Vitamin D's Role in Neuronal Development, Brain Morphology, Behaviour, and Inflammatory Modulation – From a Developmental Perspective

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Abstract

Vitamin D (VD) is pivotal in biological functions, including prenatal neurodevelopment, yet its specific roles in neuronal differentiation and offspring behaviour is not fully understood. This thesis investigates the role of VD in neurodevelopment, particularly its influence on neuronal proliferation, differentiation, and the neurobehavioral development of offspring in relation to maternal VD status.

Firstly, we refined an *in-vitro* SH-SY5Y neuroblastoma cell culture model to mimic neurogenesis, then examined VD's effect on these cells' proliferation and differentiation in a dose- and time-specific manner. Proliferation was assessed using DNA and BrdU incorporation assays, while differentiation was measured by immunocytochemistry for β3-Tubulin and gene expression analysis using rt-qPCR of MAPT, MAP2, NRXN1, and NeuroD1. Concurrently, *In-vivo* experiments in mice investigated maternal VD deficiency's (VDD) effect on offspring's body and brain morphology and behaviour, including anxiety and memory assessments, evaluated through the elevated plus maze and Y maze tests. Lastly, a scoping review of existing literature compiled and evaluated the current evidence regarding the relationship between maternal VD levels, inflammation, and offspring neurodevelopment, focusing on studies that linked maternal VD status with neurobehavioral outcomes in offspring.

Our *in-vitro* investigations in SH-SY5Y cells revealed that VD reduces proliferation (at a calcitriol concentration of 10^{-7} M) while having variable dose and time-dependent effects on differentiation. In our in-vivo experiment in mice, we observed that maternal VDD resulted in unfavourable neurodevelopmental outcomes in offspring, including larger brain sizes in 3-week-old offspring and reduced memory and increased anxiety. Our scoping review also reflected these behavioural impairments, which examined the connection between maternal VDD, inflammatory processes, and neurodevelopmental progress in offspring. The review found that optimal maternal VD levels are consistently associated with decreased levels of inflammatory markers such as IL-10 and TNF-α and correspond with enhanced socialisation and cognitive performance in mice and human offspring.

This investigation underscores maternal VD's significance in supporting optimal prenatal brain development and shaping positive behavioural outcomes in offspring. These findings prompt further research to refine VD intake recommendations for pregnant individuals, to prevent any adverse neurological and behavioural effects on their offspring associated with VDD.

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Declaration

I hereby confirm that the work presented in this thesis was conducted by myself as a registered student at the University of Nottingham, under the BBSRC doctoral training programme. The only exception to my sole authorship is the in vivo study (Chapter 5), which was completed with the assistance of Kathryn Alliband, Preeti Jethwa, John Brameld, and Matthew Elmes.

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Abbreviations

1,25(OH)2D	1,25-dihydroxycholecalciferol/Calcitriol
ADHD	Attention-deficit/hyperactivity disorder
Akt	Protein Kinase B
ASD	Autism Spectrum Disorder
BBB	Blood-Brain Barrier
BDNF	Brain-Derived Neurotrophic Factor
BMP	Bone Morphogenetic Protein
BrdU	5'-bromo-2'-deoxyuridine
CNS	Central Nervous System
CYPs	Cytochrome P450 mixed-function oxidases
CVD	Cardiovascular Disease
CypA	Cyclophilin A
cAMP	Cyclic Adenosine Monophosphate
DAPI	4',6-diamidino-2-phenylindole
DCs	Dendritic Cells
DMEM	Dulbecco's Modified Eagle's Medium
DOHaD	Developmental Origins of Health and Disease
EAE	Experimental Autoimmune Encephalomyelitis

ECACC	European Collection of Authenticated Cell Cultures
ER	Endoplasmic Reticulum
FBS	Foetal Bovine Serum
FGF23	Fibroblast Growth Factor 23
GAP43	Growth Associated Protein 43
GDNF	Glial cell line-derived Neurotrophic Factor
GDM	Gestational Diabetes Mellitus
GFP	Green Fluorescent Protein
GP	Glutathione Peroxidase
GR	Glutathione Reductase
GSH	Glutathione
GW	Gestational Week
HIE	Hypoxic-Ischemic Encephalopathy
HVDRR	Hereditary Vitamin D-resistant Rickets
IL	Interleukin
IKKβ	IκB kinase β
LPS	Lipopolysaccharide
MDA	Malondialdehyde
MAG	Myelin Associated Glycoprotein

MAP2	Microtubule-Associated Protein 2
MAPT	Microtubule-Associated Protein Tau
MIA	Maternal Immune Activation
MS	Multiple Sclerosis
MOG	Myelin-Oligodendrocyte Glycoprotein
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NF-κB	Nuclear Factor kappa-light-chain-enhancer of activated B cells
NIC	Intermediatory Progenitor Cell
NPC	Neural Progenitor Cell
NRXN1	Neurexin 1
Nrf2	Nuclear factor (erythroid-derived 2)-like 2
NRF2	Nuclear Factor Erythroid 2-Related Factor 2
NT-3	Neurotrophin-3
NTDs	Neural Tube Defects
NGF	Neuronal Growth Factor
NeuroD1	Neurogenic Differentiation 1
NK	Natural Killer cells
NSC	Neural Stem Cells

OS	Oxidative Stress
PAMPs	Pathogen-Associated Molecular Patterns
PND	Postnatal Days
PBS	Phosphate-Buffered Saline
PD	Parkinson's disease
PTH	Parathyroid Hormone
PTHrP	Parathyroid hormone-related protein
PKC	Protein Kinase C
PLA2	Phospholipase A2
POD	Peroxidase
RDA	Recommended Daily Allowance
RFP	Red Fluorescent Protein
ROS	Reactive Oxygen Species
RA	Retinoic Acid
RXR	Retinoid X Receptor
SAS	Supravalvular aortic stenosis syndrome
SH-SY5Y	SH-SY5Y neuroblastoma cells
Shh	Sonic Hedgehog
SOD	Superoxide Dismutase

SOX2	SRY-Box Transcription Factor 2
TGF-β	Transforming Growth Factor beta
Th	T-Helper
TH	Tyrosine Hydroxylase
TLR4/MyD88	Toll-Like Receptor 4/Myeloid differentiation primary response 88
TNF-α	Tumour Necrosis Factor-alpha
ToRCH	Toxoplasmosis, Rubella, Cytomegalovirus, Herpes Simplex Virus
TRPV6	Transient Receptor Potential Vanilloid 6
Tregs	Regulatory T Cells
TXN	Thioredoxin
UVB	Ultraviolet B
VD	Vitamin D
VDDR-I	Vitamin D Dependent Rickets type 1
VDRE	Vitamin D Response Element
VDR	Vitamin D Receptor
VDD	Vitamin D Deficiency
hPGH	Human placental growth hormone
hPL	Human placental lactogen

hs-CRP	High Sensitivity C-Reactive Protein

1 Introduction

1.1 Burden of Mental Illnesses

According to the Global Burden of Disease Study 2019, around 12.3% of the global population is suffering from mental health disorders (1). Poor mental health in a population leads to a cascade of adverse effects, beginning with the individual and rippling out to the broader community and economy (1). At the individual level, mental health disorders severely diminish the quality of life, leading to reduced life satisfaction and personal suffering (2). The presence of mental health conditions is often correlated with an increased incidence of physical comorbidities, which can shorten lifespan and exacerbate health complications (2, 3).

On a community scale, the impacts of widespread mental health issues are profound, contributing to increased social isolation, strained family relationships, and elevated instances of substance abuse, homelessness, and criminal activity (2, 4-7). Economically, the repercussions are significant (8). Poor mental health can diminish workforce productivity through absenteeism and presenteeism, leading to a downturn in economic output (8). The financial strain extends to public health systems, which encounter heightened demands for long-term mental health care and services, placing substantial fiscal pressure on healthcare infrastructures (8).

Lately, there's been a growing focus on enhancing well-being as a strategy to prevent mental health disorders (9). Studies indicate that initiatives aimed

at mental health promotion and preventive actions are economically viable, reducing the prevalence and impact of mental health issues both for individuals and society as a whole (8, 9). Preventive psychiatry, despite being a relatively new field, has gained prominence due to advancements in understanding psychiatric disorders and the availability of neuroimaging and electrophysiological techniques (10, 11). Prevention in psychiatry is categorised into three stages: primary, secondary, and tertiary (12). Primary prevention operates before the disease occurs, focusing on health promotion and specific protection against disease development. Secondary and tertiary prevention, however, aim at early diagnosis, treatment, and reducing disease impact and rehabilitation after the onset of the illness (12).

A key focus within primary prevention is neurodevelopment, where interventions aim to reduce risk factors and bolster protective factors that influence the onset of mental illnesses (13). These interventions are designed to modify factors associated with the disease's development, applying general and specific strategies across the lifespan, including pre-birth phases (13-15). The recent meta-review by Firth et al., (16) compiled evidence from meta-analysis' and other meta-reviews that investigated lifestyle factors and their impact on mental health. The review presents strong evidence that physical activity and quality sleep serve as protective factors against various mental health issues, such as depression, anxiety, and schizophrenia, highlighting their importance in primary prevention (16). Conversely, the impact of diet on mental health is more nuanced. However, it clearly indicates that Mediterranean diets correlate with reduced depression rates, whereas high

sugar consumption is linked to increased mental health disorders, underscoring the complexity and significance of dietary influences on mental well-being (16). Brain development, influenced by genetics and environmental factors such as nutrition, can be altered by environmental effects on gene expression through epigenetic mechanisms without changing the gene sequence (15). These changes can have long-term or even heritable effects on biological functions (15).

1.2 Vitamin D

Vitamin D (VD), encompassing forms D2 (ergocalciferol) and D3 (cholecalciferol), plays an essential role in human health, notably in bone strength and calcium regulation (17). We now know VD's influence extends beyond skeletal health, impacting cellular functions and the immune system, with deficiencies linked to increased risks of mental health issues, immune disorders and certain cancers (18-20). Given these wide-ranging effects, recommendations for VD supplementation are widespread, particularly in regions with limited sunlight, yet global deficiencies persist.

Preventing deficiency might be the most beneficial even earlier than we thought – in the womb. Emerging evidence indicates that a lack of essential micronutrients, including VD, during pregnancy could detrimentally impact foetal brain development (21). There's a possibility that the foetal environment, influenced by the mother's VD levels, could be the earliest stage

for promoting healthy brain development. Exploring and discovering the evidence supporting or challenging the effectiveness of VD supplementation for brain health could open new pathways for public health strategies, making VD supplementation a critical study area for lifelong brain health.

1.3 Vitamin D Metabolism

Humans and other animals have adapted to their environments in various ways to meet their VD requirements. Originally, skin pigmentation evolved to shield against excessive sunlight and lower the risk of skin cancer (22). However, as humans migrated away from the equator, their skin pigmentation decreased, likely aiding in maintaining sufficient VD levels essential for calcium balance and healthy bones (23). Similarly, most mammals, as well as amphibians, reptiles, and birds, depend on sunlight for VD synthesis (23). Sunlight, specifically ultraviolet radiation within the 280-320nM range (UVB), transforms 7-dehydrocholesterol (7-DHC) in the epidermis into pre-vitamin D3 (23). When exposed to UVB radiation, previtamin D3 in the skin changes through nonenzymatic isomerisation, influenced by temperature and duration, to form vitamin D3 (cholecalciferol, calciol) (23, 24). Unlike 7-DHC, which is a 5,7-diene, vitamin D3 is a 5,7,19-triene, characterised by three conjugated double bonds, a typical feature of VD molecules, as seen in *Figure 1.3.1* (23, 24).

