRI of the whole brain. The lack of gross morphological changes, combined with the neurochemical, proteomic and behavioural changes associated with AVD Deficiency suggest that this model is associated with a subtle phenotype.

The findings in Chapter 7 support the hypothesis that AVD-related brain changes may exacerbate other neurological insults. This finding is consistent with previous results related to stroke models in which vitamin D deficiency led to larger infarct volumes and greater post-stroke behavioural impairments (Balden *et al.*, 2012). In addition, clinical trials of vitamin D supplementation in adults with PD suggest that low vitamin D exacerbates the progression of this neurodegenerative disorder (Suzuki *et al.*, 2013). The findings from this thesis may have implications for understanding the role of vitamin D deficiency in clinical settings. While speculative, low vitamin D alone may not be sufficient to precipitate a neurological disorder, but may be sufficient to amplify the progression of other independent disorders. Thus, low vitamin D status may contribute to greater disability and reduce chance of recovery from neurodegenerative disorders.

Although the exact mechanisms by which AVD deficiency is impacting on brain function has not been elucidated, the results from this thesis have provided some key findings on the impact of AVD deficiency in glutathione synthesis, synaptic plasticity and glutamate and GABA signaling.

8.2 Glutamate and GABA signaling

We have previously shown that AVD deficiency in male mice leads to reduced glutamate levels and increased GABA levels, measured by HPLC in whole brain; and a small but significant reduction in the GAD65/67 enzymes in whole brain, measured by western blot (Groves *et al.*, 2013). We have now provided convergent evidence to show that there is a significant reduction in GAD65 in the hippocampus of male BALB/c mice using proteomic analysis.

Studies in knockout mice have shown that ablation of GAD67 results in neonatal lethality and reductions in GABA levels to 7% of wildtype mice (Asada *et al.*, 1997). GABA is necessary during development to regulate neocortical neurogenesis (LoTurco *et al.*, 1995). However, in GAD65 knockout mice, there was no change to GABA levels and it was shown that GAD65 is not required for development but is important for modulating inhibitory neurotransmission in response to increased demand (Asada *et al.*, 1996). GAD65 is localized to axon terminals and is reversibly bound to the membrane of synaptic vesicles (Kaufman *et al.*, 1991; Christgau *et al.*, 1992). Further studies in the GAD65 knockout mice have shown that GAD65 mediates activity-dependent GABA synthesis (Patel *et al.*, 2006) but moreover, has a significant impact on GABA release during sustained activation, possibly through mobilization of vesicles or replenishment of vesicles at the synapse (Tian *et al.*, 1999). Therefore, it is feasible that the reduction in GAD65 protein expression associated with AVD deficiency could have an impact on GABAergic inhibitory neurotransmission.

Another important protein involved in GABA signaling that was found to be upregulated by AVD deficiency was GAT1 (or SLC6A1). GAT1 is also localized to GABAergic interneuron axons and nerve terminals and is responsible for GABA reuptake from the synaptic cleft (Minelli *et al.*, 1995). With reductions in GABA synthesis, due to lower levels of the GAD65 enzyme, it is plausible to suggest that this would lead to upregulation of GAT1 in an attempt to restore intracellular GABA levels. Reductions in GAD65 in a pathophysiological pain model, did accompany elevated levels of GAT1, although it was unclear which protein expression level change preceded the other (Ford *et al.*, 2015).

A protein identified in the proteomics involved in glutamate signaling was CACNA1E, downregulated by AVD deficiency; this protein, also called $CA_v2.3$, is the α_{1E} subunit of the R-type calcium channel. The molecular basis and physiological function of the R-type channel are still relatively unclear, due to a lack of specific blockers for the channel; however it has been identified as neuron specific (Wu *et al.*, 1998). Furthermore, R-type calcium channels do contribute to calcium influx during presynaptic action potentials and have been shown to evoke enough glutamate release on their own to initiate an action potential in the postsynaptic neuron (Wu *et al.*, 1998). $Ca_v2.3$ expression is high in the dentate granule layer and CA3 regions of the hippocampus (Day *et al.*, 1996). In addition, $Ca_v2.3$ containing calcium channels are involved in synaptic plasticity, by participating in the calcium influx during the induction phase of presynaptic LTP in mossy fibres of the hippocampus (Breustedt *et al.*, 2003).

$Ca_v2.3$ calcium channel knockout mice have impaired mossy fibre LTP (Breustedt *et al.*, 2003) but intact LTP measured in CA1 pyramidal neurons (Kubota *et al.*, 2001). These mice exhibit impaired spatial memory in the Morris water maze, with a longer latency to find the platform during training and a reduced duration in the target quadrant during the probe trial compared to wild-type mice (Kubota *et al.*, 2001). Mossy fibre synapses are required for spatial learning and drug-induced blockade of mossy fibre synapses will result in similar deficits in the Morris water maze as seen by the loss of $Ca_v2.3$ calcium channels (Lassalle *et al.*, 2000).

With a reduction in $Ca_v2.3$ expression levels within the hippocampus in the AVD-deficient mice, calcium influx into presynaptic terminals would be reduced. Reductions in calcium influx may impair glutamatergic neurotransmitter release, impact on mossy fibre LTP, and therefore, impair spatial learning. These protein level changes are not only consistent with previous findings of altered glutamate and GABA neurotransmitter changes (Groves *et al.*, 2013) but could actually lead to the impaired spatial learning and memory seen with AVD deficiency.

8.3 AVD deficiency and oxidative stress

Using both proteomics and HPLC, we have shown that the γ-glutamyl cycle has been dysregulated following AVD deficiency leading to a reduction in total glutathione levels within the brain. In vivo and in vitro studies have previously shown that the addition of $1,25(OH)_2D$ also regulates proteins involved in glutathione biosynthesis and leads to increased glutathione levels (reviewed in Chapter 1**)**. Oxidants are formed as a normal part of aerobic metabolism but can be formed at an accelerated rate during pathophysiological conditions or when the body's natural antioxidant mechanisms fail (Sies, 1997). Oxidative stress occurs when there is an imbalance between antioxidants and oxidants in favour of the oxidants, and with the reduction in glutathione, it is likely that AVD-deficient mice were exposed to chronic oxidative stress.

Oxidative stress can lead to cell membrane dysfunction, DNA damage and inactivation of proteins, eventually leading to cell death. Studies have shown that people with mild cognitive impairment have higher levels of oxidative stress compared to healthy controls (Pratico *et al.*, 2002). Furthermore, a study performed in rats showed that even in young animals (3 months of age), induction of oxidative stress led to impairments in spatial learning and memory (Fukui *et al.*, 2002). Induced oxidative stress, via exposure to 100 % oxygen for 48 h, produced an abnormal accumulation of synaptic vesicles in swollen nerve terminals and a reduction in neurotransmission (Urano *et al.*, 1997). In addition, following the induction of oxidative stress, synaptic membranes were shown to have elevated markers of oxidative stress including lipid peroxides and protein carbonyls, and a change in membrane surface potential. It was proposed that this would result in reduced membrane fusion between nerve terminal membranes and synaptic vesicles and produce the subsequent decline in neurotransmission shown previously (Urano *et al.*, 1997; Fukui *et al.*, 2002).

Exposure to chronic oxidative stress with AVD deficiency could therefore result in the cognitive impairments seen in this model, and the predicted effects on synapse formation and release, and neurotransmission obtained from the Ingenuity pathway analysis.

8.4 AVD deficiency and cognition

Lesion studies of the cortico-striatal pathways involved in attentional processes have shown that damage to the medial PFC, using quinolinic acid, replicates the findings in AVD-deficient mice with deficits in accuracy and lengthening of choice latencies (Robbins, 2002). Lesions of other cortico-striatal regions produce other deficits including impulsive responding or altered motivation, which we did not see with AVD-deficiency (Robbins, 2002).

Furthermore, the phenotype seen with AVD deficiency in males is similar to that produced following an infusion of picrotoxin, a $GABA_A$ receptor antagonist that dose-dependently reduces GABA currents, into the medial PFC of rats. Picrotoxin infusion led to deficits in accuracy and omissions, without altering impulsive or motivational behaviours on the 5C-SRT task (Pezze *et al.*, 2014).

In Sprague-Dawley rats on a modified 3C-SRT in lieu of the 5C-SRT task, using two different $GABA_A$ receptor antagonists, researchers showed consistent results with that of picrotoxin effects; reduced accuracy and increased omissions, with no other alterations (Pehrson *et al.*, 2013). They also tested the effects of GAD inhibition, with 3-mercaptopropionic acid, into the anterior cingulate cortex or systemic administration in Sprague-Dawley rats, on the 3C-SRT with no significant alterations in attention (Pehrson *et al.*, 2013). These results suggest that reductions in GAD65 seen with AVD deficiency may be secondary to GAT1 upregulation, or altered expression of other proteins involved in GABAergic neurotransmission. There is consistent evidence from these previous rodent studies that disruption of GABAergic neurotransmission within the medial PFC and other areas of the cortico-striatal pathways does impact on aspects of the 5C-SRT.

Performance on the 5C-SRT is also impaired by a variety of NMDA receptor antagonists including 3-(R)-2-carboxypiperazin-4-propyl-1-phosphonic acid, PCP and MK-801; although the effects of these drugs on attention are usually combined with a loss of executive control in the form of increased premature and perseverative responses (Smith *et al.*, 2011; Carli & Invernizzi, 2014). We did not see a loss of executive control in AVD-deficient mice, however, we have previously shown reductions in glutamate levels within whole brain (Groves *et al.*, 2013) and it is therefore plausible that alterations in glutamatergic neurotransmission plays a role in impaired attentional processes seen with AVD deficiency.

There are currently no animal studies looking directly at the effects of oxidative stress on the 5C-SRT task. However, the HPLC finding of reduced total glutathione in AVD-deficient male mice was within the whole brain and therefore, if AVD deficiency produced chronic oxidative stress across the whole brain, it is likely that cognitive impairments would not be restricted to the hippocampus.

Human studies have shown significant associations between cognitive impairments and cognitive decline with systemic oxidative stress and lower antioxidant status (Berr *et al.*, 2000; Berr, 2002; Baierle *et al.*, 2015). Although many different tests are used to examine cognition in humans, the MMSE is used frequently, which measures global cognition including aspects of attention and hippocampal-dependent learning (Folstein *et al.*, 1975; Berr *et al.*, 2000).

Using $GABA_A$ receptor antagonists in the medial PFC, such as infusions of picrotoxin do induce a similar phenotype to AVD deficiency on the 5C-SRT, however infusions of picrotoxin into the ventral hippocampus induced hyperactivity, decreased startle reactivity and disrupted prepulse inhibition in rats (Bast *et al.*, 2001). These effects were not seen in AVD-deficient mice.

Studies have shown that the ventral hippocampus has a preferential role in anxiety-related behaviours and it is the dorsal hippocampus that is involved in spatial learning and memory (Bannerman *et al.*, 2004). However, studies using $GABA_A$ receptor antagonists infused into the dorsal hippocampus suggest a beneficial role on cognition (Torkaman-Boutorabi *et al.*, 2013; Yousefi *et al.*, 2013). GABAergic neurotransmission is controlled by a complex interplay of a whole range of proteins and a number of candidate proteins that may be involved have been identified in the proteomics analysis of the hippocampus.

Analysing the differentiated proteins from the proteomics using Ingenuity provided us with interesting candidate pathways that may underpin the changes found in hippocampal-dependent learning and memory in AVD deficiency. The analysis revealed that a number of proteins downregulated with AVD deficiency were involved in synapse formation, vesicle formation and release, synapse strength and plasticity. Reductions in APP, PPFIA3, MECP2 and RASGRF1 have all previously been reported to impair synaptic plasticity and are all downregulated in AVD-deficient mice (Bredt & Nicoll, 2003; Moretti *et al.*, 2006; Senechal *et al.*, 2008; Talebian *et al.*, 2013).

RASGRF1 normally facilitates neurite outgrowth in response to NGF and BDNF (Talebian *et al.*, 2013), yet is decreased with AVD deficiency. RASGRF1 knockout mice have impaired long-term memory but intact learning and short-term memory in an emotional conditioning task (Brambilla *et al.*, 1997). In addition, hippocampal-dependent learning using the Morris water maze was impaired in these mice (Giese *et al.*, 2001). MECP2 loss-of-function mutant mice have impaired spatial learning and memory on the Morris water maze, impaired contextual conditioned fear memory and show deficits in hippocampal-dependent long-term social memory (Moretti *et al.*, 2006). In addition, they have abnormal synaptic structure and function (Moretti *et al.*, 2006). Although these studies show deficits with the complete absence of the protein of interest, they do provide evidence that these proteins are important for proper synaptic plasticity and favourable learning and memory. I have not tested AVD deficiency on the Morris water maze, however I used an alternative hippocampal-dependent learning and memory task, the APA.

There are limited numbers of studies using the APA to assess GABAergic neurotransmission on hippocampal-dependent learning and memory, although, one study using an agonist of the $GABA_B$

receptor, showed disruption of spatial avoidance using the active place avoidance (Stuchlik & Vales, 2009). Studies using the active place avoidance have been used to show impairments in glutamatergic neurotransmission with systemic administration of MK-801, a non-competitive NMDA receptor antagonist. MK-801 treatment leads to increased travel on the arena, increased errors and reduced time avoiding the shock zone in Long-Evans rats (Stuchlik & Vales, 2005).

Adult hippocampal neurogenesis is important for different aspects of the active avoidance task. For example, immature doublecortin-positive neurons are required for acquisition of spatial learning and reversal learning but not for retrieval of stored long-term memories (Vukovic *et al.*, 2013). However, another study in which adult-born neurons in the hippocampus were ablated showed no deficit in learning the initial location of the shock zone but had impaired cognitive flexibility when the rules of the task were changed (Burghardt *et al.*, 2012). Finally, stimulation of adult hippocampal neurogenesis with exercise improves performance in spatial learning and memory tasks (van Praag *et al.*, 2005). Despite showing impairments on the hippocampal-dependent active place avoidance, AVD-deficient mice do not have alterations in adult hippocampal neurogenesis, as measured by cell proliferation and newborn mature neurons.

There is a large body of research on the role of vitamin D in proliferation, differentiation and apoptosis during development (see Chapter 1 for review). One study also revealed that the absence of $1,25(OH)_2D$ led to abnormal adult hippocampal neurogenesis using 1α hydroxylase knockout mice (Zhu *et al.*, 2012). Based on this previous research, we hypothesised that the AVD-deficient mice would have alterations in adult hippocampal neurogenesis, with expected results to show increased proliferation but decreased survival of newly born neurons. However, we did not see any significant changes in the numbers of new neurons. We did show an absence of a correlation between neurogenesis and behaviour in AVD-deficient mice that was evident in control mice (Chapter 4). Therefore, it is likely the function of these neurons that have been altered by AVD deficiency, possibly through alterations in dendritic branching or synaptic plasticity.