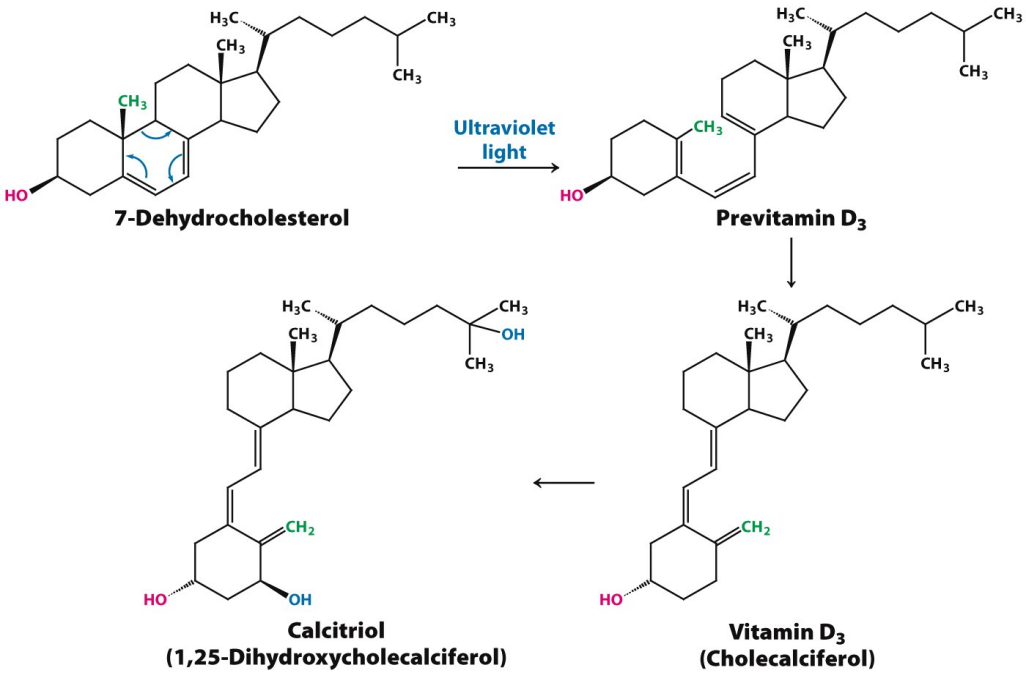


Figure 1.3.1. Chemical Structures of Vitamin D Metabolites

Adapted from Biochemistry, Seventh Edition (25).

Studies show that around 50% of previtamin D3 in the skin can convert to vitamin D3 in about 2.5 hours (24). This conversion leads to a swift increase in vitamin D3 levels in the blood following UVB exposure. The peak levels of circulating vitamin D3 are reached within 12–24 hours after exposure (24). Additionally, keratinocytes, the primary cells in the epidermis, have an autonomous vitamin D3 pathway because they contain enzymes which metabolise VD into its active form, 1,25 dihydroxyvitamin D (1,25$(OH)_2$D) (23).

VD metabolism is facilitated by cytochrome P450 mixed-function oxidases (CYPs), a set of enzymes that catalyse VD to its active forms (26). These enzymes are strategically located within different compartments of the cell:

CYP2R1 is found in the endoplasmic reticulum (ER), while CYP27B1, CYP27A1, and CYP24A1 are housed in the mitochondria (27). Once the skin produces Vitamin D3, most of the Vitamin D3 undergoes its first transformation in the liver, where the enzyme CYP2R1, and to a lesser extent CYP27A1, catalyses the 25-hydroxylation to form 25-hydroxyvitamin D (25(OH)D), otherwise known as calcidiol (27). The next crucial hydroxylation occurs primarily in the kidneys, where CYP27B1, the mitochondrial 1α-hydroxylase, converts 25(OH)D to the hormonal and active form 1,25-dihydroxyvitamin D (1,25(OH)2D), commonly known as calcitriol. This hormonal form is biologically active and crucial for calcium homeostasis and bone health (28). Finally, the catabolism of 25(OH)D and 1,25(OH)2D is carried out by CYP24A1, another mitochondrial enzyme, ensuring a negative feedback loop to maintain VD homeostasis (27, 29).

Dietary sources of VD include animal-based foods like fish, egg yolk, and liver, which contain cholecalciferol (Vitamin D3). Certain wild mushrooms provide ergocalciferol (Vitamin D2), derived from ergosterol (30). However, there are differences in the metabolism between Vitamin D3 (VD3) and Vitamin D2 (VD2), whereby VD3 has a longer half-life than VD2 due to its more effective binding to binding proteins (31). Despite the multiple sources of VD from food, the actual dietary intake of VD is often inadequate to compensate for the lack of sunlight (32). While some foods are fortified with VD, particularly with Vitamin D2 in North America, there is a shift towards using Vitamin D3 due to its superior absorption and efficacy (31, 33). Common fortified foods include

milk, cereals, margarine, and orange juice (30). Assessment of VD intake from diet is complex as many food databases lack comprehensive VD content information, and people do not consistently consume high-VD foods daily (30).

Vitamin D Binding Protein (DBP) is the principal carrier for VD in the circulatory system. Synthesised by the liver, DBP binds to VD metabolites, including 25(OH)D and 1,25$(OH)_2$D, stabilising these molecules and prolonging their circulatory half-life (34). This binding is crucial for regulating VD's bioavailability, as it dictates the distribution of VD to various bodily tissues (35). Most cells absorb VD in an unbound state, yet in specific cells, such as in the kidney and potentially in the parathyroid gland and placenta, the entry of VD is aided by DBP through the megalin-cubilin complex (27, 36, 37). Megalin aids in absorbing DBP in the kidneys, facilitating the transformation of 25(OH)D3 into its active form, 1,25$(OH)_2$D3 (27, 36, 37). This discovery challenges the free hormone hypothesis, which suggests that a hormone must be unbound to enter the cell (37). Additionally, cubilin, another receptor, is involved in DBP uptake, with its dysfunction leading to decreased VD levels in humans and dogs (38). Beyond transport, DBP contributes to immune system functions (39). It has actin-scavenging capabilities, which protect tissues from damage by binding to actin from disrupted cells, and it plays a role in macrophage activation, essential for pathogen clearance (39). Genetic variability can influence individual DBP concentrations, affecting overall serum VD levels. Despite this variability, the homeostatic mechanisms typically maintain

constant levels of bioavailable VD (34). During pregnancy, DBP is essential for the transference of VD to the placenta and developing foetus, a process critical for developmental outcomes (40). DBP levels notably increase during pregnancy, impacting the concentration of free VD, which is thought to indicate VD status more accurately in pregnant individuals than the total 25(OH)D levels (41, 42). This protein is gaining attention for its potential as a biomarker for pregnancy outcomes, with associations found with several adverse conditions, including gestational diabetes, pre-eclampsia, preterm labour and immunoregulation (40, 43-45).

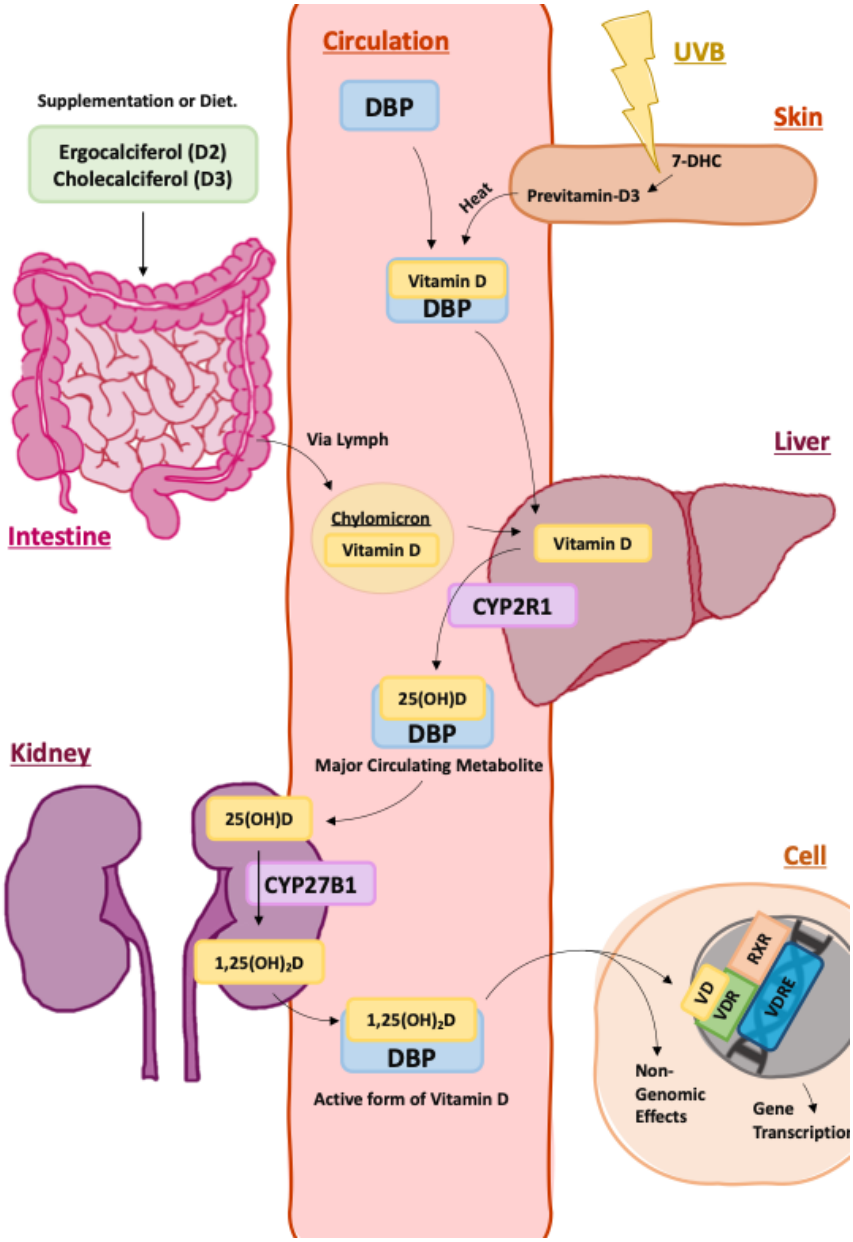


Figure 1.3.2. Summary of the Vitamin D Synthesis Pathway

Adapted from the visual summary of the pathway by *Hurst et al., (46)*. The synthesis of VD is initiated by ultraviolet-B (UVB) radiation converting 7-dehydrocholesterol in the skin to pre-vitamin D3, subsequently transformed to vitamin D3. Dietary sources provide VD as vitamin D2 and D3, absorbed alongside dietary fats into the bloodstream. VD circulates primarily bound to vitamin D-binding protein (DBP), with a smaller amount carried by albumin. In the liver, VD is metabolized to 25-hydroxyvitamin D (25(OH)D) by the enzyme CYP2R1, and in the kidneys, it is converted to its active form, 1,25-dihydroxyvitamin D (1,25(OH)2D), primarily through the action of CYP27B1 enzyme. This activation also occurs in other tissues, reflecting VD's widespread influence. VD functions akin to a hormone, modulating various cellular processes through both genomic and non-genomic pathways.