8.5 AVD deficiency and social defeat

When exposed to a social stress paradigm, AVD-deficient mice had greater impairments in a social avoidance test when exposed to both a mild separated housing stress or a more intensive social defeat paradigm. Previously, group housed male BALB/c mice showed no social interaction deficits with AVD deficiency (Groves *et al.*, 2013), yet the addition of a secondary insult once mice were already vitamin D deficient, led to greater avoidance behaviour during social interaction.

Previous studies have suggested that vitamin D deficiency may contribute to vulnerability to secondary insults or disorders, such as stroke or Parkinson's disease symptoms (Balden *et al.*, 2012; Suzuki *et al.*, 2013). The results from the Chapter 7 on social avoidance adds to literature suggesting that vitamin D deficiency does exacerbate secondary insults, including chronic social stress.

Chronic social defeat is considered an animal model of depression (Hollis & Kabbaj, 2014), because it induces anhedonia, learned helplessness, anxiety, social avoidance and freezing behaviours (Venzala *et al.*, 2012). In addition, chronic antidepressant treatment with venlafaxine has been shown to reduce the depressive effects of chronic social defeat, although not with fluoxetine treatment (Venzala *et al.*, 2012).

Higher levels of BDNF in the hippocampus are both necessary and sufficient for resilience to chronic stress (Bergstrom *et al.*, 2008) and reductions in BDNF induce depressive-like behaviours and anhedonia (Taliaz *et al.*, 2011). Furthermore, other neurotrophic factors are also reduced in rats susceptible to chronic stress (Bergstrom *et al.*, 2008). Vitamin D has been shown to stimulate a range of neurotrophic factors as reviewed by (Garcion *et al.*, 2002) and therefore, vitamin D deficiency may lead to reductions in neurotrophic factors important for neuroprotection against chronic stress. Furthermore, chronic stress has been shown to induce elevated levels of oxidative stress (Fontella *et al.*, 2005; Patki *et al.*, 2013). With lower levels of total glutathione in AVD-deficient mice, they would be more vulnerable to the effects of oxidative stress and therefore, this may have led to greater deficits following chronic social defeat, as seen in the social avoidance test.

Altered glutamate synaptic transmission, in the form of reduced vesicular glutamate transporter 1 (VGLUT1) also enhances vulnerability to stress-induced social avoidance and enhanced anxiety and depressive-like behaviours (Tordera *et al.*, 2007; Venzala *et al.*, 2012). VGLUT1 heterozygous knockout mice also have impairments in long-term memory (Tordera *et al.*, 2007). A wide range of components involved in glutamatergic neurotransmission including receptors and transporters and pre- and post-synaptic components have been associated with susceptibility to chronic stress as reviewed by Franklin *et al.* (2012).

AVD-deficient mice showed greater susceptibility to chronic social stress compared to control mice and although the socially defeated mice were not tested for depressive-like behaviours other than social avoidance, it is plausible to suggest, based on previous research (Venzala *et al.*, 2012), that AVD-deficient mice might be more vulnerable to the induction of a depressive-like phenotype following social stress. However, AVD deficiency on its own may be insufficient to induce the phenotype because previous studies using the FST as a measure of behavioural despair have shown

that group-housed male AVD-deficient mice showed similar levels of immobility to controls (Groves *et al.*, 2013). Furthermore, individually housed male and female AVD-deficient BALB/c mice have reduced immobility (decrease in behavioural despair) compared to control mice (Chapter 4).

Drugs that impact on glutamate neurotransmission have been shown to reduce immobility in the FST. For example, the NMDA antagonist, ketamine leads to rapid and robust antidepressant effects (Maeng & Zarate, 2007), as do AMPA receptor agonists (Farley *et al.*, 2010). The analysis of protein expression in the hippocampus suggested that AVD deficiency impacts on several aspects of neurotransmission, both glutamatergic and GABAergic. Therefore, this result may be more suggestive of altered neurotransmission, than a specific antidepressant-related outcome.

AVD-deficient male BALB/c mice were impaired on both a PFC-striatal circuitry requiring attention task and a hippocampal-dependent spatial task. We have also previously shown effects of AVD deficiency on an anxiety measure and response to external stimuli (Groves *et al.*, 2013), suggesting possible involvement of the amygdala and brain stem. Furthermore, we have shown altered social behaviour following exposure to a fear-mediated social defeat and altered behavioural despair. These combined results provide evidence for global effects of AVD deficiency on behaviour and brain function.

8.6 Sex-dependent AVD-deficient changes

Female mice were included for analysis when measuring attentional processes and adult hippocampal neurogenesis, including the FST. Overall, both female and male AVD-deficient mice exhibited a decrease in behavioural despair (as measured by immobility on the FST), with no change in adult hippocampal neurogenesis. However, the results from the 5C-SRT task showed that only male AVD-deficient mice had impairments in attention and only female AVD-deficient mice had an enhanced motivation for reward. These results suggest that there is an interaction between sex and AVD deficiency.

Previous human studies have shown that vitamin D status may differentially impact on sex hormones. For example, vitamin D deficiency has been associated with lower testosterone levels in men (Wehr *et al.*, 2010) and vitamin D supplementation has been shown to increase testosterone levels in men (Pilz *et al.*, 2011). Yet, higher levels of vitamin D have been associated with lower progesterone and oestradiol in young women (Knight *et al.*, 2010).

Sex-specific alterations in behaviour when using animal models is common (Curry, 2001; Kokras & Dalla, 2014), and it is not unusual for neuropsychiatric disorders and neurodegenerative diseases to

have sex-related difference in behavioural and neurobiological outcomes (Sagud *et al.*, 2002; Canuso & Pandina, 2007). For example, prevalence of mild cognitive impairment has been shown to be higher in men (Petersen *et al.*, 2010). Furthermore, oestradiol treatment has been shown to enhance cognition in animals and humans (Luine, 2014). Depression is another disorder with gender differences with higher incidence in women compared to men, but moreover, prevalence differs during the life span. Incidence is the same between males and females in children and the elderly but differs during childbearing years, suggesting a possible role of female sex hormones (Sagud *et al.*, 2002).

It is plausible to suggest that the female BALB/c mice might be protected from some of the detrimental effects of AVD deficiency and more susceptible to other effects due to the presence of oestrogen. However, this was beyond the scope of this thesis and would need to be investigated in future experiments.

8.7 Limitations and Future Directions

The finding of reduced glutathione with AVD deficiency suggests that future experiments should investigate the extent of oxidative stress in this model. For example, studies analysing markers of oxidative damage both at a tissue level and at the level of the synapse could indicate if the glutathione deficit seen with AVD deficiency is enough to induce sufficient oxidative damage to alter neurotransmission. Furthermore, treatment with N-acetyl cysteine, a molecule known to increase glutathione levels, may reverse the effects of AVD deficiency on cognition by restoring oxidative balance. There is also evidence from the proteomic analysis to suggest that AVD deficiency may be altering the expression of proteins necessary for optimal neurotransmission and synapse formation and this may be unrelated to oxidative stress.

Using proteomics the effects of AVD deficiency on protein expression in the hippocampus was analysed and a number of candidate proteins were identified, however this was limited to one brain region, ideally future experiments should include analysis of other brain regions. Furthermore, the results from the proteomics analysis are preliminary and require further validation, via technical or proteomic replication. Although, proteomics is useful to generate hypotheses based on protein expression changes, it cannot identify which protein changes are a direct result of vitamin D deficiency compared to proteins that may be altered due to compensatory changes. To enable us to unravel the mechanism by which AVD deficiency impacts on the brain, it is important to differentiate between the two forms of protein expression changes.

The active from of vitamin D, 1,25$(OH)_2$D, is highly pleotropic with over 2700 VDREs within the genome that are able to alter the transcription of wide range of genes in the presence of vitamin D (Ramagopalan *et al.*, 2010). Ideally, an experiment to analyse the genome upstream of the proteins altered by AVD deficiency for VDREs could highlight those genes that vitamin D, or the absence of vitamin D, directly regulates at a transcriptional level.

Furthermore, a number of biological processes were identified by proteomic pathway analysis that could be altered by AVD deficiency and these should be followed up. For example, a number of proteins involved in synapse formation, synapse plasticity, neurotransmission and dendritic arborisation were highlighted as being differentially expressed with AVD deficiency. Although we have shown that hippocampal-dependent learning and memory are altered by AVD deficiency, we have not as yet, identified which key biological processes may be responsible for the deficit seen. Further research at the level of the synapse needs to be undertaken in the future to unravel mechanisms of the impairments seen with AVD deficiency.

We have shown that AVD deficiency leaves male BALB/c mice vulnerable to the effect of social stress and is suggestive that they are also more vulnerable to depressive-like behaviours compared to controls. Therefore, future experiments should follow up the two-hit exposures with a range of behavioural tests including measures of depression and cognition. Furthermore, these results also have important implications for clinical research. For example, a randomised, double-blind, controlled trial in PD's patients has recently shown that vitamin D_3 supplementation stabilised and prevented deterioration of PD symptoms (Suzuki *et al.*, 2013). However, more clinical trials are needed in a wider range of disorders, including AD.

There is growing evidence for an association between vitamin D deficiency and AD in epidemiology (Littlejohns *et al.*, 2014; Keeney & Butterfield, 2015). Furthermore, animal studies have shown that vitamin D treatment reduces the load of Aβ and aids in the clearance of Aβ (Briones & Darwish, 2012). Future experiments should explore the combined effects of AVD deficiency and AD pathology to determine if vitamin D deficiency exacerbates the symptoms of AD. Furthermore, experiments in aging mice are now warranted, particularly since a vast majority of epidemiological findings are from studies in elderly populations. There may be much greater effects of AVD deficiency in more vulnerable populations, such as aged animals, or after a longer time period on the vitamin D-deficient diet because 10 weeks is still a relatively short period of dietary manipulation in mice.

Some of the research for this thesis has been performed only in males, where ideally it should all be performed in both males and females. The proteomic analysis was performed on a small scale due

to the limitations of the iTRAQ method and therefore only male mice were analysed. Furthermore, the experiments that followed up hypotheses generated by the proteomics were restricted to males, because it was unknown if female mice might have different unpredicted outcomes due to different protein expression patterns with AVD deficiency. Finally, the social defeat paradigm used in the two-hit exposure could not be effectively performed in females. Future experiments should expand to include both male and females. Of particular interest, would be to determine if female AVD-deficient BALB/c were impaired on the hippocampal-dependent active place avoidance or protected from impairments in a similar way to the attentional 5C-SRT task results. Furthermore, it would be an interesting finding to determine if protein expression changes were different between males and females, to help explain why there may be cognitive differences between the sexes.

8.8 Conclusion

The results from the research covered in this thesis indicate that exposure to vitamin D deficiency during adulthood in mice is sufficient to impair cognition, alter brain function and leave mice more vulnerable to a secondary exposure. The results from the two-hit study have confirmed that BALB/c mice are more susceptible to the effects of AVD deficiency compared to C57BL/6J mice, even when an additional insult was added, indicating that differing genetics most likely plays a role in the adverse outcomes associated with AVD deficiency, and this may be relevant to human populations.

The findings from this thesis support the epidemiological link between vitamin D deficiency in adulthood and adverse outcomes including cognitive impairment and vulnerability to secondary insults. Furthermore, the results from the thesis have provided new clues that may lead us to unravel the mechanisms as to how the absence of vitamin D can lead to such detrimental outcomes.

References

Abrahams, B.S. & Geschwind, D.H. (2008) Advances in autism genetics: on the threshold of a new neurobiology. *Nat Rev Genet*, **9**, 341-355.

Afzal, S., Bojesen, S.E. & Nordestgaard, B.G. (2013) Reduced 25-hydroxyvitamin D and risk of Alzheimer's disease and vascular dementia. *Alzheimers Dement*.

Ahlgren, C., Oden, A. & Lycke, J. (2011) High nationwide prevalence of multiple sclerosis in Sweden. *Mult Scler*, **17**, 901-908.

Almeras, L., Eyles, D., Benech, P., et al (2007) Developmental vitamin D deficiency alters brain protein expression in the adult rat: implications for neuropsychiatric disorders. *Proteomics*, **7**, 769-780.

Altemus, K.L., Finger, S., Wolf, C. & Birge, S.J. (1987) Behavioral correlates of vitamin D deficiency. *Physiol Behav*, **39**, 435-440.

Amato, R., Pinelli, M., Monticelli, A., Miele, G. & Cocozza, S. (2010) Schizophrenia and vitamin D related genes could have been subject to latitude-driven adaptation. *BMC Evol Biol*, **10**, 351.

Anglin, R.E., Samaan, Z., Walter, S.D. & McDonald, S.D. (2013) Vitamin D deficiency and depression in adults: systematic review and meta-analysis. *Br J Psychiatry*, **202**, 100-107.

Annweiler, C., Herrmann, F.R., Fantino, B., Brugg, B. & Beauchet, O. (2012a) Effectiveness of the combination of memantine plus vitamin D on cognition in patients with Alzheimer disease: a pre-post pilot study. *Cogn Behav Neurol*, **25**, 121-127.

Annweiler, C., Llewellyn, D.J. & Beauchet, O. (2013) Low serum vitamin D concentrations in Alzheimer's disease: a systematic review and meta-analysis. *J Alzheimers Dis*, **33**, 659-674.

Annweiler, C., Rolland, Y., Schott, A.M., et al (2012b) Higher vitamin D dietary intake is associated with lower risk of alzheimer's disease: a 7-year follow-up. *J Gerontol A Biol Sci Med Sci*, **67**, 1205-1211.

Annweiler, C., Schott, A.M., Allali, G., et al (2010) Association of vitamin D deficiency with cognitive impairment in older women: cross-sectional study. *Neurology*, **74**, 27-32.

Asada, H., Kawamura, Y., Maruyama, K., et al (1996) Mice lacking the 65 kDa isoform of glutamic acid decarboxylase (GAD65) maintain normal levels of GAD67 and GABA in their brains but are susceptible to seizures. *Biochem Biophys Res Commun*, **229**, 891-895.

Asada, H., Kawamura, Y., Maruyama, K., et al (1997) Cleft palate and decreased brain gamma-aminobutyric acid in mice lacking the 67-kDa isoform of glutamic acid decarboxylase. *Proc Natl Acad Sci U S A*, **94**, 6496-6499.

Ashburner, J. & Friston, K.J. (2000) Voxel-based morphometry--the methods. *Neuroimage*, **11**, 805-821.

Avants, B.B., Tustison, N.J., Song, G., et al (2011) A reproducible evaluation of ANTs similarity metric performance in brain image registration. *Neuroimage*, **54**, 2033-2044.