1.3.1 Genomic and non-Genomic Mechanisms of Action

VD is able to influence gene expression (genomic actions), and when VD is bound to the VDR, this complex can also induce activation of enzymes (non-genomic actions); a summary of how it does so can be seen in *Figure 1.3.3.*

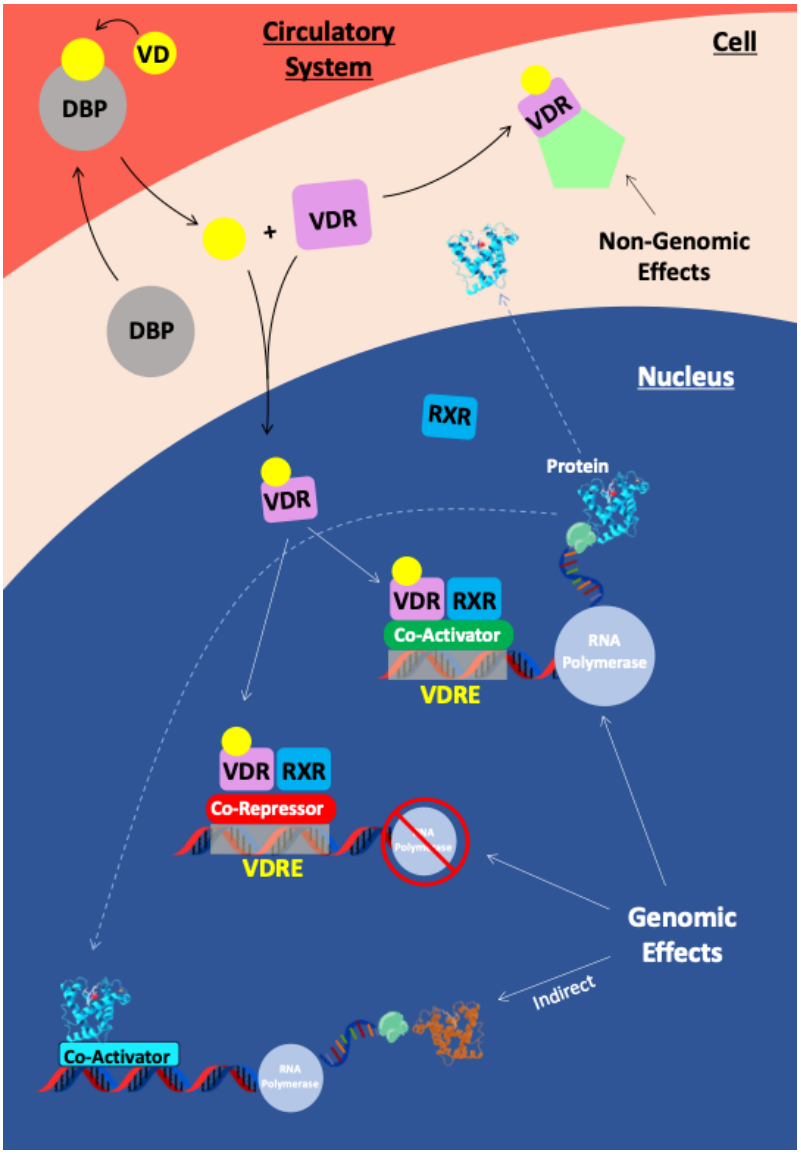


Figure 1.3.3. Genomic and Non-Genomic Mechanisms

This figure illustrates the genomic and non-genomic mechanisms of Vitamin D (VD) action within cells. In the genomic pathway, VD interacts with the Vitamin D Receptor (VDR) to form a heterodimer with the retinoid X receptor (RXR). This complex then binds to the vitamin D response element (VDRE) in gene promoter regions, regulating the transcription, with a few hundred being consistently VDR-engaged. VD can also indirectly influence gene transcription by initiating transcription of transcription factors, which themselves initiate gene transcription. Non-genomic pathways involve VD's interaction with membrane-bound VDRs or nuclear VDRs,

affecting cellular signalling through Protein Kinase C (PKC), IκB kinase β (IKKβ), and other molecules, impacting cell growth, immune function, and homeostasis.

1.3.2 Genomic Mechanisms

The cellular signalling mechanism initiated by VD starts when VD enters the cell and interacts with the Vitamin D Receptor (VDR). The VDR is a transcription factor that typically functions as part of a dimeric complex, which enhances its DNA-binding capacity (47). Its primary partnering protein for forming a heterodimer is the retinoid X receptor (RXR). Upon VD binding, the VDR-RXR heterodimer relocates to the cell nucleus and associates with the vitamin D response element (VDRE) located in the promoter region. The VDRE is essential for modulating gene expression by upregulating or downregulating gene transcription (47). A diagrammatic view can be seen in *Figure 1.3.3.*

In the human genome, over 20,000 genomic sites are identified where VDR can potentially bind (48). Only a few hundred sites are consistently occupied by VDR, while most are transiently engaged following the ligand activation (48, 49). This VD genomic signalling mechanism is uniform across all primary target genes and occurs in all tissues and cell types that express the VDR (48, 49). For example, in enterocytes, VDR induces the upregulation of the TRPV6 gene, which increases calcium absorption in the small intestine and in osteoclasts, VD stimulates osteocalcin release, which improves insulin responsiveness (50). Given the wide expression of the VDR gene in human tissues, the physiological impact of VD is extensive. In addition to the primary target genes of VD, many secondary target genes are indirectly regulated by VD (49). These genes do not

necessitate direct VDR binding to their regulatory regions. Instead, they are controlled by transcription factors that are products of primary VD target genes (49). For example in a study on monocytes, VD, and subsequent VDR activation, was found to increase NFE2, ELF4, POU4F2 and BCL6 transcription factors, which then themselves upregulate hundreds of genes related to innate immunity (51)

1.3.3 Non-Genomic Mechanisms

The non-genomic effects of VD can vary depending on the type of cell, the presence of specific membrane receptors, and the cellular context (52). This variability contributes to various VD actions in different tissues and organs. VD can interact with a specific VDR receptor on the cell membrane, which differs from the nuclear VDR involved in genomic actions (52). This type of receptor is known as the Membrane-Associated Rapid Response Steroid (MARRS) binding protein, which, when bound with calcitriol, likely activates the caveolin-1 signalling pathway, although the evidence is still emerging (52). However, evidence indicates that the nuclear VDR may also participate in non-genomic activities. VDR and VD complex induce the activation of protein kinase C (PKC) and IκB kinase β (IKKβ), increased intracellular calcium levels, and the modulation of other signalling molecules like cAMP, Akt, and phospholipase A2 (PLA2) (52, 53). These responses are crucial for various physiological processes, including those related to cell growth, immune function, and regulation of cellular homeostasis (52, 53).

1.4 Physiological Functions of Vitamin D

VD is integral to maintaining calcium equilibrium and skeletal integrity (54). It enhances intestinal calcium uptake by upregulating calcium channels like TRPV6 and transport proteins such as calbindin, enabling efficient calcium absorption and entry into the bloodstream (55). VD also facilitates renal calcium reabsorption, a key step in conserving bodily calcium and ensuring its availability for bone mineralisation (54, 55). Deficiency in VD impairs calcium absorption, leading to diminished serum calcium levels, which can precipitate secondary hyperparathyroidism (56, 57). This condition increases parathyroid hormone (PTH) levels, which, in turn, stimulate osteoclastic activity, potentially resulting in bone demineralisation diseases like osteomalacia in adults and rickets in children (56, 57).

In muscle physiology, VD plays a role beyond bone health. VDRs located in muscle tissue regulate muscle function, with VD deficiency linked to muscle weakness and a decrease in type II muscle fibres, characteristics often seen in sarcopenia associated with ageing (58). While VD's role in muscle strength and fall prevention in the elderly is recognised, its efficacy in muscle health across various age groups remains to be conclusively determined (58, 59).

In relation to cardiovascular health, VDD correlates with hypertension, a significant contributor to cardiovascular disease (CVD) (60). VDR expression in cardiovascular tissues implies VD's involvement in modulating vascular tone and providing cardioprotective effects, such as antifibrotic and antihypertrophic actions (60). Nevertheless, the direct causative effects of VD

on human cardiovascular health through supplementation are not yet firmly established in clinical trials (61).

In oncology, epidemiological studies have found associations between lower VD levels and increased cancer risk (62, 63). VD's role extends to modulating cell cycle regulation, apoptosis, and immune response—mechanisms central to cancer suppression (62, 63). The mechanisms are thought to be primarily through controlling innate immune defences and promoting an anti-inflammatory macrophage phenotype, contributing to an environment less conducive to cancer development (63). VD also impacts gene expression related to cell survival, division, and differentiation, potentially inhibiting cancer cell proliferation. Despite these insights, the comprehensive impact of VD on cancer prevention and therapy is still under extensive research scrutiny (63).

1.4.1 Vitamin D and the Immune System

Immune cells including microglia, macrophages and monocytes, T-helper 2 cells, natural killer cells (NK), and dendritic cells (DCs) widely express the VDR and can convert the inactive form of VD (25(OH)D) to its active form (1,25(OH)3), as they express the CYP27B1 (1α-hydroxylase) enzyme (64-66). This suggests that the immune system is not only influenced by the classical endocrine actions of VD, but there may also be autocrine and/or paracrine actions regulating the production of inflammatory cytokines such as interleukins (IL)-6, IL-2 and Tumour Necrosis Factor alpha (TNF-α) (67, 68).

Microglia, the primary immune cells of the central nervous system (CNS), are responsible for detecting and responding to pathogens and injuries within the brain and spinal cord (69). When VD binds to VDR, microglial cells show a decreased production of proinflammatory cytokines, including IL-1β, TNF-α, and IL-6 (70). In contrast, VD increases the secretion of anti-inflammatory mediators such as IL-4 and IFN-γ from NK cells (71). The regulatory role of VD in inflammation appears to be closely associated with the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) pathway. Specifically, VD treatment suppresses NF-κB activation in human adipocytes in response to macrophage stimulation (72). This effect is mediated by an increased NF-κB polypeptide gene enhancer in B-cells inhibitor, alpha (IκBα) expression and a reduction in NF-κB p65 phosphorylation. Indeed, a study by Jiang et al., (73) found that rats with traumatic brain injuries exhibited significantly reduced brain inflammation and oxidative stress when treated with VD. Furthermore, the study provided evidence indicating that VD treatment suppresses the pro-inflammatory TLR4/MyD88/NF-κB signalling pathway. Importantly, VD's immune system modulation does not appear to increase susceptibility to infections under optimal VD levels. Instead, a healthy VD status has been shown to increase the synthesis of antimicrobial peptides in macrophages, immune cells that engulf and destroy pathogens and have been linked to a reduced risk of infection in both pregnancy and children (74). This suggests that optimal VD status may simultaneously enhance innate immunity and promote an anti-inflammatory environment, which is crucial during pregnancy. The role of VD on inflammation is explored at greater length in Chapter 4.

1.5 Vitamin D Deficiency and Toxicity

For routine measurement of VD status, calcidiol (25(OH)D) is usually measured for a number of reasons. The circulating levels of the active form of VD, calcitriol (1,25(OH)D3) are 1000 times lower than calcidiol, therefore assays would need to be extremely sensitive. Furthermore, when a deficiency in VD occurs, levels of PTH increase. This elevation in PTH, in turn, boosts the production of calcitriol, despite the ongoing deficiency in VD (75). The SACN Vitamin D and Health report is the primary reference for establishing VD recommendations in the UK (76). This report provides specific guidelines regarding the levels of serum VD and their associated diagnostic criteria, which can be seen in *Table 1.5.1.*

Table 1.5.1 Serum Vitamin D Reference Values for Diagnosis of Toxicity and Deficiency

Serum 25(OH)D	Diagnosis
< 25 nmol/L.	Deficient
25 – 50 nmol/L	Inadequate for some people
> 50 nmol/L	Adequate
> **375 nmol/L**	Toxic

Without regular consumption of fortified foods or dietary supplements, achieving the recommended intake levels of VD through diet can be challenging for many individuals (22, 25). Hence, most developed countries in the northern hemisphere suggest supplementation in the winter months. However, there is much discrepancy in the Recommended Daily Average (RDA) between

countries, as seen in Table 1.1, adapted from the review by EFSA Panel on Dietetic Products et al., (77). The effectiveness of these recommendations will be discussed in section 1.5.2.

Table 1.5.2. Vitamin D RDA's Across Countries.

Government Body	Age (years)	RDA (IU/day)
SACN (2016)	≥ 18	400
	≥ 75	800
D-A-CH (2015)	≥ 19	800
NCM (2014)	18–74	400
	≥ 75	800
NL (2012)	18–69	400
	≥ 70	800
IOM (2011)	19–70	600
	≥ 71	800
WHO/FAO (2004)	19–50	200
	51–65	400
	≥ 66	600-800
Afssa (2001)	20–74	200
	≥ 75	400-600
SCF (1993)	18–64	0–400
DH (1991)	19–64	0
	≥ 65	400

SACN: Scientific Advisory Committee on Nutrition (UK). D-A-CH: Germany (D for Deutschland), Austria (A), and Switzerland (CH). NCM: Nordic Council of Ministers. NL: The Netherlands. IOM: Institute of Medicine (US). WHO/FAO: World Health Organization. Afssa: Agence française de sécurité sanitaire des aliments (France). SCF: Scientific Committee on Food (European Union). DH: Department of Health (UK)

1.5.1 Toxicity

Toxicity resulting from excessive VD intake was notably documented in the 1940s and 1950s, primarily in patients receiving mega-doses for tuberculosis treatment and through the fortification of milk with high VD levels, which unfortunately led to fatalities or permanent harm in infants and children (78). The predominant consequence of VD toxicity is hypercalcemia, which is when the concentration of calcium reaches above safe limits (79). This is due to VD's role in increasing calcium absorption from the intestine (79). Hypercalcemia results in many adverse symptoms (78, 79). Manifestations in the nervous system can include symptoms of fatigue, disorientation, mood swings, despondency, visual and auditory delusions, and in severe situations, a state of near-unconsciousness or coma (78, 79). Digestive issues include loss of appetite, feelings of sickness, regurgitation, and bowel irregularity (78, 79). There are also cardiac symptoms like abnormal heart rhythms; and urinary symptoms including frequent urination and kidney stone-related abdominal pain, in more severe cases, vascular calcification occurs which is thought to have life-long repercussions (78-80). It is imperative to underscore that VD toxicity, in modern medical practice, is a rare occurrence, is generally manageable, and seldom results in significant complications in the modern-day (81).

Despite indications that hypercalcemia due to VD supplementation is now rare, there exists an unwarranted apprehension regarding VD supplementation during pregnancy, as articulated by Hollis et al., (82). This unease largely stems from outdated studies conducted in 1969, which associated VD

supplementation with supravalvular aortic stenosis syndrome (81). It is crucial to emphasise that concerns about toxicity should not overshadow current scientific research and enhanced comprehension of VD, as individuals would need to be ingesting 750 times the amount of the recommended dose for months before toxicity would be evident (83).

1.5.2 Deficiency

Symptoms of VDD are commonly musculoskeletal. Children primarily suffer from rickets, whereas adults experience osteomalacia (84). A prevalent symptom among all ages is muscle pain (85). However, burgeoning evidence suggests the symptomatology of VDD may extend beyond these manifestations. At present, VD levels are not routinely evaluated by healthcare providers in patients manifesting symptoms of depression, preeclampsia, or inflammation (86-88). Yet, there is evidence that VD supplementation may substantially benefit the treatment of these conditions, which will be discussed further in subsequent chapters (86-88).

A recent meta-analysis by Cui et al., (89) found that as much as 15.7 % of the global population has less than 30 nmol/l serum hydroxyvitamin D, which is deemed as deficient, but also that 47.9% of the global population was estimated to be below 50 nmol/l, which the scientific advisory committee on nutrition (SACN) deems as inadequate for some people (76, 89-91). This widespread deficiency could be attributed to modern lifestyle trends, such as spending more time indoors, increased use of sunscreen, and clothing choices during sunny weather. Despite the 2011 findings, recent studies, including one

in 2016 by Cashman et al., (92), indicate that VD deficiency remains a significant concern. In Europe alone, 40% of the population is deficient in VD, a staggering figure considering the widespread recommendations for VD supplementation and food fortification in these regions (92, 93). Determining the optimum levels of VD is complicated due to multiple VD metabolites in the blood (94). With a significant portion of the population facing VDD, recent research indicates that the 1997 guidelines for VD intake, particularly in low sunlight conditions, are insufficient (23). The consensus is that at least 1000 IU of vitamin D3 daily is essential to maintain blood levels of 25(OH)D at or above 30 ng/ml (23).

The prevalence of VD deficiency is notably higher among certain demographic groups, including people with lower socioeconomic status, those with obesity and dark skin, and individuals who cover their bodies for religious reasons (95-99). This demographic skew introduces a significant health inequity, particularly for those already at a heightened risk of poor health outcomes. While the link between socioeconomic status and VD deficiency is not thoroughly explored, research indicates that factors such as exposure to pollution, affordability of VD-rich foods like fish, and a higher likelihood of obesity contribute to this deficiency (95). Interestingly, despite the strong correlation between education and diet quality, education does not appear to be a reliable predictor of VD status (100). This may be because VD is synthesised in the skin in response to UV light exposure, not solely obtained through diet. Instead, factors like deprivation and personal income show a closer association with VDD, influenced by variables such as limited access to sunny holidays, fish consumption, supplements, or safe outdoor spaces (98).

Skin pigmentation also plays a critical role in VD synthesis. Melanin, the pigment that determines skin colour, is protective against UV rays but simultaneously reduces the skin's ability to produce VD (101). This is particularly problematic for dark-skinned individuals living in regions far from the equator, especially during the winter months, where UV exposure is minimal (102-104). There's an observed "VD paradox" in this context; despite similar deficiency levels, people with darker skin have a lower incidence of osteomalacia than their white counterparts. However, they are at an increased risk for conditions like adverse birth outcomes and schizophrenia, which are associated with VD deficiency (105-107).

Covering the skin for religious or cultural reasons also impacts VD status. Research focusing on immigrants, who often have more pigmented skin and wear skin-covering clothing, shows a higher risk of VDD (108). This risk is compounded by low VD supplementation and fish consumption (95, 108). Intriguingly, the longer an immigrant resides in a new country, the higher their risk of deficiency becomes, underscoring the inadequacy of current interventions in addressing the needs of these populations (109).

The succeeding sections of this thesis will explore further and in more detail the role of VD on brain development, drawing upon research to explore how VDD affects neurobiological processes and, consequently, behavioural outcomes. Such findings underscore the crucial role of adequate VD levels for optimal brain development, particularly during the prenatal stage (110-112).

1.6 Vitamin D Regulation during Pregnancy

During the early stages of pregnancy, serum VD concentrations (specifically the active form calcitriol) are elevated to between double and triple the amount before pregnancy (113, 114). This elevation is not solely for foetal skeletal development and calcium homeostasis, as the upregulated levels of 1,25(OH)2D early in pregnancy precede substantial calcium demand, indicating additional roles for this hormone (115). In foetal bone and mineral metabolism, PTH is suppressed, and Parathyroid hormone-related protein (PTHrP) increases, but the involvement of VD is less straightforward (57). Unlike the usual decrease in calcitriol levels following reduced PTH levels, pregnancy maintains high calcitriol levels, indicating a unique regulatory mechanism (114). PTHrP is thought to be central to this regulation, suggesting its potential to elevate calcitriol during pregnancy (114). Although this is only a hypothesis, investigating PTHrP's role requires further investigation in both human and animal models (114). Hormonal changes during pregnancy, particularly increased oestradiol levels, further stimulate PTHrP production and activation of Cyp27b1, consequently increasing calcitriol levels (116). Indeed, findings from studies in postmenopausal women found that oestrogen replacement therapy increases serum calcitriol levels (117). Interestingly, progesterone, which is also elevated during pregnancy, does not impact calcitriol concentrations (118). However, it has been shown to increase VDR expression, specifically in T-cells, making them highly sensitive to calcitriol (118). Given that calcitriol is known to have an anti-inflammatory effect on T-cells, progesterone may thus play a crucial role in regulating the foetal-maternal immune interface.

Therefore, during pregnancy, the increase in hormones oestrogen and PTHrP increases calcitriol concentrations while the simultaneous increase in progesterone acts to make key cells, such as T-cells, more receptive to the calcitriol, thereby enhancing VD signalling during pregnancy. Despite higher production and transfer of calcitriol's precursor, 25-hydroxyvitamin D (25(OH)D), to the foetus, 25(OH)D levels remain stable throughout pregnancy, including in twin pregnancies (114). This highlights a regulated balance in maternal-foetal VD metabolism, though the mechanisms behind renal Cyp27b1 stimulation during pregnancy are yet to be fully understood (114).

The placenta ensures a consistent supply of essential minerals to the foetus, (119). The placenta's expression of VD pathway components, including megalin /cubilin and various CYP enzymes, indicates its potential contribution to maternal circulating VD metabolites (120). Maternal 25(OH)D crosses the placenta to become a significant source for the foetus, achieving cord blood levels that closely match maternal levels, thus highlighting the importance of maternal VD status (57). The placenta, an active endocrine organ in this context, synthesises 1,25(OH)2D through CYP27B1 enzyme activity. The emerging evidence suggests that this localised synthesis is to maintain a maternal-foetal interface by modulating inflammation, facilitating the differentiation of endometrial cells, limiting oxidative stress, and promoting angiogenesis (121-125).

During healthy human pregnancy, adipose tissue expansion serves as an adaptive mechanism to support foetal development and prepare for lactation (126). This involves significant alterations in maternal body composition and metabolism, enabling a consistent nutrient supply to the foetus (126, 127). The pregnancy begins with an anabolic phase, where maternal fat stores expand, reaching a peak by the end of the second trimester through adipocyte hyperplasia and enhanced lipogenesis (128). By mid-gestation, metabolism shifts to a catabolic state, marked by increased fatty acid turnover and lipolysis in adipose tissue (128). This upsurge in lipolysis is propelled by maternal insulin resistance induced by pregnancy hormones including Human placental lactogen (hPL) and human placental growth hormone (hPGH), leading to diminished insulin-mediated lipolysis inhibition in late pregnancy (129). Fat mass is a known VD reservoir, and most likely the reason for the association between obesity and VDD (130). Therefore, the pregnancy-related fat accumulation could serve as a VD source for lactation when that fat deposition is utilised after pregnancy (131). This is supported by the observation that fat loss can elevate serum VD levels, even without dietary supplementation, indicating that the stored calcitriol during pregnancy could be mobilised during lactation (132).

1.6.1 Vitamin D's Immunomodulatory Influences During Pregnancy

During the first trimester, the placenta contains many immune cells that maintain inflammatory balance in a healthy pregnancy (68, 133). However, as pregnancy progresses, there is a shift from a pro-inflammatory state to an anti-

inflammatory state in which Th2 and T-regulator (Treg) cells are recruited to prevent rejection via the release of anti-inflammatory cytokines IL-4 and IL-10 (68, 133). Interestingly, VD has been shown to regulate and aid this shift; studies have shown that VD suppresses the differentiation of dendritic cells and reduces the capacity to secrete pro-inflammatory cytokines such as IL-12 and IL-8 while promoting the expression of anti-inflammatory cytokines such as IL-10 (134-136). In support, Barrera et al., (68) showed VD treatment dose-dependently downregulated pro-inflammatory cytokines in placental cells *in vitro* (134-136), suggesting that VDD during pregnancy could increase the exposure of the developing foetus to pro-inflammatory cytokines potentially predisposing it to disease. Increased exposure to pro-inflammatory cytokines *in utero* has been associated with an increased incidence of schizophrenia (137). At the same time, injections of IL-1β or TNFα to neonatal rats lead to neuronal cell death, delayed myelination, and white matter damage (138-140). Furthermore, there is evidence that VD supplementation reduces the risk of pre-eclampsia via reduced blood pressure, immunomodulation and regulation of genes related to angiogenesis, placental implantation, and placental invasion, (141-143). However, the timing of supplementation may be critical, since MirazFakhani et al (144) reported that a key factor in preventing pre-eclampsia was VD status in early pregnancy. The role of VD on inflammation during pregnancy is explored further in Chapter 4.