Baeke, F., Takiishi, T., Korf, H., Gysemans, C. & Mathieu, C. (2010) Vitamin D: modulator of the immune system. *Curr Opin Pharmacol*, **10**, 482-496.

Baierle, M., Nascimento, S.N., Moro, A.M., et al (2015) Relationship between Inflammation and Oxidative Stress and Cognitive Decline in the Institutionalized Elderly. *Oxidative Medicine and Cellular Longevity*.

Baksi, S.N. & Hughes, M.J. (1982) Chronic vitamin D deficiency in the weanling rat alters catecholamine metabolism in the cortex. *Brain Res*, **242**, 387-390.

Balden, R., Selvamani, A. & Sohrabji, F. (2012) Vitamin D Deficiency Exacerbates Experimental Stroke Injury and Dysregulates Ischemia-Induced Inflammation in Adult Rats. *Endocrinology*.

Balion, C., Griffith, L.E., Strifler, L., et al (2012) Vitamin D, cognition, and dementia: a systematic review and meta-analysis. *Neurology*, **79**, 1397-1405.

Banerjee, P. & Chatterjee, M. (2003) Antiproliferative role of vitamin D and its analogs--a brief overview. *Mol Cell Biochem*, **253**, 247-254.

Bannerman, D.M., Rawlins, J.N., McHugh, S.B., et al (2004) Regional dissociations within the hippocampus--memory and anxiety. *Neurosci Biobehav Rev*, **28**, 273-283.

Bao, B.Y., Ting, H.J., Hsu, J.W. & Lee, Y.F. (2008) Protective role of 1 alpha, 25-dihydroxyvitamin D3 against oxidative stress in nonmalignant human prostate epithelial cells. *Int J Cancer*, **122**, 2699-2706.

Barchetta, I., Carotti, S., Labbadia, G., et al (2012) Liver vitamin D receptor, CYP2R1, and CYP27A1 expression: relationship with liver histology and vitamin D3 levels in patients with nonalcoholic steatohepatitis or hepatitis C virus. *Hepatology*, **56**, 2180-2187.

Bari, A., Dalley, J.W. & Robbins, T.W. (2008) The application of the 5-choice serial reaction time task for the assessment of visual attentional processes and impulse control in rats. *Nat Protoc*, **3**, 759-767.

Bashir, Z.I., Bortolotto, Z.A., Davies, C.H., et al (1993) Induction of LTP in the hippocampus needs synaptic activation of glutamate metabotropic receptors. *Nature*, **363**, 347-350.

Bast, T., Zhang, W.N. & Feldon, J. (2001) Hyperactivity, decreased startle reactivity, and disrupted prepulse inhibition following disinhibition of the rat ventral hippocampus by the GABA(A) receptor antagonist picrotoxin. *Psychopharmacology (Berl)*, **156**, 225-233.

Beatty, W.W. & Aupperle, R.L. (2002) Sex differences in cognitive impairment in multiple sclerosis. *Clin Neuropsychol*, **16**, 472-480.

Becker, A., Eyles, D.W., McGrath, J.J. & Grecksch, G. (2005) Transient prenatal vitamin D deficiency is associated with subtle alterations in learning and memory functions in adult rats. *Behav Brain Res*, **161**, 306-312.

Beecham, G.W., Martin, E.R., Li, Y.J., et al (2009) Genome-wide association study implicates a chromosome 12 risk locus for late-onset Alzheimer disease. *Am J Hum Genet*, **84**, 35-43.

Bergstrom, A., Jayatissa, M.N., Mork, A. & Wiborg, O. (2008) Stress sensitivity and resilience in the chronic mild stress rat model of depression; an in situ hybridization study. *Brain Res*, **1196**, 41-52.

Berr, C. (2002) Oxidative stress and cognitive impairment in the elderly. *J Nutr Health Aging*, **6**, 261-266.

Berr, C., Balansard, B., Arnaud, J., et al (2000) Cognitive decline is associated with systemic oxidative stress: The EVA study. *Journal of the American Geriatrics Society*, **48**, 1285-1291.

Bikle, D. (2009) Nonclassic actions of vitamin D. *J Clin Endocrinol Metab*, **94**, 26-34.

Black, C.N., Bot, M., Scheffer, P.G., Cuijpers, P. & Penninx, B.W. (2015) Is depression associated with increased oxidative stress? A systematic review and meta-analysis. *Psychoneuroendocrinology*, **51**, 164-175.

Bonnet, A.M. & Houeto, J.L. (1999) Pathophysiology of Parkinson's disease. *Biomed Pharmacother*, **53**, 117-121.

Bouillon, R., Carmeliet, G., Verlinden, L., et al (2008) Vitamin D and human health: lessons from vitamin D receptor null mice. *Endocr Rev*, **29**, 726-776.

Bowers, D., Miller, K., Mikos, A., et al (2006) Startling facts about emotion in Parkinson's disease: blunted reactivity to aversive stimuli. *Brain*, **129**, 3356-3365.

Brambilla, R., Gnesutta, N., Minichiello, L., et al (1997) A role for the Ras signalling pathway in synaptic transmission and long-term memory. *Nature*, **390**, 281-286.

Bratt, A.M., Cassel, J.C., Neufang, B., et al (1995) Behavioural and neurochemical effects of superior cervical ganglionectomy in rats with septo-hippocampal lesions. *Exp Brain Res*, **102**, 429-444.

Bredt, D.S. & Nicoll, R.A. (2003) AMPA receptor trafficking at excitatory synapses. *Neuron*, **40**, 361-379.

Breustedt, J., Vogt, K.E., Miller, R.J., Nicoll, R.A. & Schmitz, D. (2003) Alpha1E-containing Ca2+ channels are involved in synaptic plasticity. *Proc Natl Acad Sci U S A*, **100**, 12450-12455.

Brewer, L.D., Thibault, V., Chen, K.C., et al (2001) Vitamin D hormone confers neuroprotection in parallel with downregulation of L-type calcium channel expression in hippocampal neurons. *J Neurosci*, **21**, 98-108.

Briones, T.L. & Darwish, H. (2012) Vitamin D mitigates age-related cognitive decline through the modulation of pro-inflammatory state and decrease in amyloid burden. *J Neuroinflammation*, **9**, 244.

Brondum-Jacobsen, P., Nordestgaard, B.G., Schnohr, P. & Benn, M. (2013) 25-hydroxyvitamin D and symptomatic ischemic stroke: an original study and meta-analysis. *Ann Neurol*, **73**, 38-47.

Brouwer-Brolsma, E.M., Schuurman, T., de Groot, L.C.P.M.G., et al (2014) No role for vitamin D or a moderate fat diet in aging induced cognitive decline and emotional reactivity in C57BL/6 mice. *Behav Brain Res*, **267**, 133-143.

Brown, J., Bianco, J.I., McGrath, J.J. & Eyles, D.W. (2003) 1,25-dihydroxyvitamin D3 induces nerve growth factor, promotes neurite outgrowth and inhibits mitosis in embryonic rat hippocampal neurons. *Neurosci Lett*, **343**, 139-143.

Brown, T.H., Chapman, P.F., Kairiss, E.W. & Keenan, C.L. (1988) Long-term synaptic potentiation. *Science*, **242**, 724-728.

Buell, J.S., Dawson-Hughes, B., Scott, T.M., et al (2010) 25-Hydroxyvitamin D, dementia, and cerebrovascular pathology in elders receiving home services. *Neurology*, **74**, 18-26.

Buell, J.S., Scott, T.M., Dawson-Hughes, B., et al (2009) Vitamin D Is Associated With Cognitive Function in Elders Receiving Home Health Services. *Journals of Gerontology Series a-Biological Sciences and Medical Sciences*, **64**, 888-895.

Burghardt, N.S., Park, E.H., Hen, R. & Fenton, A.A. (2012) Adult-born hippocampal neurons promote cognitive flexibility in mice. *Hippocampus*, **22**, 1795-1808.

Burket, R., McGrath, J. & Eyles, D. (2003) Vitamin D receptor expression in the embryonic rat brain. *Neuroscience Research Communications*, **33**, 63-71.

Burne, T.H., Becker, A., Brown, J., et al (2004a) Transient prenatal Vitamin D deficiency is associated with hyperlocomotion in adult rats. *Behav Brain Res*, **154**, 549-555.

Burne, T.H., Feron, F., Brown, J., et al (2004b) Combined prenatal and chronic postnatal vitamin D deficiency in rats impairs prepulse inhibition of acoustic startle. *Physiol Behav*, **81**, 651-655.

Byrne, J.H., Voogt, M., Turner, K.M., et al (2013) The impact of adult vitamin d deficiency on behaviour and brain function in male sprague-dawley rats. *PLoS One*, **8**, e71593.

Cabungcal, J.H., Steullet, P., Kraftsik, R., Cuenod, M. & Do, K.Q. (2013a) Early-life insults impair parvalbumin interneurons via oxidative stress: reversal by N-acetylcysteine. *Biol Psychiatry*, **73**, 574-582.

Cabungcal, J.H., Steullet, P., Morishita, H., et al (2013b) Perineuronal nets protect fast-spiking interneurons against oxidative stress. *Proc Natl Acad Sci U S A*, **110**, 9130-9135.

Cahill, L.S., Steadman, P.E., Jones, C.E., et al (2015) MRI-detectable changes in mouse brain structure induced by voluntary exercise. *Neuroimage*, **113**, 175-183.

Cantor-Graae, E. & Selten, J.P. (2005) Schizophrenia and migration: a meta-analysis and review. *Am J Psychiatry*, **162**, 12-24.

Cantorna, M.T., Hayes, C.E. & DeLuca, H.F. (1996) 1,25-Dihydroxyvitamin D3 reversibly blocks the progression of relapsing encephalomyelitis, a model of multiple sclerosis. *Proc Natl Acad Sci U S A*, **93**, 7861-7864.

Cantorna, M.T., Woodward, W.D., Hayes, C.E. & DeLuca, H.F. (1998) 1,25-dihydroxyvitamin D3 is a positive regulator for the two anti-encephalitogenic cytokines TGF-beta 1 and IL-4. *J Immunol*, **160**, 5314-5319.

Canuso, C.M. & Pandina, G. (2007) Gender and schizophrenia. *Psychopharmacol Bull*, **40**, 178-190.

Carli, M. & Invernizzi, R.W. (2014) Serotoninergic and dopaminergic modulation of cortico-striatal circuit in executive and attention deficits induced by NMDA receptor hypofunction in the 5-choice serial reaction time task. *Front Neural Circuits*, **8**, 58.

Cass, W.A., Peters, L.E., Fletcher, A.M. & Yurek, D.M. (2012) Evoked dopamine overflow is augmented in the striatum of calcitriol treated rats. *Neurochem Int*, **60**, 186-191.

Cass, W.A., Smith, M.P. & Peters, L.E. (2006) Calcitriol protects against the dopamine- and serotonin-depleting effects of neurotoxic doses of methamphetamine. *Ann N Y Acad Sci*, **1074**, 261-271.

Cekic, M., Cutler, S.M., VanLandingham, J.W. & Stein, D.G. (2011) Vitamin D deficiency reduces the benefits of progesterone treatment after brain injury in aged rats. *Neurobiol Aging*, **32**, 864-874.

Chao, H.T., Zoghbi, H.Y. & Rosenmund, C. (2007) MeCP2 controls excitatory synaptic strength by regulating glutamatergic synapse number. *Neuron*, **56**, 58-65.

Chao, M.V. (1994) The p75 neurotrophin receptor. *J Neurobiol*, **25**, 1373-1385.

Chel, V.G., Ooms, M.E., Popp-Snijders, C., et al (1998) Ultraviolet irradiation corrects vitamin D deficiency and suppresses secondary hyperparathyroidism in the elderly. *J Bone Miner Res*, **13**, 1238-1242.

Chen, J., Olivares-Navarrete, R., Wang, Y., et al (2010) Protein-disulfide isomerase-associated 3 (Pdia3) mediates the membrane response to 1,25-dihydroxyvitamin D3 in osteoblasts. *J Biol Chem*, **285**, 37041-37050.

Chen, T.C., Chimeh, F., Lu, Z., et al (2007) Factors that influence the cutaneous synthesis and dietary sources of vitamin D. *Arch Biochem Biophys*, **460**, 213-217.

Christgau, S., Aanstoot, H.J., Schierbeck, H., et al (1992) Membrane anchoring of the autoantigen GAD65 to microvesicles in pancreatic beta-cells by palmitoylation in the NH2-terminal domain. *J Cell Biol*, **118**, 309-320.

Christiansen, C., Rodbro, P. & Sjo, O. (1974) "Anticonvulsant action" of vitamin D in epileptic patients? A controlled pilot study. *Br Med J*, **2**, 258-259.

Cimadevilla, J.M., Kaminsky, Y., Fenton, A. & Bures, J. (2000) Passive and active place avoidance as a tool of spatial memory research in rats. *J Neurosci Methods*, **102**, 155-164.

Cimadevilla, J.M., Wesierska, M., Fenton, A.A. & Bures, J. (2001) Inactivating one hippocampus impairs avoidance of a stable room-defined place during dissociation of arena cues from room cues by rotation of the arena. *Proc Natl Acad Sci U S A*, **98**, 3531-3536.

Clark, P.J., Kohman, R.A., Miller, D.S., et al (2011) Genetic influences on exercise-induced adult hippocampal neurogenesis across 12 divergent mouse strains. *Genes Brain Behav*, **10**, 345-353.

Clemens, Z., Hollo, A., Kelemen, A., et al (2013) Seasonality in Epileptic seizures. *J Neurol Transl Neurosci*.

Correale, J., Ysrraelit, M.C. & Gaitan, M.I. (2009) Immunomodulatory effects of Vitamin D in multiple sclerosis. *Brain*, **132**, 1146-1160.

Cui, X., Groves, N.J., Burne, T.H., Eyles, D.W. & McGrath, J.J. (2013a) Low vitamin D concentration exacerbates adult brain dysfunction. *Am J Clin Nutr*.

Cui, X., McGrath, J.J., Burne, T.H., Mackay-Sim, A. & Eyles, D.W. (2007) Maternal vitamin D depletion alters neurogenesis in the developing rat brain. *Int J Dev Neurosci*, **25**, 227-232.

Cui, X., Pelekanos, M., Burne, T.H., McGrath, J.J. & Eyles, D.W. (2010) Maternal vitamin D deficiency alters the expression of genes involved in dopamine specification in the developing rat mesencephalon. *Neurosci Lett*, **486**, 220-223.

Cui, X., Pelekanos, M., Liu, P.Y., et al (2013b) The vitamin D receptor in dopamine neurons; its presence in human substantia nigra and its ontogenesis in rat midbrain. *Neuroscience*, **236**, 77-87.