1.6.2 The Role of Vitamin D on Oxidative Stress During Pregnancy

Oxidative stress and inflammation are closely interlinked, each capable of inducing the other (145). As mentioned previously, VD exhibits anti-inflammatory properties by downregulating the production of pro-inflammatory cytokines and inhibiting the activation of inflammatory cells, which are also sources of ROS (134-136). Thus, by mitigating inflammation, VD indirectly reduces ROS production (134-136, 145). Upon activation, immune cells such as macrophages and T lymphocytes enhance glycolysis to rapidly generate ATP, supporting their immediate energy needs (146). This is mediated by the upregulation of enzymes such as hexokinase, phosphofructokinase (PFK), and lactate dehydrogenase (LDH), alongside glucose transporters like GLUT1 (146, 147). These cellular processes, particularly mitochondrial energy production, generate highly reactive molecules known as reactive oxygen species (ROS) (148). These include superoxide ($O2^{-}\bullet$), hydroxyl radical ($OH^{-}\bullet$), and hydrogen peroxide (H_2O_2) (148). At controlled levels, ROS act as signalling molecules, influencing pathways like NF-κB and MAPKs, which are involved in gene expression, cellular proliferation, and apoptosis (149, 150). Excessive ROS, however, can induce cell death in neighbouring tissues by activating pathways involving proteins such as p53, Bax, and caspases (151, 152). However, excessive ROS production can be detrimental, causing damage to tissues through various mechanisms such as lipid peroxidation, protein and amino acid modifications, and DNA oxidation (151, 153, 154). The uterus's low-oxygen environment and the embryo's low antioxidant capacity make the embryo highly sensitive to oxidant molecules (155). The placenta is a major source of

ROS (156). During normal pregnancy, placental oxidative stress (increase in circulating levels ROS) is normal and present throughout the pregnancy, as it is required to activate redox-sensitive transcription factors and protein kinases (157). However, the overproduction of ROS by immune cells is central to the progression of the inflammatory process leading to injury in the developing brain via the creation of reactive by-products from lipid peroxidation, such as Malondialdehyde (MDA), consequently leading to membrane damage and cell death of proliferating and differentiating neurones (158, 159). An increase in antioxidants can counterbalance this increase in ROS (148). The glutathione system, including glutathione peroxidase (GP), glutathione reductase (GR), and glutathione itself (GSH), plays a central role in detoxifying ROS (160). GP converts H2O2 into water, using GSH as a substrate, which is then regenerated from its oxidised form (GSSG) by GR, using Nicotinamide adenine dinucleotide phosphate (NADPH) from the Pentose Phosphate Pathway (160). Superoxide Dismutases (SODs) are critical enzymes that convert superoxide radicals into H2O2 (160). The three types of SODs, located in different cellular compartments (SOD1 in the cytoplasm, SOD2 in the mitochondria, and SOD3 extracellularly), ensure comprehensive protection against ROS, mostly through catalysing the dismutation of the superoxide radical into H2O2 and O2 (160). This reaction is critical because H2O2 is a less reactive species that can be further degraded by other antioxidant enzymes (like GP), thus effectively reducing the oxidative burden (160). Still, if oxidative stress exceeds these antioxidant defence in the placenta, this could damage distal tissues, including the developing foetal brain (148, 157).

In vitro and in vivo work has revealed that VD can reduce oxidative stress in various cells through multiple mechanisms. In a study with 1α(OH)ase−/− null mice, supplementation with calcitriol was found to increase nuclear factor (erythroid-derived 2)-like 2 (Nrf2) expression; in addition, the authors confirmed that the Nrf2 gene's promoter with VDR binding sites sufficiently promotes transcription, supporting the idea that VDR mediates Nrf2 activation (161). Nrf2 is a transcription factor in the nucleus that regulates and synchronises the activation of various genes, including components of the GSH and thioredoxin (TXN) antioxidant system and enzymes involved in (NADPH) regeneration (162). The VDRE was also identified upstream of the klotho gene in mice and human renal cells, and it was shown that calcitriol upregulates the transcription of klotho, which is known for its anti-ageing and antioxidant defence (163). Transcription of the klotho gene has also been shown to lead to an increase in antioxidant enzymes like SOD, and further (in addition to VD's effects) upregulates the transcription of Nrf2, which further increases anti-oxidants (163, 164).

1.7 Brain Development

1.7.1 Brain Development in Humans

Neural progenitor cells (NPCs) are the precursor cells within the central nervous system (CNS) responsible for generating the vast majority, if not all, of the glial and neuronal cell types found within the CNS (165). Neurons are cells specialised for signal transmission via electrochemical impulses, serving as the nervous system's primary functional units (166). Glial cells, in contrast, support, protect, and maintain neuronal function, lacking impulse conduction capabilities (167). The cellular mechanisms involved in brain development have specific stages summarised in *Figure* 1.7.1. Neural tube formation, commencing around the third gestational week (GW), is governed by the orchestrated expression of genes such as Sonic hedgehog (Shh) and Bone Morphogenetic Proteins (BMPs) (168, 169). This phase is characterised by the symmetrical division of NPCs, where each NPC divides into two NPCs (170). Neurogenesis in humans starts around the GW 5, driven by the proliferation of NPCs in the ventricular zone (171). As development progresses, a shift towards asymmetrical division occurs, where one daughter cell remains a progenitor, and the other is converted into an intermediatory progenitor cell (NIC) (171). These NICs eventually differentiate into neurons, guided by transcription factors like Neurogenin and Mash1 (172). Interestingly, the expression of Neurogenin not only promotes the formation of neurons but also plays a role in suppressing the development of glial cells (172). This dual function of Neurogenin ensures that the brain first focuses on generating neurons (neurogenesis) before shifting to the production of glial cells (gliogenesis)

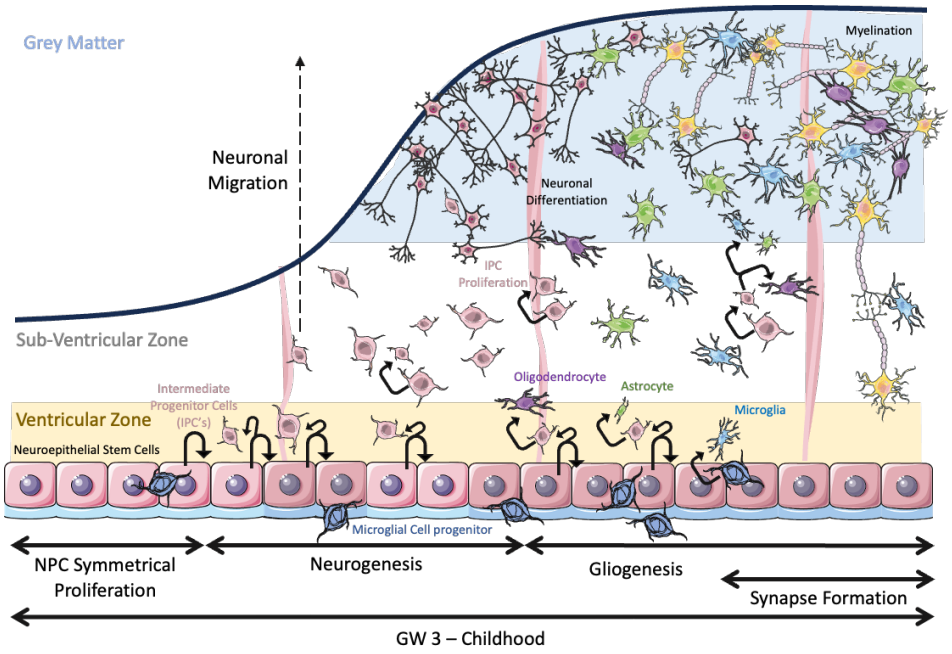
(172). Neuronal production reaches its peak intensity up until approximately GW 20, after which the emphasis gradually shifts towards the differentiation and maturation of the NIC's (171). Concurrently, neurones migrate inwards from the Ventricular Zone to the Subventricular zone, eventually differentiating within the grey matter of the brain (173).

Gliogenesis starts in late embryo development, around the first trimester ,but intensifies postnatally, following the peak of neurogenesis (174). The differentiation of NPC's into glial cells is facilitated by the JAK-STAT signalling pathway for astrocyte formation and Olig1 and Olig2 transcription factors for oligodendrocytes (175, 176). Microglia, distinct from other glial cells, are thought to originate from the mesoderm, initially produced in the yolk sac of the developing embryo, akin to immune cells such as macrophages and monocytes (177, 178). These microglial progenitors migrate into the brain, and by around gestational week (GW) 5, the microglial precursors are observed near the early forms of meninges, concurrent with the commencement of cerebrospinal fluid synthesis (177). From GW 5 to 12, there is a significant increase in the number of microglial precursors within the brain, indicating their potential role in the vascular development of the foetal brain (178). Between GW 12 and 16, the microglia mature and are found in large numbers within the grey and white matter of the brain (177). During this stage, microglia begin to execute their functions, including debris clearance, synaptic pruning, controlling apoptosis, and promoting angiogenesis (179-182). This process illustrates the brain's evolving needs, transitioning from creating neurons to producing the supportive cells essential for neural function and integrity.

Synaptogenesis is the process by which neurons form synapses, the specialised junctions through which they communicate with other neurons or effector cells. Synapse formation begins as early as gestational week 9 but mainly occurs postnatally, involving both genetic and environmental factors (183). Cell-adhesion molecules like neuroligins and neurexins mediate this process, significantly increasing synaptic formation during early childhood (184, 185). This stage reflects a shift towards functional maturation, emphasising the role of neuronal activity and neurotrophic factors like Brain Derived Neutrophic Factors (BDNF) in shaping synaptic connections and brain plasticity (186). Following birth, the formation of synapses lays the groundwork for intricate neural networks, which are then refined through synapse pruning (187). This critical process eliminates superfluous connections, enhancing brain efficiency (188). Informed by neural activity and experiences, pruning complements initial synapse formation, ensuring the stabilisation and optimisation of functional synaptic circuits essential for learning and memory (188).

Myelination, beginning around the 30th GW and continuing into adulthood, underscores a late developmental focus on optimising neural transmission (189, 190). This is facilitated by differentiated oligodendrocyte precursor cells, and myelination is characterised by myelin protein expression, including Myelin-Associated Glycoprotein (MAG) and Myelin-Oligodendrocyte Glycoprotein (MOG) (189, 190).

It is evident, that there are many intricate stages to brain development, and a very fine balance between proliferation and differentiation exists.



STAGE	START GW	END GW	NOTES
NEURAL TUBE FORMATION	GW 3	GW 4	Symmetrical division of NPCs
NEUROGENESIS	GW 5	GW 20	Shift towards asymmetrical division
NPC TO NIC DIVISION	GW 5	GW 20	NICs differentiate into neurons
PEAK NEURONAL PRODUCTION	GW 5	GW 20	Emphasis shifts towards differentiation
MIGRATION TO GREY MATTER	GW 5	Ongoing	From Ventricular Zone to Subventricular zone
GLIOGENESIS (GENERAL)	Late 1st Trimester	Postnatal	JAK-STAT signalling for astrocytes; Olig1 & Olig2 for oligodendrocytes
MICROGLIA PRECURSORS MIGRATION	GW 5	GW 12	Migrate from yolk sac; role in vascular development
MICROGLIA MATURATION	GW 12	GW 16	Functions: debris clearance, synaptic pruning, apoptosis, angiogenesis
SYNAPTOGENESIS	GW 9	Postnatal	Mainly postnatal; involves genetic and environmental factors
SYNAPSE PRUNING	Birth	Ongoing	Eliminates excess connections; complements synapse formation
MYELINATION	GW 30	Adulthood	Characterised by MAG and MOG expression

Figure 1.7.1. Neurogenesis and Gliogenesis in Humans, with Key Time Points Summary.