Cunha, M.P., Oliveira, A., Pazini, F.L., et al (2013) The antidepressant-like effect of physical activity on a voluntary running wheel. *Med Sci Sports Exerc*, **45**, 851-859.

Curry, B.B. (2001) Animal models used in identifying gender-related differences. *International Journal of Toxicology*, **20**, 153-160.

Daly, R.M., Gagnon, C., Lu, Z.X., et al (2012) Prevalence of vitamin D deficiency and its determinants in Australian adults aged 25 years and older: a national, population-based study. *Clin Endocrinol (Oxf)*, **77**, 26-35.

Davis, J., Eyre, H., Jacka, F.N., et al (2016) A review of vulnerability and risks for schizophrenia: Beyond the two hit hypothesis. *Neurosci Biobehav Rev*, **65**, 185-194.

Davis, M., Walker, D.L. & Lee, Y. (1997) Amygdala and bed nucleus of the stria terminalis: differential roles in fear and anxiety measured with the acoustic startle reflex. *Philos Trans R Soc Lond B Biol Sci*, **352**, 1675-1687.

Day, N.C., Shaw, P.J., McCormack, A.L., et al (1996) Distribution of alpha 1A, alpha 1B and alpha 1E voltage-dependent calcium channel subunits in the human hippocampus and parahippocampal gyrus. *Neuroscience*, **71**, 1013-1024.

de Abreu, D.A., Nivet, E., Baril, N., et al (2010) Developmental vitamin D deficiency alters learning in C57Bl/6J mice. *Behav Brain Res*, **208**, 603-608.

De Rosa, S.C., Zaretsky, M.D., Dubs, J.G., et al (2000) N-acetylcysteine replenishes glutathione in HIV infection. *Eur J Clin Invest*, **30**, 915-929.

Dealberto, M.J. (2010) Ethnic origin and increased risk for schizophrenia in immigrants to countries of recent and longstanding immigration. *Acta Psychiatr Scand*.

Dichter, G.S. & Tomarken, A.J. (2008) The chronometry of affective startle modulation in unipolar depression. *J Abnorm Psychol*, **117**, 1-15.

Ding, H., Dhima, K., Lockhart, K.C., et al (2013) Unrecognized vitamin D3 deficiency is common in Parkinson disease: Harvard Biomarker Study. *Neurology*, **81**, 1531-1537.

Dringen, R. (2000) Metabolism and functions of glutathione in brain. *Prog Neurobiol*, **62**, 649-671.

Duman, C.H., Schlesinger, L., Russell, D.S. & Duman, R.S. (2008) Voluntary exercise produces antidepressant and anxiolytic behavioral effects in mice. *Brain Res*, **1199**, 148-158.

Dursun, E., Gezen-Ak, D. & Yilmazer, S. (2011) A novel perspective for Alzheimer's disease: vitamin D receptor suppression by amyloid-beta and preventing the amyloid-beta induced alterations by vitamin D in cortical neurons. *J Alzheimers Dis*, **23**, 207-219.

Dursun, E., Gezen-Ak, D. & Yilmazer, S. (2013) A new mechanism for Amyloid-β induction of iNOS: Vitamin D-VDR Pathway Disruption. *Journal of Alzheimer's Disease*.

Eisman, J.A., Barkla, D.H. & Tutton, P.J. (1987) Suppression of in vivo growth of human cancer solid tumor xenografts by 1,25-dihydroxyvitamin D3. *Cancer Res*, **47**, 21-25.

Elgersma, Y. & Silva, A.J. (1999) Molecular mechanisms of synaptic plasticity and memory. *Curr Opin Neurobiol*, **9**, 209-213.

Engelsen, O., Brustad, M., Aksnes, L. & Lund, E. (2005) Daily duration of vitamin D synthesis in human skin with relation to latitude, total ozone, altitude, ground cover, aerosols and cloud thickness. *Photochem Photobiol*, **81**, 1287-1290.

Etgen, T., Sander, D., Bickel, H., Sander, K. & Forstl, H. (2012) Vitamin D deficiency, cognitive impairment and dementia: a systematic review and meta-analysis. *Dement Geriatr Cogn Disord*, **33**, 297-305.

Evatt, M.L., Delong, M.R., Khazai, N., et al (2008) Prevalence of vitamin d insufficiency in patients with Parkinson disease and Alzheimer disease. *Arch Neurol*, **65**, 1348-1352.

Eyles, D., Almeras, L., Benech, P., et al (2007) Developmental vitamin D deficiency alters the expression of genes encoding mitochondrial, cytoskeletal and synaptic proteins in the adult rat brain. *J Steroid Biochem Mol Biol*, **103**, 538-545.

Eyles, D., Anderson, C., Ko, P., et al (2009a) A sensitive LC/MS/MS assay of 25OH vitamin D3 and 25OH vitamin D2 in dried blood spots. *Clin Chim Acta*, **403**, 145-151.

Eyles, D., Brown, J., Mackay-Sim, A., McGrath, J. & Feron, F. (2003) Vitamin D3 and brain development. *Neuroscience*, **118**, 641-653.

Eyles, D., Burne, T. & McGrath, J. (2011) Vitamin D: A Neurosteroid Affecting Brain Development and Function; Implications for Neurological and Psychiatric Disorders *Vitamin D (Third Edition)*. Academic Press, pp. 565-582.

Eyles, D.W., Burne, T.H. & McGrath, J.J. (2013) Vitamin D, effects on brain development, adult brain function and the links between low levels of vitamin D and neuropsychiatric disease. *Front Neuroendocrinol*, **34**, 47-64.

Eyles, D.W., Feron, F., Cui, X., et al (2009b) Developmental vitamin D deficiency causes abnormal brain development. *Psychoneuroendocrinology*, **34 Suppl 1**, S247-257.

Eyles, D.W., Smith, S., Kinobe, R., Hewison, M. & McGrath, J.J. (2005) Distribution of the vitamin D receptor and 1 alpha-hydroxylase in human brain. *J Chem Neuroanat*, **29**, 21-30.

Falkenstein, E., Tillmann, H.C., Christ, M., Feuring, M. & Wehling, M. (2000) Multiple actions of steroid hormones--a focus on rapid, nongenomic effects. *Pharmacol Rev*, **52**, 513-556.

Farley, S., Apazoglou, K., Witkin, J.M., Giros, B. & Tzavara, E.T. (2010) Antidepressant-like effects of an AMPA receptor potentiator under a chronic mild stress paradigm. *Int J Neuropsychopharmacol*, **13**, 1207-1218.

Fatemi, S.H. & Folsom, T.D. (2009) The neurodevelopmental hypothesis of schizophrenia, revisited. *Schizophr Bull*, **35**, 528-548.

Fernandes de Abreu, D.A., Eyles, D. & Feron, F. (2009) Vitamin D, a neuro-immunomodulator: implications for neurodegenerative and autoimmune diseases. *Psychoneuroendocrinology*, **34 Suppl 1**, S265-277.

Fernandes de Abreu, D.A., Ibrahim, E.C., Boucraut, J., Khrestchatisky, M. & Feron, F. (2010a) Severity of experimental autoimmune encephalomyelitis is unexpectedly reduced in mice born to vitamin D-deficient mothers. *J Steroid Biochem Mol Biol*, **121**, 250-253.

Fernandes de Abreu, D.A., Landel, V., Barnett, A.G., et al (2012) Prenatal vitamin d deficiency induces an early and more severe experimental autoimmune encephalomyelitis in the second generation. *Int J Mol Sci*, **13**, 10911-10919.

Fernandes de Abreu, D.A., Nivet, E., Baril, N., et al (2010b) Developmental vitamin D deficiency alters learning in C57Bl/6J mice. *Behav Brain Res*, **208**, 603-608.

Fernell, E., Barnevik-Olsson, M., Bagenholm, G., et al (2010) Serum levels of 25-hydroxyvitamin D in mothers of Swedish and of Somali origin who have children with and without autism. *Acta Paediatrica*, **99**, 743-747.

Feron, F., Burne, T.H., Brown, J., et al (2005) Developmental Vitamin D3 deficiency alters the adult rat brain. *Brain Res Bull*, **65**, 141-148.

Fisher, R.S., van Emde Boas, W., Blume, W., et al (2005) Epileptic seizures and epilepsy: definitions proposed by the International League Against Epilepsy (ILAE) and the International Bureau for Epilepsy (IBE). *Epilepsia*, **46**, 470-472.

Folstein, M.F., Folstein, S.E. & McHugh, P.R. (1975) "Mini-mental state". A practical method for grading the cognitive state of patients for the clinician. *J Psychiatr Res*, **12**, 189-198.

Fontella, F.U., Siqueira, I.R., Vasconcellos, A.P., et al (2005) Repeated restraint stress induces oxidative damage in rat hippocampus. *Neurochem Res*, **30**, 105-111.

Forbes, E.E., Miller, A., Cohn, J.F., Fox, N.A. & Kovacs, M. (2005) Affect-modulated startle in adults with childhood-onset depression: relations to bipolar course and number of lifetime depressive episodes. *Psychiatry Res*, **134**, 11-25.

Ford, A., Castonguay, A., Cottet, M., et al (2015) Engagement of the GABA to KCC2 signaling pathway contributes to the analgesic effects of A3AR agonists in neuropathic pain. *J Neurosci*, **35**, 6057-6067.

Franklin, G.P.a.K.B.J. (2012) *Paxinos and Franklin's the Mouse Brain in Stereotaxic Coordinates*. Elsevier Academic Press.

Franklin, T.B., Saab, B.J. & Mansuy, I.M. (2012) Neural mechanisms of stress resilience and vulnerability. *Neuron*, **75**, 747-761.

Frim, D.M., Yee, W.M. & Isacson, O. (1993) NGF reduces striatal excitotoxic neuronal loss without affecting concurrent neuronal stress. *Neuroreport*, **4**, 655-658.

Frith, C. (1996) Neuropsychology of schizophrenia, what are the implications of intellectual and experiential abnormalities for the neurobiology of schizophrenia? *Br Med Bull*, **52**, 618-626.

Fukui, K., Omoi, N.O., Hayasaka, T., et al (2002) Cognitive impairment of rats caused by oxidative stress and aging, and its prevention by vitamin E. *Ann N Y Acad Sci*, **959**, 275-284.

Gage, F.H. (2002) Neurogenesis in the adult brain. *J Neurosci*, **22**, 612-613.

Garcion, E., Sindji, L., Leblondel, G., Brachet, P. & Darcy, F. (1999) 1,25-dihydroxyvitamin D3 regulates the synthesis of gamma-glutamyl transpeptidase and glutathione levels in rat primary astrocytes. *J Neurochem*, **73**, 859-866.

Garcion, E., Sindji, L., Montero-Menei, C., et al (1998) Expression of inducible nitric oxide synthase during rat brain inflammation: regulation by 1,25-dihydroxyvitamin D3. *Glia*, **22**, 282-294.

Garcion, E., Sindji, L., Nataf, S., et al (2003) Treatment of experimental autoimmune encephalomyelitis in rat by 1,25-dihydroxyvitamin D3 leads to early effects within the central nervous system. *Acta Neuropathol*, **105**, 438-448.

Garcion, E., Wion-Barbot, N., Montero-Menei, C.N., Berger, F. & Wion, D. (2002) New clues about vitamin D functions in the nervous system. *Trends Endocrinol Metab*, **13**, 100-105.

Gash, D.M., Zhang, Z. & Gerhardt, G. (1998) Neuroprotective and neurorestorative properties of GDNF. *Ann Neurol*, **44**, S121-125.

Gezen-Ak, D., Dursun, E., Bilgic, B., et al (2012) Vitamin D receptor gene haplotype is associated with late-onset Alzheimer's disease. *Tohoku J Exp Med*, **228**, 189-196.

Gezen-Ak, D., Dursun, E. & Yilmazer, S. (2011) The effects of vitamin D receptor silencing on the expression of LVSCC-A1C and LVSCC-A1D and the release of NGF in cortical neurons. *PLoS One*, **6**, e17553.

Giese, K.P., Friedman, E., Telliez, J.B., et al (2001) Hippocampus-dependent learning and memory is impaired in mice lacking the Ras-guanine-nucleotide releasing factor 1 (Ras-GRF1). *Neuropharmacology*, **41**, 791-800.

Giovanoli, S., Engler, H., Engler, A., et al (2013) Stress in puberty unmasks latent neuropathological consequences of prenatal immune activation in mice. *Science*, **339**, 1095-1099.

Girgis, C.M., Clifton-Bligh, R.J., Mokbel, N., Cheng, K. & Gunton, J.E. (2013) Vitamin D Signaling Regulates Proliferation, Differentiation and Myotube Size in C2C12 Skeletal Muscle Cells. *Endocrinology*.

Golden, S.A., Covington, H.E., 3rd, Berton, O. & Russo, S.J. (2011) A standardized protocol for repeated social defeat stress in mice. *Nat Protoc*, **6**, 1183-1191.

Gorman, J.M. (1996) Comorbid depression and anxiety spectrum disorders. *Depress Anxiety*, **4**, 160-168.

Grant, W.B. & Cannell, J.J. (2013) Autism prevalence in the United States with respect to solar UV-B doses: An ecological study. *Dermatoendocrinol*, **5**, 159-164.

Gratzner, H.G. (1982) Monoclonal antibody to 5-bromo- and 5-iododeoxyuridine: A new reagent for detection of DNA replication. *Science*, **218**, 474-475.

Grecksch, G., Ruthrich, H., Hollt, V. & Becker, A. (2009) Transient prenatal vitamin D deficiency is associated with changes of synaptic plasticity in the dentate gyrus in adult rats. *Psychoneuroendocrinology*, **34 Suppl 1**, S258-264.

Griffin, M.D., Lutz, W., Phan, V.A., et al (2001) Dendritic cell modulation by 1alpha,25 dihydroxyvitamin D3 and its analogs: a vitamin D receptor-dependent pathway that promotes a persistent state of immaturity in vitro and in vivo. *Proc Natl Acad Sci U S A*, **98**, 6800-6805.

Groves, N.J., Kesby, J.P., Eyles, D.W., et al (2013) Adult vitamin D deficiency leads to behavioural and brain neurochemical alterations in C57BL/6J and BALB/c mice. *Behav Brain Res*, **241**, 120-131.

Groves, N.J., McGrath, J.J. & Burne, T.H. (2014) Vitamin D as a neurosteroid affecting the developing and adult brain. *Annu Rev Nutr*, **34**, 117-141.

Hafner, H. (2003) Gender differences in schizophrenia. *Psychoneuroendocrinology*, **28 Suppl 2**, 17-54.