Adapted from the diagram by Figarella-Branger et al., (191)

1.7.2 Brain Development in Mice

In mice, the process is slightly different from humans, mostly due to peak brain growth occurring after birth (192). The rodent brain development stage between postnatal days (PND) 2 to 7 aligns with the human third trimester, and PND 7 to 10 is comparable to that of a newborn human, as seen in *Figure 1.7.2* (192). While the fundamental processes of brain development are alike in rodents and humans, the timelines for specific regions such as cerebellum, hippocampus, and olfactory bulb development differ significantly between the species (192). For example, at birth, the human hippocampus has 80% of the granule cell population, compared to only 20% in rodents, which continues to develop throughout the rodent's life (192).

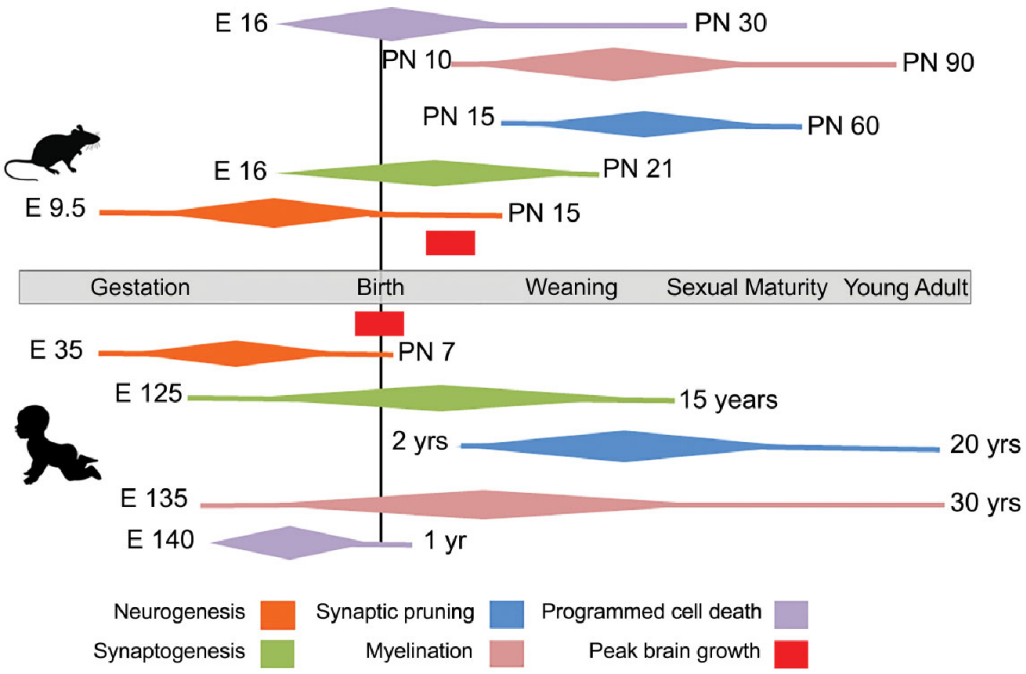


Figure 1.7.2. Comparison between Human and Rodent CNS development

Taken from Zeiss (192), Comparative Milestones in Rodent and Human Postnatal Central Nervous System Development.

1.7.3 How does the Environment Affect Brain Development?

Environmental factors like viral infections, maternal stress, substance use, and hypoxia during pregnancy influence neurogenesis and gliogenesis (193). For instance, Zika virus infection has been shown to disrupt NSC division in brain organoids, causing developmental defects (193). The transmission of infectious diseases from mother to foetus is usually rare but is primarily through transplacental or intrauterine routes and is mostly limited to ToRCH pathogens (toxoplasmosis, rubella, cytomegalovirus, herpes simplex virus, and others) (193). These diseases differ from other infections during pregnancy, where the damage is often a result of maternal inflammatory response rather than the virus itself (193).

Maternal malnutrition, including both undernutrition and overnutrition (obesity), can negatively affect brain development. Specific nutrients, such as folate and omega-3 fatty acids, are particularly important during critical periods of brain growth (194-196). Folate deficiency can lead to altered brain structure and function, while maternal obesity can increase oxidative stress and inflammation, impairing neurogenesis and gliogenesis (194). The timing, duration, and severity of malnutrition all play a role in the extent of its impact (197). Early malnutrition can have long-lasting effects, while the degree of recovery following malnutrition depends on the quality and intensity of intervention (197). *Table 1.7.1* summarises the more studied nutrients in relation to maternal deficiency and brain outcomes in offspring, excluding VD, which will be discussed in future sections.

Table 1.7.1. Nutrient Deficiencies and Impact on Brain Development

Nutrient	*Effects of deficiency*	*Ref*
Folate	Smaller total brain volume and cerebral white matter, altered cortical morphology, neurogenesis, and neuronal apoptosis	(194)
Omega-3 fatty acids	Impaired cognitive function, decreased visual acuity, and attention problems	(195, 196)
Iron	Anaemia, which can lead to cognitive delays and impaired motor function	(198)
Zinc	Impaired cognitive function, weakened immune system, and increased risk of infection	(199-201)
Iodine	Intellectual disability, stunted growth, and impaired motor function	(202, 203)

1.7.4 Vitamin D and Brain

VD possesses the unique ability to passively traverse the blood-brain barrier (BBB), and the VDR is present in both neurones and glial cells within human and murine brains, highlighting its potential influence on brain function and development (110-112). The review by Eyles (110) summated evidence relating to developmental VDD in mice and rats, and brain gene expression, accompanied by observable behavioural changes. They found that studies in both foetuses and neonates from VDD mothers displayed increased proliferation in comparison to the controls and a reduction in markers of

neuronal differentiation (Nurr1, TGF-β1 and p57kip2a), suggesting that maternal VDD influenced the sensitive redox balance between proliferation and differentiation in these offspring (110). This imbalance in cellular processes leads to larger ventricles and an increase in mitotic cells within the brain, and in studies which have measured the behaviour of these offspring showed reduced social behaviour, memory and increased anxiety compared to control offspring (110). Studies which have induced deficiency in mice and rats have also been done during adulthood, however, the effects of VDD are more pronounced when VDD occurs during critical periods of development, specifically during pregnancy (204, 205). *In-vitro* analysis has also revealed similar findings. In the study by Pertile et al., (206), a human neuroblastoma cell line (SH-SY5Y) was transfected to overexpress the VDR. They found that treating those cells with calcitriol led to significant increases in neuron length, improved neurotransmitter release and increased levels of presynaptic neurones, all of which suggest increased differentiation (206).

The correlation between VDD during pregnancy and its impact on the developing human brain is nuanced and not yet fully understood, primarily due to ethical limitations in human research. However, insights are emerging, and in a recent meta-analysis by García-Serna et al., (207) the researchers aimed to aggregate findings across studies. This review indicates that low levels of maternal serum 25-hydroxyvitamin D (25(OH)D)—a marker of VD status—correlate with suboptimal neurodevelopmental outcomes in offspring, including challenges in cognitive development and an elevated risk of attention-deficit/hyperactivity disorder (ADHD) and autism spectrum disorders

(ASD) (207). The potential underlying biological processes affected by VDD in humans are mostly inferred from animal models, suggesting that VD plays a role in regulating the balance between the proliferation and differentiation of neural cells—a critical aspect of brain formation and function (207). A deficit in VD may disrupt this balance, leading to neurodevelopmental issues. Moreover, the review proposes that the anti-inflammatory properties of VD are significant for brain development (207). Inflammation during critical periods of brain development has been associated with neurodevelopmental disorders, mirroring the consequences of VDD. Therefore, VDs potential to mitigate inflammatory responses might be a mechanism that supports proper neural development.

In another review the authors found a positive association between low maternal VD levels during pregnancy and a risk of psychiatric disorders during childhood and adulthood such as ASD, ADHD, and schizophrenia in the offspring (208). This evidence suggests that maintaining adequate VD levels in expectant mothers had long term impacts on the behavioural and mental health outcomes (208). It highlights the need for a careful consideration of maternal VD status as a potentially modifiable factor in prenatal care, with the aim of improving neurodevelopmental health in the next generation (208).

It is critical to acknowledge that the majority of human studies examining the association between maternal VD levels and offspring mental health are observational. Consequently, establishing a causal relationship from these studies is not possible. Furthermore, many of these studies have measured

maternal VD levels at a single point in time, which varied across different stages of gestation among the participants. This inconsistency means that any potential stage-specific effects of VD on foetal brain development may not have been adequately captured. Despite these limitations, the available evidence does suggest an association between sufficient maternal VD levels during pregnancy and better mental health outcomes in the offspring. This association may stem from the role of VD in brain development, although the precise mechanisms remain to be fully elucidated.

1.9 Aims of the Thesis

The current evidence highlights VD's pivotal role during pregnancy and its role in neurogenesis. Despite this, the detailed mechanisms of VD's interaction with these neuronal processes and its subsequent impact on behaviour, particularly from prenatal exposure, remain inadequately understood. To address this gap, this thesis aimed to investigate the effects of VD on brain development and behaviour, focusing on the prenatal period and its implications for neuronal differentiation and mental health outcomes.

We hypothesised that VD reduces proliferation and increases markers of differentiation in neurones. To test this, we aimed to optimise (Chapter 2) and use *in-vitro* studies to examine the impact of VD on neuronal differentiation and proliferation using the SH-SY5Y neuroblastoma cell line (Chapter 3). To shed light on the cellular mechanisms underpinning VD's role, we measured DNA, BrdU incorporation and the gene expression of SOX2 to measure proliferation and measure differentiation by quantifying ß3-Tubulin protein and MAPT, MAP2, NeuroD1 and NRXN1 gene expression.

Given the established connection between VD and inflammation and the emerging evidence linking VD to brain development, we aimed to explore the potential interrelation of these concepts. We hypothesised that a healthy VD status in pregnant mothers reduces inflammation and oxidative stress,

promoting optimal brain development in their offspring. To examine this hypothesis, we conducted a scoping review of in-vivo studies that assess brain development in offspring of mothers supplemented with VD or those deficient in VD (VDD), alongside measurements of inflammation or oxidative stress in either the offspring or the mother. We hypothesised that VD supplementation, or a healthy VD status during pregnancy, is protective against inflammation or oxidative stress, leading to healthy brain development and behaviour.

Due to the vast literature suggesting that maternal VDD induces abnormal brain development in the offspring, which in turn causes behavioural changes, we wanted to investigate if this would be true at multiple stages of the offspring's growth. Therefore, we conducted an *in-vivo* study in mice to assess the consequences of maternal VD status on foetal brain development, measuring brain size, ventricle volume, proliferation and differentiation, locomotion, anxiety, and memory (Chapter 5). In addition to these measurements, body weight, food intake, insulin tolerance and fat mass were also measured to evaluate the effects of VDD on other aspects of development, which could be potentially linked to the brain. We hypothesise that maternal VDD leads to brain size, ventricle volume, and body composition alterations in the offspring. We also hypothesise that the offspring of VDD mothers will have altered behaviour, including increased locomotion and anxiety, and reduced memory. In addition, we think the VDD offspring will also have a higher ratio of proliferating cells to differentiated cells, which leads to increased brain volume.