Hansen, C.F., Torgalsboen, A.K., Melle, I. & Bell, M.D. (2009) Passive/apathetic social withdrawal and active social avoidance in schizophrenia: difference in underlying psychological processes. *J Nerv Ment Dis*, **197**, 274-277.

Harbo, H.F., Gold, R. & Tintore, M. (2013) Sex and gender issues in multiple sclerosis. *Ther Adv Neurol Disord*, **6**, 237-248.

Hardingham, G.E. & Do, K.Q. (2016) Linking early-life NMDAR hypofunction and oxidative stress in schizophrenia pathogenesis. *Nat Rev Neurosci*, **17**, 125-134.

Harms, L.R., Burne, T.H., Eyles, D.W. & McGrath, J.J. (2011) Vitamin D and the brain. *Best Pract Res Clin Endocrinol Metab*, **25**, 657-669.

Harms, L.R., Cowin, G., Eyles, D.W., et al (2012a) Neuroanatomy and psychomimetic-induced locomotion in C57BL/6J and 129/X1SvJ mice exposed to developmental vitamin D deficiency. *Behav Brain Res*, **230**, 125-131.

Harms, L.R., Eyles, D.W., McGrath, J.J., Mackay-Sim, A. & Burne, T.H. (2008) Developmental vitamin D deficiency alters adult behaviour in 129/SvJ and C57BL/6J mice. *Behav Brain Res*, **187**, 343-350.

Harms, L.R., Turner, K.M., Eyles, D.W., et al (2012b) Attentional processing in C57BL/6J mice exposed to developmental vitamin D deficiency. *PLoS One*, **7**, e35896.

Harrison, P.J. (1999) The neuropathology of schizophrenia. A critical review of the data and their interpretation. *Brain*, **122 (Pt 4)**, 593-624.

Hart, P.H., Gorman, S. & Finlay-Jones, J.J. (2011) Modulation of the immune system by UV radiation: more than just the effects of vitamin D? *Nat Rev Immunol*, **11**, 584-596.

Hashimoto, T., Volk, D.W., Eggan, S.M., et al (2003) Gene expression deficits in a subclass of GABA neurons in the prefrontal cortex of subjects with schizophrenia. *J Neurosci*, **23**, 6315-6326.

Haussler, M.R., Whitfield, G.K., Haussler, C.A., et al (1998) The nuclear vitamin D receptor: biological and molecular regulatory properties revealed. *J Bone Miner Res*, **13**, 325-349.

Hawes, J.E., Tesic, D., Whitehouse, A.J., et al (2015) Maternal vitamin D deficiency alters fetal brain development in the BALB/c mouse. *Behav Brain Res*, **286**, 192-200.

Heaney, R.P., Recker, R.R., Grote, J., Horst, R.L. & Armas, L.A. (2011) Vitamin D(3) is more potent than vitamin D(2) in humans. *J Clin Endocrinol Metab*, **96**, E447-452.

Hewison, M., Burke, F., Evans, K.N., et al (2007) Extra-renal 25-hydroxyvitamin D3-1alpha-hydroxylase in human health and disease. *J Steroid Biochem Mol Biol*, **103**, 316-321.

Hoang, M.T., Defina, L.F., Willis, B.L., et al (2011) Association between low serum 25-hydroxyvitamin D and depression in a large sample of healthy adults: the Cooper Center longitudinal study. *Mayo Clin Proc*, **86**, 1050-1055.

Hohman, E.E., Martin, B.R., Lachcik, P.J., et al (2011) Bioavailability and efficacy of vitamin D2 from UV-irradiated yeast in growing, vitamin D-deficient rats. *J Agric Food Chem*, **59**, 2341-2346.

Holick, K.A., Lee, D.C., Hen, R. & Dulawa, S.C. (2008) Behavioral effects of chronic fluoxetine in BALB/cJ mice do not require adult hippocampal neurogenesis or the serotonin 1A receptor. *Neuropsychopharm*, **33**, 406-417.

Holick, M.F. (1995) Environmental factors that influence the cutaneous production of vitamin D. *Am J Clin Nutr*, **61**, 638S-645S.

Holick, M.F. (2007) Vitamin D Deficiency Medical Progress:. *The New Eng J of Med*, **357**, 266.

Holick, M.F., Binkley, N.C., Bischoff-Ferrari, H.A., et al (2012) Guidelines for preventing and treating vitamin D deficiency and insufficiency revisited. *J Clin Endocrinol Metab*, **97**, 1153-1158.

Hollis, F. & Kabbaj, M. (2014) Social defeat as an animal model for depression. *ILAR J*, **55**, 221-232.

Hollo, A., Clemens, Z., Kamondi, A., Lakatos, P. & Szucs, A. (2012) Correction of vitamin D deficiency improves seizure control in epilepsy: a pilot study. *Epilepsy Behav*, **24**, 131-133.

Hoogendijk, W.J., Lips, P., Dik, M.G., et al (2008) Depression is associated with decreased 25-hydroxyvitamin D and increased parathyroid hormone levels in older adults. *Arch Gen Psychiatry*, **65**, 508-512.

Horst, R.L., Napoli, J.L. & Littledike, E.T. (1982) Discrimination in the metabolism of orally dosed ergocalciferol and cholecalciferol by the pig, rat and chick. *Biochem J*, **204**, 185-189.

Hua, F., Reiss, J.I., Tang, H., et al (2012) Progesterone and low-dose vitamin D hormone treatment enhances sparing of memory following traumatic brain injury. *Horm Behav*, **61**, 642-651.

Jack, C.R., Jr., Petersen, R.C., Xu, Y.C., et al (1999) Prediction of AD with MRI-based hippocampal volume in mild cognitive impairment. *Neurology*, **52**, 1397-1403.

Jain, S.K. & Micinski, D. (2013) Vitamin D upregulates glutamate cysteine ligase and glutathione reductase, and GSH formation, and decreases ROS and MCP-1 and IL-8 secretion in high-glucose exposed U937 monocytes. *Biochem Biophys Res Commun*, **437**, 7-11.

Janoutova, J., Janackova, P., Sery, O., et al (2016) Epidemiology and risk factors of schizophrenia. *Neuro Endocrinol Lett*, **37**, 1-8.

Jiang, P., Zhang, W.Y., Li, H.D., et al (2013) Stress and vitamin D: Altered vitamin D metabolism in both the hippocampus and myocardium of chronic unpredictable mild stress exposed rats. *Psychoneuroendocrinology*.

Jorde, R., Sneve, M., Figenschau, Y., Svartberg, J. & Waterloo, K. (2008) Effects of vitamin D supplementation on symptoms of depression in overweight and obese subjects: randomized double blind trial. *J Intern Med*, **264**, 599-609.

Jorde, R., Strand Hutchinson, M., Kjaergaard, M., Sneve, M. & Grimnes, G. (2013) Supplementation with High Doses of Vitamin D to Subjects without Vitamin D Deficiency May Have Negative Effects: Pooled Data from Four Intervention Trials in Tromso. *ISRN Endocrinol*, **2013**, 348705.

Joseph, B., Wallen-Mackenzie, A., Benoit, G., et al (2003) p57(Kip2) cooperates with Nurr1 in developing dopamine cells. *Proc Natl Acad Sci U S A*, **100**, 15619-15624.

Kaelber, C.T., Moul, D.E. & Farmer, M.E. (1995) Epidemiology of depression. In Beckham, E.E., Leber, W.R. (eds) *Handbood of depression*. Guilford Press, New York, NY, US.

Kampman, M.T., Wilsgaard, T. & Mellgren, S.I. (2007) Outdoor activities and diet in childhood and adolescence relate to MS risk above the Arctic Circle. *J Neurol*, **254**, 471-477.

Karolewicz, B. & Paul, I.A. (2001) Group housing of mice increases immobility and antidepressant sensitivity in the forced swim and tail suspension tests. *Eur J Pharmacol*, **415**, 197-201.

Kaufman, D.L., Houser, C.R. & Tobin, A.J. (1991) Two forms of the gamma-aminobutyric acid synthetic enzyme glutamate decarboxylase have distinct intraneuronal distributions and cofactor interactions. *J Neurochem*, **56**, 720-723.

Kearns, C.M., Cass, W.A., Smoot, K., Kryscio, R. & Gash, D.M. (1997) GDNF protection against 6-OHDA: time dependence and requirement for protein synthesis. *J Neurosci*, **17**, 7111-7118.

Keeney, J.T. & Butterfield, D.A. (2015) Vitamin D deficiency and Alzheimer disease: Common links. *Neurobiol Dis*, **84**, 84-98.

Kehrer, C., Maziashvili, N., Dugladze, T. & Gloveli, T. (2008) Altered Excitatory-Inhibitory Balance in the NMDA-Hypofunction Model of Schizophrenia. *Front Mol Neurosci*, **1**, 6.

Keilhoff, G., Grecksch, G. & Becker, A. (2010) Haloperidol normalized prenatal vitamin D depletion-induced reduction of hippocampal cell proliferation in adult rats. *Neurosci Lett*, **476**, 94-98.

Kempermann, G., Kuhn, H.G. & Gage, F.H. (1997) Genetic influence on neurogenesis in the dentate gyrus of adult mice. *Proc Natl Acad Sci U S A*, **94**, 10409-10414.

Kesby, J.P., Burne, T.H., McGrath, J.J. & Eyles, D.W. (2006) Developmental vitamin D deficiency alters MK 801-induced hyperlocomotion in the adult rat: An animal model of schizophrenia. *Biol Psychiatry*, **60**, 591-596.

Kesby, J.P., Cui, X., Burne, T.H. & Eyles, D.W. (2013) Altered dopamine ontogeny in the developmentally vitamin D deficient rat and its relevance to schizophrenia. *Front Cell Neurosci*, **7**, 111.

Kesby, J.P., Cui, X., Ko, P., et al (2009) Developmental vitamin D deficiency alters dopamine turnover in neonatal rat forebrain. *Neurosci Lett*, **461**, 155-158.

Kesby, J.P., Cui, X., O'Loan, J., et al (2010) Developmental vitamin D deficiency alters dopamine-mediated behaviors and dopamine transporter function in adult female rats. *Psychopharmacology (Berl)*, **208**, 159-168.

Khan, A., Khan, M.I., Iqbal, Z., et al (2011) A new HPLC method for the simultaneous determination of ascorbic acid and aminothiols in human plasma and erythrocytes using electrochemical detection. *Talanta*, **84**, 789-801.

Khanal, R.C. & Nemere, I. (2007) The ERp57/GRp58/1,25D3-MARRS receptor: multiple functional roles in diverse cell systems. *Curr Med Chem*, **14**, 1087-1093.

Khoraminya, N., Tehrani-Doost, M., Jazayeri, S., Hosseini, A. & Djazayery, A. (2013) Therapeutic effects of vitamin D as adjunctive therapy to fluoxetine in patients with major depressive disorder. *Aust N Z J Psychiatry*, **47**, 271-275.

Kinney, D.K., Barch, D.H., Chayka, B., Napoleon, S. & Munir, K.M. (2010) Environmental risk factors for autism: do they help cause de novo genetic mutations that contribute to the disorder? *Med Hypotheses*, **74**, 102-106.

Kirkby, D.L. & Higgins, G.A. (1998) Characterization of perforant path lesions in rodent models of memory and attention. *Eur J Neurosci*, **10**, 823-838.

Kjaergaard, M., Waterloo, K., Wang, C.E., et al (2012) Effect of vitamin D supplement on depression scores in people with low levels of serum 25-hydroxyvitamin D: nested case-control study and randomised clinical trial. *Br J Psychiatry*, **201**, 360-368.

Knekt, P., Kilkkinen, A., Rissanen, H., et al (2010) Serum vitamin D and the risk of Parkinson disease. *Arch Neurol*, **67**, 808-811.

Knight, J.A., Wong, J., Blackmore, K.M., Raboud, J.M. & Vieth, R. (2010) Vitamin D association with estradiol and progesterone in young women. *Cancer Causes Control*, **21**, 479-483.

Ko, P., Burkert, R., McGrath, J. & Eyles, D. (2004) Maternal vitamin D3 deprivation and the regulation of apoptosis and cell cycle during rat brain development. *Brain Res Dev Brain Res*, **153**, 61-68.

Kocovska, E., Andorsdottir, G., Weihe, P., et al (2014) Vitamin d in the general population of young adults with autism in the faroe islands. *J Autism Dev Disord*, **44**, 2996-3005.

Koh, P.O. (2014) Estradiol alleviates the ischemic brain injury-induced decrease of neuronal calcium sensor protein hippocalcin. *Neurosci Lett*, **582**, 32-37.

Kokras, N. & Dalla, C. (2014) Sex differences in animal models of psychiatric disorders. *Br J Pharmacol*, **171**, 4595-4619.

Korsching, S., Auburger, G., Heumann, R., Scott, J. & Thoenen, H. (1985) Levels of nerve growth factor and its mRNA in the central nervous system of the rat correlate with cholinergic innervation. *EMBO J*, **4**, 1389-1393.

Kubota, M., Murakoshi, T., Saegusa, H., et al (2001) Intact LTP and fear memory but impaired spatial memory in mice lacking Ca(v)2.3 (alpha(IE)) channel. *Biochem Biophys Res Commun*, **282**, 242-248.

Kume, T., Nishikawa, H., Tomioka, H., et al (2000) p75-mediated neuroprotection by NGF against glutamate cytotoxicity in cortical cultures. *Brain Res*, **852**, 279-289.

Kurniawan, N.D., Richards, K.L., Yang, Z., et al (2014) Visualization of mouse barrel cortex using ex-vivo track density imaging. *Neuroimage*, **87**, 465-475.

Kurt, M.A., Kafa, M.I., Dierssen, M. & Davies, D.C. (2004) Deficits of neuronal density in CA1 and synaptic density in the dentate gyrus, CA3 and CA1, in a mouse model of Down syndrome. *Brain Res*, **1022**, 101-109.

Lassalle, J.M., Bataille, T. & Halley, H. (2000) Reversible inactivation of the hippocampal mossy fiber synapses in mice impairs spatial learning, but neither consolidation nor memory retrieval, in the Morris navigation task. *Neurobiol Learn Mem*, **73**, 243-257.

Latimer, C.S., Brewer, L.D., Searcy, J.L., et al (2014) Vitamin D prevents cognitive decline and enhances hippocampal synaptic function in aging rats. *Proc Natl Acad Sci U S A*, **111**, E4359-4366.

Lee, K.J., Lee, Y., Rozeboom, A., et al (2011) Requirement for Plk2 in orchestrated ras and rap signaling, homeostatic structural plasticity, and memory. *Neuron*, **69**, 957-973.

Lee, S.J., Lee, H.K., Kweon, Y.S., Lee, C.T. & Lee, K.U. (2009) The impact of executive function on emotion recognition and emotion experience in patients with schizophrenia. *Psychiatry Investig*, **6**, 156-162.

Lefebvre d'Hellencourt, C., Montero-Menei, C.N., Bernard, R. & Couez, D. (2003) Vitamin D3 inhibits proinflammatory cytokines and nitric oxide production by the EOC13 microglial cell line. *J Neurosci Res*, **71**, 575-582.

Leggio, M.G., Mandolesi, L., Federico, F., et al (2005) Environmental enrichment promotes improved spatial abilities and enhanced dendritic growth in the rat. *Behav Brain Res*, **163**, 78-90.

Lehmann, B. & Meurer, M. (2010) Vitamin D metabolism. *Dermatol Ther*, **23**, 2-12.

Lemire, J.M. & Adams, J.S. (1992) 1,25-Dihydroxyvitamin D3 inhibits the passive transfer of cellular immunity by a myelin basic protein-specific T cell clone. *J Bone Miner Res*, **7**, 171-177.

Lemire, J.M. & Archer, D.C. (1991) 1,25-dihydroxyvitamin D3 prevents the in vivo induction of murine experimental autoimmune encephalomyelitis. *J Clin Invest*, **87**, 1103-1107.

Lewis, D.A., Hashimoto, T. & Volk, D.W. (2005) Cortical inhibitory neurons and schizophrenia. *Nat Rev Neurosci*, **6**, 312-324.

Lewis, D.A. & Moghaddam, B. (2006) Cognitive dysfunction in schizophrenia: convergence of gamma-aminobutyric acid and glutamate alterations. *Arch Neurol*, **63**, 1372-1376.

Lips, P. (2010) Worldwide status of vitamin D nutrition. *J Steroid Biochem Mol Biol*, **121**, 297-300.

Littlejohns, T.J., Henley, W.E., Lang, I.A., et al (2014) Vitamin D and the risk of dementia and Alzheimer disease. *Neurology*, **83**, 920-928.

Liu, T., Sun, L., Xiong, Y., et al (2011) Calcium triggers exocytosis from two types of organelles in a single astrocyte. *J Neurosci*, **31**, 10593-10601.

Llewellyn, D.J., Lang, I.A., Langa, K.M., et al (2010) Vitamin D and risk of cognitive decline in elderly persons. *Arch Intern Med*, **170**, 1135-1141.

Llewellyn, D.J., Langa, K.M. & Lang, I.A. (2009) Serum 25-hydroxyvitamin D concentration and cognitive impairment. *J Geriatr Psychiatry Neurol*, **22**, 188-195.

Long, P., Mercer, A., Begum, R., et al (2009) Nerve Terminal GABAA Receptors Activate Ca2+/Calmodulin-dependent Signaling to Inhibit Voltage-gated Ca2+ Influx and Glutamate Release. *J Biol Chem*, **284**, 8726-8737.

Lorr, M., Sonn, T.M. & Katz, M.M. (1967) Toward a definition of depression. *Arch Gen Psychiatry*, **17**, 183-186.

LoTurco, J.J., Owens, D.F., Heath, M.J., Davis, M.B. & Kriegstein, A.R. (1995) GABA and glutamate depolarize cortical progenitor cells and inhibit DNA synthesis. *Neuron*, **15**, 1287-1298.

Luine, V.N. (2014) Estradiol and cognitive function: past, present and future. *Horm Behav*, **66**, 602-618.

Ma, Y., Hof, P.R., Grant, S.C., et al (2005) A three-dimensional digital atlas database of the adult C57BL/6J mouse brain by magnetic resonance microscopy. *Neuroscience*, **135**, 1203-1215.

Maeng, S. & Zarate, C.A., Jr. (2007) The role of glutamate in mood disorders: results from the ketamine in major depression study and the presumed cellular mechanism underlying its antidepressant effects. *Curr Psychiatry Rep*, **9**, 467-474.

Mahon, B.D., Gordon, S.A., Cruz, J., Cosman, F. & Cantorna, M.T. (2003) Cytokine profile in patients with multiple sclerosis following vitamin D supplementation. *J Neuroimmunol*, **134**, 128-132.

Maisonpierre, P.C., Belluscio, L., Squinto, S., et al (1990) Neurotrophin-3: a neurotrophic factor related to NGF and BDNF. *Science*, **247**, 1446-1451.

Manolagas, S.C., Provvedini, D.M. & Tsoukas, C.D. (1985) Interactions of 1,25-dihydroxyvitamin D3 and the immune system. *Mol Cell Endocrinol*, **43**, 113-122.

Marniemi, J., Alanen, E., Impivaara, O., et al (2005) Dietary and serum vitamins and minerals as predictors of myocardial infarction and stroke in elderly subjects. *Nutr Metab Cardiovasc Dis*, **15**, 188-197.

Mates, J.M., Perez-Gomez, C., Nunez de Castro, I., Asenjo, M. & Marquez, J. (2002) Glutamine and its relationship with intracellular redox status, oxidative stress and cell proliferation/death. *Int J Biochem Cell Biol*, **34**, 439-458.

Maynard, T.M., Sikich, L., Lieberman, J.A. & LaMantia, A.S. (2001) Neural development, cell-cell signaling, and the "two-hit" hypothesis of schizophrenia. *Schizophr Bull*, **27**, 457-476.

Mayne, C.G., Spanier, J.A., Relland, L.M., Williams, C.B. & Hayes, C.E. (2011) 1,25-Dihydroxyvitamin D3 acts directly on the T lymphocyte vitamin D receptor to inhibit experimental autoimmune encephalomyelitis. *Eur J Immunol*, **41**, 822-832.

Mazdeh, M., Seifirad, S., Kazemi, N., et al (2013) Comparison of Vitamin D3 Serum Levels in New Diagnosed Patients with Multiple Sclerosis versus Their Healthy Relatives. *Acta Med Iran*, **51**, 289-292.

McDonald, C. & Murray, R.M. (2000) Early and late environmental risk factors for schizophrenia. *Brain Res Brain Res Rev*, **31**, 130-137.

McGrath, J. (1999) Hypothesis: is low prenatal vitamin D a risk-modifying factor for schizophrenia? *Schizophr Res*, **40**, 173-177.

McGrath, J., Iwazaki, T., Eyles, D., et al (2008) Protein expression in the nucleus accumbens of rats exposed to developmental vitamin D deficiency. *PLoS One*, **3**, e2383.

McGrath, J., Scragg, R., Chant, D., et al (2007) No association between serum 25-hydroxyvitamin D3 level and performance on psychometric tests in NHANES III. *Neuroepidemiology*, **29**, 49-54.

McGrath, J.J., Burne, T.H., Feron, F., Mackay-Sim, A. & Eyles, D.W. (2010a) Developmental vitamin D deficiency and risk of schizophrenia: a 10-year update. *Schizophr Bull*, **36**, 1073-1078.

McGrath, J.J., Eyles, D.W., Pedersen, C.B., et al (2010b) Neonatal vitamin D status and risk of schizophrenia: a population-based case-control study. *Arch Gen Psychiatry*, **67**, 889-894.

McKenna, M.J. (1992) Differences in vitamin D status between countries in young adults and the elderly. *Am J Med*, **93**, 69-77.

Medina, D., DeToledo-Morrell, L., Urresta, F., et al (2006) White matter changes in mild cognitive impairment and AD: A diffusion tensor imaging study. *Neurobiol Aging*, **27**, 663-672.

Meguid, N.A., Hashish, A.F., Anwar, M. & Sidhom, G. (2010) Reduced serum levels of 25-hydroxy and 1,25-dihydroxy vitamin D in Egyptian children with autism. *J Altern Complement Med*, **16**, 641-645.

Menegaz, D., Mizwicki, M.T., Barrientos-Duran, A., et al (2011) Vitamin D receptor (VDR) regulation of voltage-gated chloride channels by ligands preferring a VDR-alternative pocket (VDR-AP). *Mol Endocrinol*, **25**, 1289-1300.

Milaneschi, Y., Bandinelli, S., Penninx, B.W., et al (2011) Depressive symptoms and inflammation increase in a prospective study of older adults: a protective effect of a healthy (Mediterranean-style) diet. *Mol Psychiatry*, **16**, 589-590.

Milaneschi, Y., Shardell, M., Corsi, A.M., et al (2010) Serum 25-hydroxyvitamin D and depressive symptoms in older women and men. *J Clin Endocrinol Metab*, **95**, 3225-3233.

Miller, B.R. & Hen, R. (2015) The current state of the neurogenic theory of depression and anxiety. *Curr Opin Neurobiol*, **30**, 51-58.

Miller, J.W., Harvey, D.J., Beckett, L.A., et al (2015) Vitamin D Status and Rates of Cognitive Decline in a Multiethnic Cohort of Older Adults. *JAMA Neurol*, **72**, 1295-1303.

Minelli, A., Brecha, N.C., Karschin, C., DeBiasi, S. & Conti, F. (1995) GAT-1, a high-affinity GABA plasma membrane transporter, is localized to neurons and astroglia in the cerebral cortex. *J Neurosci*, **15**, 7734-7746.

Miyaura, C., Abe, E., Nomura, H., Nishii, Y. & Suda, T. (1982) 1 alpha,25-Dihydroxyvitamin D3 suppresses proliferation of murine granulocyte-macrophage progenitor cells (CFU-C). *Biochem Biophys Res Commun*, **108**, 1728-1733.

Moon, Y., Moon, W.J., Kwon, H., Lee, J.M. & Han, S.H. (2015) Vitamin D deficiency disrupts neuronal integrity in cognitively impaired patients. *J Alzheimers Dis*, **45**, 1089-1096.

Mora, J.R., Iwata, M. & von Andrian, U.H. (2008) Vitamin effects on the immune system: vitamins A and D take centre stage. *Nat Rev Immunol*, **8**, 685-698.

Moretti, P., Levenson, J.M., Battaglia, F., et al (2006) Learning and memory and synaptic plasticity are impaired in a mouse model of Rett syndrome. *J Neurosci*, **26**, 319-327.

Moriwaki, M., Kishi, T., Takahashi, H., et al (2009) Prepulse inhibition of the startle response with chronic schizophrenia: a replication study. *Neurosci Res*, **65**, 259-262.

Moskovitz, R.A. (1978) Seasonality in Schizophrenia. *Lancet*, **1**, 664-664.

Mostafa, G.A. & Al-Ayadhi, L.Y. (2012) Reduced serum concentrations of 25-hydroxy vitamin D in children with autism: relation to autoimmunity. *J Neuroinflammation*, **9**, 201.

Moussavi, S., Chatterji, S., Verdes, E., et al (2007) Depression, chronic diseases, and decrements in health: results from the World Health Surveys. *Lancet*, **370**, 851-858.

Mozaffari-Khosravi, H., Nabizade, L., Yassini-Ardakani, S.M., Hadinedoushan, H. & Barzegar, K. (2013) The effect of 2 different single injections of high dose of vitamin d on improving the depression in depressed patients with vitamin d deficiency: a randomized clinical trial. *J Clin Psychopharmacol*, **33**, 378-385.

Muir, J.L., Everitt, B.J. & Robbins, T.W. (1996) The cerebral cortex of the rat and visual attentional function: dissociable effects of mediofrontal, cingulate, anterior dorsolateral, and parietal cortex lesions on a five-choice serial reaction time task. *Cereb Cortex*, **6**, 470-481.

Mullen, R.J., Buck, C.R. & Smith, A.M. (1992) NeuN, a neuronal specific nuclear protein in vertebrates. *Development*, **116**, 201-211.

Munger, K.L., Levin, L.I., Hollis, B.W., Howard, N.S. & Ascherio, A. (2006) Serum 25-hydroxyvitamin D levels and risk of multiple sclerosis. *JAMA*, **296**, 2832-2838.

Munger, K.L., Zhang, S.M., O'Reilly, E., et al (2004) Vitamin D intake and incidence of multiple sclerosis. *Neurology*, **62**, 60-65.

Nataf, S., Garcion, E., Darcy, F., et al (1996) 1,25 Dihydroxyvitamin D3 exerts regional effects in the central nervous system during experimental allergic encephalomyelitis. *J Neuropathol Exp Neurol*, **55**, 904-914.

National Research Council (1995) *Nutrient Requirements of Laboratory Animals: Fourth Revised Edition, 1995,* Washington, DC.

Naveilhan, P., Neveu, I., Baudet, C., et al (1996a) 1,25-Dihydroxyvitamin D3 regulates the expression of the low-affinity neurotrophin receptor. *Brain Res Mol Brain Res*, **41**, 259-268.

Naveilhan, P., Neveu, I., Wion, D. & Brachet, P. (1996b) 1,25-Dihydroxyvitamin D3, an inducer of glial cell line-derived neurotrophic factor. *Neuroreport*, **7**, 2171-2175.

Nelson, E.D., Kavalali, E.T. & Monteggia, L.M. (2006) MeCP2-dependent transcriptional repression regulates excitatory neurotransmission. *Curr Biol*, **16**, 710-716.

Nemere, I., Garbi, N., Hammerling, G. & Hintze, K.J. (2012) Role of the 1,25D3-MARRS receptor in the 1,25(OH)2D3-stimulated uptake of calcium and phosphate in intestinal cells. *Steroids*, **77**, 897-902.

Neveu, I., Naveilhan, P., Baudet, C., Brachet, P. & Metsis, M. (1994a) 1,25-dihydroxyvitamin D3 regulates NT-3, NT-4 but not BDNF mRNA in astrocytes. *Neuroreport*, **6**, 124-126.

Neveu, I., Naveilhan, P., Jehan, F., et al (1994b) 1,25-dihydroxyvitamin D3 regulates the synthesis of nerve growth factor in primary cultures of glial cells. *Brain Res Mol Brain Res*, **24**, 70-76.

Nguyen, M.V., Du, F., Felice, C.A., et al (2012) MeCP2 is critical for maintaining mature neuronal networks and global brain anatomy during late stages of postnatal brain development and in the mature adult brain. *J Neurosci*, **32**, 10021-10034.

Nichols, T.E. & Holmes, A.P. (2002) Nonparametric permutation tests for functional neuroimaging: a primer with examples. *Hum Brain Mapp*, **15**, 1-25.

Nissou, M.F., Brocard, J., El Atifi, M., et al (2013) The transcriptomic response of mixed neuron-glial cell cultures to 1,25-dihydroxyvitamin d3 includes genes limiting the progression of neurodegenerative diseases. *J Alzheimers Dis*, **35**, 553-564.

O'Gorman, C., Lucas, R. & Taylor, B. (2012) Environmental risk factors for multiple sclerosis: a review with a focus on molecular mechanisms. *Int J Mol Sci*, **13**, 11718-11752.