In conclusion, this thesis aims to establish a link between maternal VD status, neurogenesis, and offspring brain development and behaviour. We investigated VD's role in modulating neuronal proliferation and differentiation and its potential to mitigate prenatal inflammation and affect behaviour and body composition. The scope includes *in-vitro* and *in-vivo* studies designed to provide an understanding of the impact of VD at various developmental stages. The ultimate goal is to contribute to the body of knowledge, potentially encouraging targeted interventions for VD deficiency during pregnancy and potentially improving neurodevelopmental outcomes across the lifespan.

2 Optimisation of In Vitro Growth Protocols for SH-SY5Y Neuroblastoma Cells: A Focus on Differentiation Methods

2.1 Introduction

In vitro cell culture models have revolutionised scientific research, offering controlled environments to investigate cellular mechanisms, disease pathways, and therapeutic interventions (209). Among these models, immortalised cell lines, such as the SH-SY5Y neuroblastoma cells, have played fundamental roles in advancing our understanding of cellular biology and neurodevelopment and have undeniably been valuable tools in scientific investigations (209, 210). However, it is evident that cell lines, including SH-SY5Y cells, possess limitations that must be addressed for their effective utilisation (211)

Immortalised cells often diverge significantly from primary cell culture counterparts regarding genetic, phenotypic, and functional characteristics (212). For example, SH-SY5Y cells genetically do not possess the neuroendocrine characteristics which primary neurones do (212, 213). Phenotypically, SH-SY5Y cells assume a neurone-like morphology upon differentiation, but they remain less complex than primary neurones (214). One of the significant drawbacks is their genetic instability, resulting from most immortalised cell stocks being derived from cancer cells, which can lead to aberrant cellular behaviours that do not faithfully recapitulate normal physiological responses (214, 215). Moreover, immortalised cell lines lack the context of the multicellular, three-dimensional environment found in living

organisms, although recently, there have been great advances in 3D cell culture (216). Immortalised cell lines often fail to replicate the intricate cellular interactions, gradients of signalling molecules, and spatial cues that profoundly influence cellular behaviour *in vivo* (217). For example, a systematic review investigating the SH-SY5Y cell lines' applicability to Parkinson's disease (PD) research concluded that although the SH-SY5Y model is a "valuable asset" in PD research, animal models and human studies are required to validate any findings from SH-SY5Y results (214). However, when used in context and when results are reproducible and consistent, immortalised cell lines can be the first step in scientific discovery.

Reproducibility is often cited to be one of the advantages of using immortalised cell lines, however, these cells can display variations not only between experiments but also between different passages (218). This variation can confound results, making it challenging to replicate findings consistently. Prolonged passages can alter cell metabolism and phenotype, potentially skewing experimental outcomes (218). Another concern is the authenticity of the cells being studied. Cross-contamination or misidentification of cell lines can occur, meaning researchers may inadvertently study a different cell type than the intended (219).

To ensure the reliability of our experiments and the suitability of SH-SY5Y cells as a model for studying the effects of VD on neurogenesis, we needed to address these complexities. This involved optimising our methods to confirm

that these cells could differentiate into neuron-like cells, a crucial step for accurately measuring this differentiation process. There are variations in SH-SY5Y maintenance methods between studies. For example, the original culture conditions were described by Biedler et al., (220) to use Eagles Minimum Essential Medium supplemented with Eagles formulation amino acids, whereas European Collection of Authenticated Cell Cultures suggests Hams F12 Nutrient mix; both media are suggested to be supplemented with 15% foetal Bovine Serum (FBS) (221). However, most studies use F12 Dulbecco's-Modified Eagles Medium (DMEM) plus 10% FBS to maintain their media (222). There is also great variation in the way in which differentiation is achieved in these cells, with most experiments using 10 μM Retinoic Acid (RA) to differentiate SH-SY5Y cells and some with the addition of either Neuronal Growth Factor (NGF) or BDNF or after five days of treatment with RA (211, 222, 223). The recent reviews by dos Santos et al., (224) and Xicoy et al., (214) outline the great variability and inconsistency in passage and differentiation protocols concerning SH-SY5Y cells.

2.1.1 SH-SY5Y Sub-Types

SH-SY5Y cells differentiate into S-type (endothelial-like cells) and N-type (neuronal) cells (225). The N-type cell subtype is neurone-like and expresses proteins related to neurones, such as β-iii-tubulin, Microtubule-Associated Protein 2 (MAP2) and Growth Associated Protein 43 (GAP43) (225). The S-type cells do not possess the neuronal type (N-type) characteristics. Instead, they are thought to be like Schwann cells, glial cells, and melanocyte precursors,

expressing vimentin and fibronectin (225, 226). Their presence may dilute any changes in gene expression in the N-type cells, which are the cells of interest for neurogenesis. Indeed, immunofluorescence staining work by Bell et al., (226) and data using flow cytometry by Ferlemann et al., (227) found that there is a difference between the proteins expressed in the N-type and S-type cells, suggesting that there are also differences in gene expression. If the SHSY-5Y cells differentiate into S and N-type, the results would not indicate brain development, as some of the data would be influenced by endothelial-like cells. The reliability of results could be hindered, resulting in a lack of confidence in outcomes.

2.1.2 Neutrophic Factors

Neutrophic factors, or neutrophins, are growth factors which regulate differentiation and proliferation (228). In both developmental and adult stages, NGF and BDNF stimulate neuronal growth, synapse formation and improve brain function (229). Their addition to SH-SY5Y cell culture medium has been shown to increase neuronal projections, by increasing differentiation and reducing proliferation (224). Studies by LoPresti et al., (230), Lewis et al., (231) and Agholme et al., (232) found that the addition of NGF also increased markers of differentiation. Encinas et al., (233), Agholme et al., (232) and Teppola et al., (234), to name a few, all found that BDNF increases markers of differentiation in SH-SY5Y cells.

2.1.3 Neuronal Maturation Markers in SH-SY5Y Cell Differentiation

The study of differentiation and proliferation in SH-SY5Y neuroblastoma cells can be approached through various methodologies, with morphological examination being the most apparent indicator. As SH-SY5Y cells differentiate, they exhibit an increased number and length of neurites, which are the projections which contain synapses and link to other neurones and are indicative of neuronal maturation (233). Molecular markers often parallel this morphological change. Beta-3-Tubulin (ß3-Tubulin) is a neuron-specific protein that serves as a component of the microtubules in the cytoskeleton of neurones and is mostly expressed in neurites (235). An increase in ß3-Tubulin typically indicates neuronal differentiation as it reflects the development of the cellular architecture characteristic of neurones (235).

SOX2 is a transcription factor associated with the maintenance of neural progenitor/stem cell properties and is inversely correlated with neuronal differentiation (236). Typically, as neurones begin to differentiate, SOX2 levels decrease, signifying a transition from a proliferative to a mature state, although there is also evidence that SOX2 is essential for differentiation (237). NeuroD1 is another transcription factor, but it is directly involved in the differentiation process of neurones (238). It activates the expression of genes necessary for the mature neuronal phenotype and is upregulated during neuronal differentiation (239). MAPT (Microtubule-Associated Protein Tau) and MAP2 (Microtubule-Associated Protein 2) are both involved in stabilising microtubules but in different parts of the neurones (240). MAPT is primarily found in axons, while MAP2 is associated with dendrites (240). These proteins'

expression also reflects neuronal maturation, with MAP2 often as a marker for dendritic development (235, 240). Neurexins, including Neurexin1, are presynaptic proteins that are critical for the formation and function of synapses (241). They interact with post-synaptic neuroligins to bridge the synaptic cleft and facilitate synaptic signalling. The expression of Neurexin1 is a marker of synaptogenesis, and its presence can indicate the formation of functional neuronal networks (241, 242).

During the differentiation of SH-SY5Y cells, there is typically a sequential regulation of these genes and proteins. Initially, proliferation markers like SOX2 are high, and as differentiation progresses, these levels decrease while differentiation markers like B-III-Tubulin, NeuroD1, MAPT, MAP2, and Neurexin1 increase.

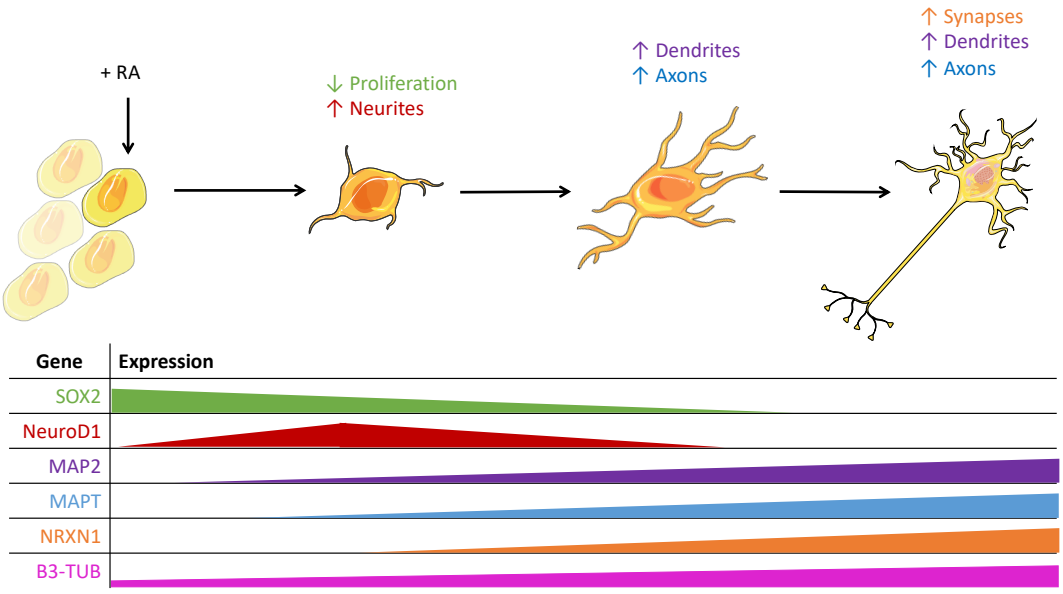


Figure 2.1.1. Differential Expression of Molecular Markers during SH-SY5Y Neuroblastoma Cell Differentiation and Proliferation

This figure illustrates the dynamic changes in key molecular markers as SH-SY5Y cells transition from proliferative to differentiated neurones when exposed to RA. High levels of SOX2, a marker for neural progenitor/stem cells, signify a proliferative phase. As differentiation progresses, SOX2 levels decrease, indicating a shift towards maturation. Concurrently, there's an upsurge in neuronal-specific markers, including NeuroD1, a transcription factor promoting neuronal differentiation; MAPT, associated with axonal microtubules; and MAP2, indicative of dendritic development. The expression of Neurexin1, a presynaptic protein essential for synaptogenesis, marks the formation of functional neuronal networks. These molecular changes mirror the morphological transformation of SH-SY5Y cells, characterised by increased neurite number and length, reflecting neuronal maturation.

2.2 Aims

The primary goal of this chapter is to develop an effective model for differentiation and proliferation. Our objectives to accomplish this include:

1. To Evaluate the Impact of RA on the Proliferation of SH-SY5Y Cells:
    a. Investigate the time-dependent effects of RA on cellular DNA content and proliferation, as indicated by BrdU incorporation and DNA assay, to understand how RA treatment correlates with decreased proliferation.
2. To Analyse the Influence of RA on Gene Expression Related to Neuronal Differentiation:
    a. Examine the changes in gene expression profiles, particularly focusing on neuronal markers, to assess the degree of differentiation in SH-SY5Y cells under RA treatment.
3. To Determine the Optimal Cell Splitting Method for Promoting Neuronal Differentiation:
    a. Compare the effects of different splitting methods on the morphology and gene expression of SH-SY5Y cells, including trypsin and EDTA, to identify the method that best supports differentiation.
4. To Assess the Combined Effects of RA and Neurotrophic Factors (NGF, BDNF) on Differentiation and Proliferation:
    a. Investigate the role of neurotrophic factors, both alone and in combination with RA, on the differentiation and proliferation of SH-SY5Y cells, using gene expression and immunocytochemistry as a measure.