Patel, A.B., de Graaf, R.A., Martin, D.L., Battaglioli, G. & Behar, K.L. (2006) Evidence that GAD(65) mediates increased GABA synthesis during intense neuronal activity in vivo. *J Neurochem*, **97**, 385-396.

Patki, G., Solanki, N., Atrooz, F., Allam, F. & Salim, S. (2013) Depression, anxiety-like behavior and memory impairment are associated with increased oxidative stress and inflammation in a rat model of social stress. *Brain Res*, **1539**, 73-86.

Pearlson, G.D. (2000) Neurobiology of schizophrenia. *Ann Neurol*, **48**, 556-566.

Pehrson, A.L., Bondi, C.O., Totah, N.K. & Moghaddam, B. (2013) The influence of NMDA and GABA(A) receptors and glutamic acid decarboxylase (GAD) activity on attention. *Psychopharmacology (Berl)*, **225**, 31-39.

Pendyala, G., Ninemire, C. & Fox, H.S. (2012) Protective role for the disulfide isomerase PDIA3 in methamphetamine neurotoxicity. *PLoS One*, **7**, e38909.

Petersen, R.C., Roberts, R.O., Knopman, D.S., et al (2010) Prevalence of mild cognitive impairment is higher in men. The Mayo Clinic Study of Aging. *Neurology*, **75**, 889-897.

Peterson, A., Mattek, N., Clemons, A., et al (2012) Serum vitamin D concentrations are associated with falling and cognitive function in older adults. *J Nutr Health Aging*, **16**, 898-901.

Pezze, M., McGarrity, S., Mason, R., Fone, K.C. & Bast, T. (2014) Too little and too much: hypoactivation and disinhibition of medial prefrontal cortex cause attentional deficits. *J Neurosci*, **34**, 7931-7946.

Pfefferbaum, A., Sullivan, E.V., Hedehus, M., et al (2000) Age-related decline in brain white matter anisotropy measured with spatially corrected echo-planar diffusion tensor imaging. *Magn Reson Med*, **44**, 259-268.

Pilz, S., Dobnig, H., Fischer, J.E., et al (2008) Low vitamin d levels predict stroke in patients referred to coronary angiography. *Stroke*, **39**, 2611-2613.

Pilz, S., Frisch, S., Koertke, H., et al (2011) Effect of vitamin D supplementation on testosterone levels in men. *Horm Metab Res*, **43**, 223-225.

Pols, H.A., Birkenhager, J.C., Foekens, J.A. & van Leeuwen, J.P. (1990) Vitamin D: a modulator of cell proliferation and differentiation. *J Steroid Biochem Mol Biol*, **37**, 873-876.

Pompili, A., Arnone, B. & Gasbarri, A. (2012) Estrogens and memory in physiological and neuropathological conditions. *Psychoneuroendocrinology*, **37**, 1379-1396.

Porsolt, R.D., Bertin, A. & Jalfre, M. (1977) Behavioral despair in mice: a primary screening test for antidepressants. *Arch Int Pharmacodyn Ther*, **229**, 327-336.

Pozuelo-Moyano, B., Benito-Leon, J., Mitchell, A.J. & Hernandez-Gallego, J. (2013) A systematic review of randomized, double-blind, placebo-controlled trials examining the clinical efficacy of vitamin D in multiple sclerosis. *Neuroepidemiology*, **40**, 147-153.

Pratico, D., Clark, C.M., Liun, F., et al (2002) Increase of brain oxidative stress in mild cognitive impairment: a possible predictor of Alzheimer disease. *Arch Neurol*, **59**, 972-976.

Prins, N.D. & Scheltens, P. (2015) White matter hyperintensities, cognitive impairment and dementia: an update. *Nat Rev Neurol*, **11**, 157-165.

Procopio, M. & Marriott, P.K. (1998) Seasonality of birth in epilepsy: a Danish study. *Acta Neurol Scand*, **98**, 297-301.

Procopio, M., Marriott, P.K. & Davies, R.J. (2006) Seasonality of birth in epilepsy: a Southern Hemisphere study. *Seizure*, **15**, 17-21.

Procopio, M., Marriott, P.K. & Williams, P. (1997) Season of birth: aetiological implications for epilepsy. *Seizure*, **6**, 99-105.

Prufer, K. & Barsony, J. (2002) Retinoid X receptor dominates the nuclear import and export of the unliganded vitamin D receptor. *Mol Endocrinol*, **16**, 1738-1751.

Prufer, K., Veenstra, T.D., Jirikowski, G.F. & Kumar, R. (1999) Distribution of 1,25-dihydroxyvitamin D3 receptor immunoreactivity in the rat brain and spinal cord. *J Chem Neuroanat*, **16**, 135-145.

Przybelski, R.J. & Binkley, N.C. (2007) Is vitamin D important for preserving cognition? A positive correlation of serum 25-hydroxyvitamin D concentration with cognitive function. *Arch Biochem Biophys*, **460**, 202-205.

Pugliatti, M., Harbo, H.F., Holmoy, T., et al (2008) Environmental risk factors in multiple sclerosis. *Acta Neurol Scand Suppl*, **188**, 34-40.

Pulliam, J.V., Dawaghreh, A.M., Alema-Mensah, E. & Plotsky, P.M. (2010) Social defeat stress produces prolonged alterations in acoustic startle and body weight gain in male Long Evans rats. *J Psychiatr Res*, **44**, 106-111.

Quintero, E.M., Willis, L.M., Zaman, V., et al (2004) Glial cell line-derived neurotrophic factor is essential for neuronal survival in the locus coeruleus-hippocampal noradrenergic pathway. *Neuroscience*, **124**, 137-146.

Ramagopalan, S.V., Dyment, D.A., Cader, M.Z., et al (2011) Rare variants in the CYP27B1 gene are associated with multiple sclerosis. *Ann Neurol*, **70**, 881-886.

Ramagopalan, S.V., Heger, A., Berlanga, A.J., et al (2010) A ChIP-seq defined genome-wide map of vitamin D receptor binding: associations with disease and evolution. *Genome Res*, **20**, 1352-1360.

Razzoli, M., Carboni, L., Andreoli, M., Ballottari, A. & Arban, R. (2011) Different susceptibility to social defeat stress of BalbC and C57BL6/J mice. *Behav Brain Res*, **216**, 100-108.

Richer, J. (1976) The social-avoidance behaviour of autistic children. *Anim Behav*, **24**, 898-906.

Robbins, T.W. (2002) The 5-choice serial reaction time task: behavioural pharmacology and functional neurochemistry. *Psychopharmacology (Berl)*, **163**, 362-380.

Saaf, M., Fernell, E., Kristiansson, F., et al (2011) Severe vitamin D deficiency in pregnant women of Somali origin living in Sweden. *Acta Paediatr*, **100**, 612-614.

Sagud, M., Hotujac, L., Mihaljevic-Peles, A. & Jakovljevic, M. (2002) Gender differences in depression. *Coll Antropol*, **26**, 149-157.

Salami, M., Talaei, S.A., Davari, S. & Taghizadeh, M. (2012) Hippocampal long term potentiation in rats under different regimens of vitamin D: an in vivo study. *Neurosci Lett*, **509**, 56-59.

Sanchez, B., Lopez-Martin, E., Segura, C., Labandeira-Garcia, J.L. & Perez-Fernandez, R. (2002) 1,25-Dihydroxyvitamin D(3) increases striatal GDNF mRNA and protein expression in adult rats. *Brain Res Mol Brain Res*, **108**, 143-146.

Sanchez, B., Relova, J.L., Gallego, R., Ben-Batalla, I. & Perez-Fernandez, R. (2009) 1,25-Dihydroxyvitamin D3 administration to 6-hydroxydopamine-lesioned rats increases glial cell line-derived neurotrophic factor and partially restores tyrosine hydroxylase expression in substantia nigra and striatum. *J Neurosci Res*, **87**, 723-732.

Sanders, K.M., Stuart, A.L., Williamson, E.J., et al (2011) Annual high-dose vitamin D3 and mental well-being: randomised controlled trial. *Br J Psychiatry*, **198**, 357-364.

Sato, Y., Honda, Y., Iwamoto, J., Kanoko, T. & Satoh, K. (2005) Abnormal bone and calcium metabolism in immobilized Parkinson's disease patients. *Mov Disord*, **20**, 1598-1603.

Savettieri, G., Messina, D., Andreoli, V., et al (2004) Gender-related effect of clinical and genetic variables on the cognitive impairment in multiple sclerosis. *J Neurol*, **251**, 1208-1214.

Sawcer, S. & Hellenthal, G. & Pirinen, M., et al (2011) Genetic risk and a primary role for cell-mediated immune mechanisms in multiple sclerosis. *Nature*, **476**, 214-219.

Schoch, S., Castillo, P.E., Jo, T., et al (2002) RIM1alpha forms a protein scaffold for regulating neurotransmitter release at the active zone. *Nature*, **415**, 321-326.

Schrader, M., Kahlen, J.P. & Carlberg, C. (1997) Functional characterization of a novel type of 1 alpha,25-dihydroxyvitamin D3 response element identified in the mouse c-fos promoter. *Biochem Biophys Res Commun*, **230**, 646-651.

Schuster, I. (2011) Cytochromes P450 are essential players in the vitamin D signaling system. *Biochim Biophys Acta*, **1814**, 186-199.

Senechal, Y., Kelly, P.H. & Dev, K.K. (2008) Amyloid precursor protein knockout mice show age-dependent deficits in passive avoidance learning. *Behav Brain Res*, **186**, 126-132.

Shahbeigi, S., Pakdaman, H., Fereshtehnejad, S.M., et al (2013) Vitamin d3 concentration correlates with the severity of multiple sclerosis. *Int J Prev Med*, **4**, 585-591.

Shirazi, H.A., Rasouli, J., Ciric, B., Rostami, A. & Zhang, G.X. (2015) 1,25-Dihydroxyvitamin D3 enhances neural stem cell proliferation and oligodendrocyte differentiation. *Exp Mol Pathol*, **98**, 240-245.

Sies, H. (1997) Oxidative stress: oxidants and antioxidants. *Exp Physiol*, **82**, 291-295.

Simpson, S., Jr., Blizzard, L., Otahal, P., Van der Mei, I. & Taylor, B. (2011) Latitude is significantly associated with the prevalence of multiple sclerosis: a meta-analysis. *J Neurol Neurosurg Psychiatry*, **82**, 1132-1141.

Singh, M., Gupta, S., Singhal, U., Pandey, R. & Aggarwal, S.K. (2013) Evaluation of the oxidative stress in chronic alcoholics. *J Clin Diagn Res*, **7**, 1568-1571.

Slinin, Y., Paudel, M.L., Taylor, B.C., et al (2010) 25-Hydroxyvitamin D levels and cognitive performance and decline in elderly men. *Neurology*, **74**, 33-41.

Smith, J.W., Gastambide, F., Gilmour, G., et al (2011) A comparison of the effects of ketamine and phencyclidine with other antagonists of the NMDA receptor in rodent assays of attention and working memory. *Psychopharmacology (Berl)*, **217**, 255-269.

Smith, R.J. & Wilmore, D.W. (1990) Glutamine nutrition and requirements. *JPEN J Parenter Enteral Nutr*, **14**, 94S-99S.

Smith, S.M., Jenkinson, M., Johansen-Berg, H., et al (2006) Tract-based spatial statistics: voxelwise analysis of multi-subject diffusion data. *Neuroimage*, **31**, 1487-1505.

Sonnenberg, J., Luine, V.N., Krey, L.C. & Christakos, S. (1986) 1,25-Dihydroxyvitamin D3 treatment results in increased choline acetyltransferase activity in specific brain nuclei. *Endocrinology*, **118**, 1433-1439.

Stephensen, C.B., Zerofsky, M., Burnett, D.J., et al (2012) Ergocalciferol from mushrooms or supplements consumed with a standard meal increases 25-hydroxyergocalciferol but decreases 25-hydroxycholecalciferol in the serum of healthy adults. *J Nutr*, **142**, 1246-1252.

Steullet, P., Cabungcal, J.H., Kulak, A., et al (2010) Redox dysregulation affects the ventral but not dorsal hippocampus: impairment of parvalbumin neurons, gamma oscillations, and related behaviors. *J Neurosci*, **30**, 2547-2558.

Stuchlik, A. & Vales, K. (2005) Systemic administration of MK-801, a non-competitive NMDA-receptor antagonist, elicits a behavioural deficit of rats in the Active Allothetic Place Avoidance (AAPA) task irrespectively of their intact spatial pretraining. *Behav Brain Res*, **159**, 163-171.

Stuchlik, A. & Vales, K. (2009) Baclofen dose-dependently disrupts learning in a place avoidance task requiring cognitive coordination. *Physiol Behav*, **97**, 507-511.

Suzuki, M., Yoshioka, M., Hashimoto, M., et al (2013) Randomized, double-blind, placebo-controlled trial of vitamin D supplementation in Parkinson disease. *Am J Clin Nutr*, **97**, 1004-1013.

Taghizadeh, M., Talaei, S.A. & Salami, M. (2013) Vitamin D deficiency impairs spatial learning in adult rats. *Iran Biomed J*, **17**, 42-48.

Talebian, A., Robinson-Brookes, K., MacDonald, J.I. & Meakin, S.O. (2013) Ras guanine nucleotide releasing factor 1 (RasGrf1) enhancement of Trk receptor-mediated neurite outgrowth requires activation of both H-Ras and Rac. *J Mol Neurosci*, **49**, 38-51.

Taliaz, D., Loya, A., Gersner, R., et al (2011) Resilience to chronic stress is mediated by hippocampal brain-derived neurotrophic factor. *J Neurosci*, **31**, 4475-4483.

Tekes, K., Gyenge, M., Folyovich, A. & Csaba, G. (2009a) Influence of neonatal vitamin A or vitamin D treatment on the concentration of biogenic amines and their metabolites in the adult rat brain. *Horm Metab Res*, **41**, 277-280.

Tekes, K., Gyenge, M., Hantos, M. & Csaba, G. (2009b) Transgenerational hormonal imprinting caused by vitamin A and vitamin D treatment of newborn rats. Alterations in the biogenic amine contents of the adult brain. *Brain Dev*, **31**, 666-670.

Tenenhouse, A., Warner, M. & Commissiong, J.W. (1991) Neurotransmitters in the CNS of the vitamin D deficient, hypocalcemic rat. *Neurochem Int*, **18**, 249-255.

Tian, N., Petersen, C., Kash, S., et al (1999) The role of the synthetic enzyme GAD65 in the control of neuronal gamma-aminobutyric acid release. *Proc Natl Acad Sci U S A*, **96**, 12911-12916.

Toffanello, E.D., Coin, A., Perissinotto, E., et al (2014) Vitamin D deficiency predicts cognitive decline in older men and women: The Pro.V.A. Study. *Neurology*, **83**, 2292-2298.

Tohda, C., Urano, T., Umezaki, M., Nemere, I. & Kuboyama, T. (2012) Diosgenin is an exogenous activator of 1,25D(3)-MARRS/Pdia3/ERp57 and improves Alzheimer's disease pathologies in 5XFAD mice. *Sci Rep*, **2**, 535.

Tomac, A., Widenfalk, J., Lin, L.F., et al (1995) Retrograde axonal transport of glial cell line-derived neurotrophic factor in the adult nigrostriatal system suggests a trophic role in the adult. *Proc Natl Acad Sci U S A*, **92**, 8274-8278.

Tomiyama, T., Matsuyama, S., Iso, H., et al (2010) A mouse model of amyloid beta oligomers: their contribution to synaptic alteration, abnormal tau phosphorylation, glial activation, and neuronal loss in vivo. *J Neurosci*, **30**, 4845-4856.

Tordera, R.M., Totterdell, S., Wojcik, S.M., et al (2007) Enhanced anxiety, depressive-like behaviour and impaired recognition memory in mice with reduced expression of the vesicular glutamate transporter 1 (VGLUT1). *Eur J Neurosci*, **25**, 281-290.

Torkaman-Boutorabi, A., Soltani, S., Oryan, S., et al (2013) Involvement of the dorsal hippocampal GABA-A receptors in histamine-induced facilitation of memory in the Morris water maze. *Pharmacol Biochem Behav*, **105**, 142-150.

Torres-Lista, V. & Gimenez-Llort, L. (2014) Persistence of behaviours in the Forced Swim Test in 3xTg-AD mice at advanced stages of disease. *Behav Processes*, **106C**, 118-121.

Townsend, D.M., Tew, K.D. & Tapiero, H. (2003) The importance of glutathione in human disease. *Biomed Pharmacother*, **57**, 145-155.

Tsai, M.J. & O'Malley, B.W. (1994) Molecular mechanisms of action of steroid/thyroid receptor superfamily members. *Annu Rev Biochem*, **63**, 451-486.

Tu, W.J., Zhao, S.J., Xu, D.J. & Chen, H. (2014) Serum 25-hydroxyvitamin D predicts the short-term outcomes of Chinese patients with acute ischaemic stroke. *Clin Sci (Lond)*, **126**, 339-346.

Turner, K.M., Young, J.W., McGrath, J.J., Eyles, D.W. & Burne, T.H. (2013) Cognitive performance and response inhibition in developmentally vitamin D (DVD)-deficient rats. *Behav Brain Res*, **242**, 47-53.

Tyler, C.R., Solomon, B.R., Ulibarri, A.L. & Allan, A.M. (2014) Fluoxetine treatment ameliorates depression induced by perinatal arsenic exposure via a neurogenic mechanism. *Neurotoxicology*, **44**, 98-109.

Urano, S., Asai, Y., Makabe, S., et al (1997) Oxidative injury of synapse and alteration of antioxidative defense systems in rats, and its prevention by vitamin E. *Eur J Biochem*, **245**, 64-70.

Urso, M.L. & Clarkson, P.M. (2003) Oxidative stress, exercise, and antioxidant supplementation. *Toxicology*, **189**, 41-54.

Van Cromphaut, S.J., Dewerchin, M., Hoenderop, J.G., et al (2001) Duodenal calcium absorption in vitamin D receptor-knockout mice: functional and molecular aspects. *Proc Natl Acad Sci U S A*, **98**, 13324-13329.

Van den Buuse, M. (2013) Exploring the role of 5-HT1A receptors in the regulation of prepulse inhibition in mice: implications for cross-species comparisons. *ACS Chem Neurosci*, **4**, 149-160.

van der Mei, I.A., Ponsonby, A.L., Dwyer, T., et al (2003) Past exposure to sun, skin phenotype, and risk of multiple sclerosis: case-control study. *BMJ*, **327**, 316.

van Praag, H. (2008) Neurogenesis and exercise: past and future directions. *Neuromolecular Med*, **10**, 128-140.

van Praag, H., Shubert, T., Zhao, C. & Gage, F.H. (2005) Exercise enhances learning and hippocampal neurogenesis in aged mice. *J Neurosci*, **25**, 8680-8685.

VanAmerongen, B.M., Dijkstra, C.D., Lips, P. & Polman, C.H. (2004) Multiple sclerosis and vitamin D: an update. *Eur J Clin Nutr*, **58**, 1095-1109.

Vander Heiden, M.G., Cantley, L.C. & Thompson, C.B. (2009) Understanding the Warburg effect: the metabolic requirements of cell proliferation. *Science*, **324**, 1029-1033.

Veenstra, T.D., Prufer, K., Koenigsberger, C., et al (1998) 1,25-Dihydroxyvitamin D3 receptors in the central nervous system of the rat embryo. *Brain Res*, **804**, 193-205.

Venzala, E., Garcia-Garcia, A.L., Elizalde, N., Delagrange, P. & Tordera, R.M. (2012) Chronic social defeat stress model: behavioral features, antidepressant action, and interaction with biological risk factors. *Psychopharmacology (Berl)*, **224**, 313-325.

Vina, J.R., Palacin, M., Puertes, I.R., Hernandez, R. & Vina, J. (1989) Role of the gamma-glutamyl cycle in the regulation of amino acid translocation. *Am J Physiol*, **257**, E916-922.

Vukovic, J., Borlikova, G.G., Ruitenberg, M.J., et al (2013) Immature doublecortin-positive hippocampal neurons are important for learning but not for remembering. *J Neurosci*, **33**, 6603-6613.

Walbert, T., Jirikowski, G.F. & Prufer, K. (2001) Distribution of 1,25-dihydroxyvitamin D3 receptor immunoreactivity in the limbic system of the rat. *Horm Metab Res*, **33**, 525-531.

Walsh, R.N. & Cummins, R.A. (1976) The Open-Field Test: a critical review. *Psychol Bull*, **83**, 482-504.

Wang, J.Y., Wu, J.N., Cherng, T.L., et al (2001) Vitamin D(3) attenuates 6-hydroxydopamine-induced neurotoxicity in rats. *Brain Res*, **904**, 67-75.

Wang, L., Hara, K., Van Baaren, J.M., et al (2012a) Vitamin D receptor and Alzheimer's disease: a genetic and functional study. *Neurobiol Aging*, **33**, 1844 e1841-1849.

Wang, X., Su, H., Copenhagen, L.D., et al (2002a) Urocortin-deficient mice display normal stress-induced anxiety behavior and autonomic control but an impaired acoustic startle response. *Mol Cell Biol*, **22**, 6605-6610.

Wang, Y., Chang, C.F., Morales, M., Chiang, Y.H. & Hoffer, J. (2002b) Protective effects of glial cell line-derived neurotrophic factor in ischemic brain injury. *Ann N Y Acad Sci*, **962**, 423-437.

Wang, Y., Zhu, J. & DeLuca, H.F. (2012b) Where is the vitamin D receptor? *Arch Biochem Biophys*, **523**, 123-133.

Wehr, E., Pilz, S., Boehm, B.O., Marz, W. & Obermayer-Pietsch, B. (2010) Association of vitamin D status with serum androgen levels in men. *Clin Endocrinol (Oxf)*, **73**, 243-248.

Wergeland, S., Torkildsen, O., Myhr, K.M., et al (2011) Dietary vitamin D3 supplements reduce demyelination in the cuprizone model. *PLoS One*, **6**, e26262.

Wilkins, C.H., Sheline, Y.I., Roe, C.M., Birge, S.J. & Morris, J.C. (2006) Vitamin D deficiency is associated with low mood and worse cognitive performance in older adults. *Am J Geriatr Psychiatry*, **14**, 1032-1040.

Williams, J.H., Wellman, N.A., Geaney, D.P., et al (1998) Reduced latent inhibition in people with schizophrenia: an effect of psychosis or of its treatment. *Br J Psychiatry*, **172**, 243-249.

Wion, D., MacGrogan, D., Neveu, I., et al (1991) 1,25-Dihydroxyvitamin D3 is a potent inducer of nerve growth factor synthesis. *J Neurosci Res*, **28**, 110-114

Wray, N.R. & Visscher, P.M. (2010) Narrowing the boundaries of the genetic architecture of schizophrenia. *Schizophr Bull*, **36**, 14-23.

Wright, D.W., Kellermann, A.L., Hertzberg, V.S., et al (2007) ProTECT: a randomized clinical trial of progesterone for acute traumatic brain injury. *Ann Emerg Med*, **49**, 391-402, 402 e391-392.

Wu, L.G., Borst, J.G. & Sakmann, B. (1998) R-type Ca2+ currents evoke transmitter release at a rat central synapse. *Proc Natl Acad Sci U S A*, **95**, 4720-4725.

Wu, W.W., Wang, G., Baek, S.J. & Shen, R.F. (2006) Comparative study of three proteomic quantitative methods, DIGE, cICAT, and iTRAQ, using 2D gel- or LC-MALDI TOF/TOF. *J Proteome Res*, **5**, 651-658.

Xiao, G., Wei, J., Yan, W., Wang, W. & Lu, Z. (2008) Improved outcomes from the administration of progesterone for patients with acute severe traumatic brain injury: a randomized controlled trial. *Crit Care*, **12**, R61.

Yasuhara, T., Hara, K., Maki, M., et al (2008) Dietary supplementation exerts neuroprotective effects in ischemic stroke model. *Rejuvenation Res*, **11**, 201-214.

Yau, S.Y., Li, A. & So, K.F. (2015) Involvement of Adult Hippocampal Neurogenesis in Learning and Forgetting. *Neural Plast*, **2015**, 717958.

Ylikomi, T., Laaksi, I., Lou, Y.R., et al (2002) Antiproliferative action of vitamin D. *Vitam Horm*, **64**, 357-406.

Young, J.W., Light, G.A., Marston, H.M., Sharp, R. & Geyer, M.A. (2009a) The 5-choice continuous performance test: evidence for a translational test of vigilance for mice. *PLoS One*, **4**, e4227.

Young, J.W., Powell, S.B., Risbrough, V., Marston, H.M. & Geyer, M.A. (2009b) Using the MATRICS to guide development of a preclinical cognitive test battery for research in schizophrenia. *Pharmacol Ther*, **122**, 150-202.

Yousefi, B., Farjad, M., Nasehi, M. & Zarrindast, M.R. (2013) Involvement of the CA1 GABAA receptors in ACPA-induced impairment of spatial and non-spatial novelty detection in mice. *Neurobiol Learn Mem*, **100**, 32-40.

Yuksel, R.N., Altunsoy, N., Tikir, B., et al (2014) Correlation between total vitamin D levels and psychotic psychopathology in patients with schizophrenia: therapeutic implications for add-on vitamin D augmentation. *Ther Adv Psychopharmacol*, **4**, 268-275.

Zahr, N.M., Mayer, D., Pfefferbaum, A. & Sullivan, E.V. (2008) Low striatal glutamate levels underlie cognitive decline in the elderly: evidence from in vivo molecular spectroscopy. *Cereb Cortex*, **18**, 2241-2250.

Zanatta, L., Goulart, P.B., Goncalves, R., et al (2012) 1alpha,25-dihydroxyvitamin D(3) mechanism of action: modulation of L-type calcium channels leading to calcium uptake and intermediate filament phosphorylation in cerebral cortex of young rats. *Biochim Biophys Acta*, **1823**, 1708-1719.

Zehnder, D., Bland, R., Williams, M.C., et al (2001) Extrarenal expression of 25-hydroxyvitamin d(3)-1 alpha-hydroxylase. *J Clin Endocrinol Metab*, **86**, 888-894.

Zetterstrom, R.H., Solomin, L., Jansson, L., et al (1997) Dopamine neuron agenesis in Nurr1-deficient mice. *Science*, **276**, 248-250.

Zhang, Y., Schuff, N., Jahng, G.H., et al (2007) Diffusion tensor imaging of cingulum fibers in mild cognitive impairment and Alzheimer disease. *Neurology*, **68**, 13-19.

Zhao, Y. & Zhao, B. (2013) Oxidative stress and the pathogenesis of Alzheimer's disease. *Oxidative Medicine and Cellular Longevity*, **2013**, 316523.

Zhu, Y., Zhou, R., Yang, R., et al (2012) Abnormal neurogenesis in the dentate gyrus of adult mice lacking 1,25-dihydroxy vitamin D3 (1,25-(OH)2 D3). *Hippocampus*, **22**, 421-433.

Appendix 1

Specialty Feeds Diet - Soya Oil Maize Starch Modification of AIN93G Rodent Diet

a) Base components of the Control and AVD-deficient mouse feed

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

Values given are prior to irradiation with 25 kGy

b) Vitamin Composition of Control and AVD-deficient mouse feed

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

Values given are prior to irradiation with 25 kGy

c) Mineral Composition of Control and AVD-deficient mouse feed

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

Values given are prior to irradiation with 25 kGy

Appendix 2

Specialty Feeds Standard Mouse Chow

a) Base components of the standard mouse diet

Ingredients
A fixed formula ration using Wheat, Barley, Lupins, Soya meal, Fish meal, Mixed vegetable oils, Canola oil, Calcium carbonate, Dicalcium phosphate, Magnesium oxide, and a Vitamin and trace Mineral premix

b) Vitamin Composition of the standard mouse diet

Calculated Total Vitamins	
Vitamin A (Retinol)	19500 IU/Kg
Vitamin D (Cholecalciferol)	2000 IU/Kg
Vitamin E (α Tocopherol acetate)	110 mg/Kg
Vitamin K (Menadione)	20 mg/Kg
Vitamin C (Ascorbic acid)	No data
Vitamin B1 (Thiamine)	80 mg/Kg
Vitamin B2 (Riboflavin)	30 mg/Kg
Niacin (Nicotinic acid)	145 mg/Kg
Vitamin B6 (Pyridoxine)	28 mg/Kg
Pantothenic Acid	60 mg/Kg
Biotin	410 μg/Kg
Folic Acid	5 mg/Kg
Inositol	No data
Vitamin B12 (Cyancobalamin)	152 μg/Kg
Choline	1600 mg/Kg

Values given are prior to autoclaving

c) Mineral Composition of the standard mouse diet

Calculated Total Minerals	
Calcium	0.80%
Phosphorous	0.70%
Magnesium	0.20%
Sodium	0.20%
Potassium	0.70%
Sulphur	0.20%
Iron	590 mg/Kg
Copper	24 mg/Kg
Iodine	0.5 mg/Kg
Manganese	115 mg/Kg
Cobalt	1.0 mg/Kg
Zinc	90 mg/Kg
Molybdenum	1.2 mg/Kg
Selenium	0.4 mg/Kg
Cadmium	0.04 mg/Kg

Values given are prior to autoclaving