2.3 General Methods

2.3.1 Cell Culture and Cell Treatments

Maintenance Media comprised DMEM Nutrient Mixture F-12 Ham with 10 % Foetal Bovine Serum and 100 units/litre penicillin and 100 mg/litre streptomycin.

The vehicle for both the VD and RA was Ethanol (EtOH), which was kept at a concentration of 0.1% throughout due to preliminary results showing that a 1% EtOH concentration resulted in reduced cell numbers and that a 0.1% concentration made no difference to cell numbers. We initially assessed differentiation after treating SH-SY5Y cells with 10 μM RA in DMEM/Nutrient mixture F-12 Ham with 2.5% FBS (Sigma, UK) and 100 units/litre penicillin and 100 mg/litre streptomycin, as the reduction in serum concentration is required for differentiation. The latter experiments included either NGF or BDNF from day 5. In experiments which contained BDNF, the media was changed to 0% FBS from day 5 onwards. The specific treatments and the preparations are outlined in Table 2.3.1.

2.3.1.1 DNA Assay

SH-SY5Y cells were plated at a density of 15,000 per well in 96-well plates and left for 24 hours to ensure adherence. Treatments of either control (0.1% EtOH) or RA (10 μM) were initiated on day 0, each allocated to eight wells (*n=8*). We monitored DNA quantity on days 0, 3, 5, 7, and 10 post-treatments, changing the media every two to three days. The assay, described previously

by Rago et al., (243), began by removing the media, rinsing the wells with 100 µl PBS once (to remove the dead cells and debris), adding 100 µl of water, and conducting two freeze-thaw cycles for cell lysis. Hoechst 33258 dye (Sigma-Aldrich) was added to the resulting lysates to bind DNA, and fluorescence emission was measured at an excitation of 350 nm and emission of 460 nm using a FLUOstar® Omega plate reader (BMG, Labtech, Aylesbury). The emission directly correlates with DNA content, and the standard curve was used to measure the total DNA quantity in each well (243). We repeated the experiment three times to validate the results and pooled the data for the final analysis.

2.3.1.2 5′-bromo-2′-deoxyuridine (BrdU) Assay

To measure the proliferation rate directly and confirm the findings from the DNA assay, BrdU incorporation was measured using the cell proliferation ELISA kit (Roche, Garden City). A BrdU assay works by incorporating the thymidine analog BrdU into the DNA of proliferating cells, allowing the measurement of cell proliferation. Five days of treatment were chosen because, in our DNA assays, there was a non-significant difference in DNA between the control and RA-treated cells. After five days of treatment with either control (0.1% EtOH) or RA (10 µM), the media was removed, and 10 µl/well BrdU labelling solution was applied. The plates were incubated for 90 minutes at room temperature. The labelling solution was removed, 200 µl of the FixDenat solution was applied for 30 minutes, removed, and 100 µl of the anti-BrdU-Peroxidase (POD) working solution was applied. The plate was incubated for a further 90 minutes at room temperature. The antibody conjugate solution was removed, and the

wells were washed three times with the wash solution. 100 µl of substrate solution was then applied, and after 30 minutes of incubation at room temperature, the absorbance was measured at 370nm.

Table 2.3.1 Preparations of Treatments used for Cell Culture Experiment

Treatment	Vehicle	Preparation	Supplier
10 µM All-Trans Retinoic Acid (RA)	Ethanol, absolute 99.8% (EtOH)	The RA powder was diluted in EtOH to 10 mM. To make a working volume of 10 µM this stock solution was diluted in media 1/1000.	Fischer Scientific, Loughborough.
10^{-13} M Calcitriol (VD)	Ethanol, absolute 99.8% (EtOH)	The VD powder was diluted in EtOH to 10^{-10} M VD. To make a working volume of 10^{-13} M this stock solution was diluted in media 1/1000.	Merck, Frankfurt
10^{-10} M Calcitriol (VD)	Ethanol, absolute 99.8% (EtOH)	The VD powder was diluted in EtOH to 10^{-7} M VD. To make a working volume of 10^{-10} M this stock solution was diluted in media 1/1000.	Merck, Frankfurt
10^{-7} M Calcitriol (VD)	Ethanol, absolute 99.8% (EtOH)	The VD powder was diluted in EtOH to 10^{-3} M VD. To make a working volume of 10^{-7} M this stock solution was diluted in media 1/1000.	Merck, Frankfurt
50 ng/ml NGF	DMEM/F12 Media	NGF powder was diluted in DMEM/F12 to a concentration of 10 µg/ml. To make a working volume of 50ng/ml this stock was diluted in media 5/1000.	Fischer Scientific, Loughborough.
50 ng/ml BDNF	PBS	BDNF powder was diluted in PBS to a concentration 10 µg/ml. To make a working volume of 50ng/ml this stock was diluted in media 5/1000.	Merck, Frankfurt

2.3.1.3 Collagen Coating

Cells were grown on collagen-coated wells in 6-well plates in experiments where gene and protein quantification were the endpoint measurements. This was done as previous work on this cell line which found that a collagen coating resulted in longer neuronal projections than either poly-l-lysine or uncoated 6-well plates (244). This may suggest that the collagen improves the conditions for the neurones to differentiate.

The wells were coated with 100 µg/ml collagen (Sigma, UK) 1. Type I Collagen solution (1 mg/ml) was diluted 10-fold with sterile water to obtain a working concentration of 0.01%. The dishes were coated with 1 ml per well and left overnight at 2-8°C. After overnight incubation, the excess fluid was removed from the coated surface and dried until no moisture remained on the plastic. The plates were then placed under a UV lamp overnight. Before cells were seeded, the plates were rinsed with 3 ml of sterile PBS three times.

2.3.2 Gene Expression

The SH-SY5Y cells were seeded at 250,000 cells per well and grown on collagen-coated 6-well plates (*n=4 per time point*) and stopped at 0, 3, 5, 7, and 10-day intervals in all the experiments, adding a 13-day time point for the last gene expression experiment with BDNF. To investigate differentiation, initially, we chose the key neurogenic markers MAP2, MAPT, and NeuroD1, and a marker of proliferation, SOX2. In the last experiment, the marker of synaptogenesis, NRXN1, was also measured.

2.3.2.1 RNA Extraction

The cells were scraped into 200 µl RNAse free PBS, transferred into Eppendorfs and kept at -80°C before the RNA extraction. The RNA extraction was done using the Roche High Pure Isolation Kit (Roche Life Sciences, UK). The frozen samples were thawed on ice prior to adding 400 µl lysis-binding buffer. The Eppendorfs with the cell lysate were then vortexed for 10 seconds. The cell lysate was added to the upper reservoir of the filter tube and centrifuged at 8000 x g for 15 seconds. Once the flow through was discarded, the DNAse incubation buffer was added and left for 15 minutes at room temperature. The sample was then washed using Buffer I at 8000 g for 15 seconds and the same again using Buffer II. The sample was washed using buffer II and centrifuged for 2 minutes at 13000 x g. 20 µl of elution buffer was added, and the RNA was collected by spinning down with the upper reservoir in a new Eppendorf at 8000 x g for 1 minute. The RNA was then quantified using a Nanodrop (Themo Scientific, UK), and subsequently stored at -80°C.

The RNA quality was checked, and when it was of low quality (i.e. was either of low concentration or had a low 260 nm/280 nm absorbance ratio), the RNA was further purified. This was done by adding 10% (v/v) 3M NaAc (RNase free) and 2.5 volumes of 100% ethanol, then precipitating the mixture overnight at -80°C. The solution was then centrifuged at 13000 rpm for 15 minutes at 2-8°C, and the supernatant was discarded, with care being taken to avoid touching the pellet. The pellet was then washed with 500 µl of 75% ethanol and vortexed before being centrifuged again at 13000 rpm for 5 minutes; the ethanol was

removed by using a pipette tip and occasionally spun down to isolate the pellet further, after which the pellet was resuspended in RNAse-free water.

2.3.2.2 cDNA Synthesis

The RNA was initially diluted to 100 ng/ μl in RNAse-free water so that each sample and repeat had the same concentration of RNA. The cDNA was synthesised using Revert Aid RT Reverse Transcription Kit (Thermo Fisher, Loughborough, UK). This was done by combining 1 μl of random hexamers and 6 μl of water (per sample per well) to make the first master mix, followed by 5 μl of each sample's RNA in each well. This plate was then placed in the ABI Genecamp 9700 PCR - Thermal Cycler (Thermo Fisher, Loughborough, UK) at 65°C for 5 min, after which the plate was immediately placed on ice. The second master mix was made using the Revert Aid RT First Strand cDNA synthesis kit (Loughborough, UK). The mix used was 4 μl Reaction buffer, 2μl Deoxynucleotide Mix, 1 μl RiboLock RNAse Inhibitor, 1 μl Revert Aid RT for the total volume of 8 μl which was applied to each well. The plate was placed back into the Thermal Cycler on 42°C for 1 hour. 80 μl of RNAse free water was added to each sample to make cDNA stock. To make the standard curve, a cDNA pool was made by taking small amounts of each sample and pooling it. 20 μl aliquots of each sample were taken into a separate plate and diluted 1/8 so that the samples were more likely to fall within the mid-range of the standard curve. The plates were kept at -20 °C until further use.

2.3.2.3 RTqPCR cDNA Absolute Quantification

The master mix consisted of 7.5 μl SYBR Master (2x), 0.45 μl of both the Forward and reverse primers and 1.6 μl of molecular RNAse free water per sample to make a total volume of 10 μl. The master mix was added to each well before adding 5 μl of sample cDNA. A standard curve was generated using the cDNA pool from all the samples and taking subsequent 1:2 dilutions until the lowest concentration was 1:128. The standard curve was also blank corrected. We included four distinct biological replicates for each sample in our study to account for biological variability. Additionally, each of these biological replicates was assessed in triplicate, which were analysed in the Roche LightCycler 480 (Welwyn Garden City, UK) instrument.

The Protocol for the PCR was as follows:

Pre incubation – 1 cycle

- Temperature at 95°C for 5 min

Amplification – 45 cycles

1. Temperature at 95°C for 10 seconds
2. Temperature at 60°C for 10 seconds
3. Temperature at 75°C for 15 seconds

Melt Curve – 1 cycle

1. Temperature at 95°C for 5 seconds
2. Temperature at 65°C for 1 minute
3. Temperature at 95°C continued

Cooling – 1 cycle

- Temperature at 40°C for 10 seconds.

2.3.2.4 Oligreen

The cDNA was quantified in the 1/8 diluted samples using the Quant-iT Oligreen ssDNA fluorescent nucleic acid stain (Thermo-Fischer). The stain was diluted 1:200 in Tris/EDTA (TE) buffer, and 5 µl was added per well to 5 µl of cDNA in triplicate per sample. The plate was then incubated in the dark for 5 minutes and placed in the LightCycler480. The LightCycler480 was set on a continuous acquisition program with a 95°C target. Once the run was finished, the data acquired closest to 80°C was used. A standard curve was calculated, and cDNA concentration was subsequently quantified.

2.3.2.5 Primer Design

Cyclophilin A and β-Actin were selected as potential reference genes based on similar experiments. Primers were designed using Primer Express by importing FASTA database sequence data. Primers created by the software used the default criteria:

- Primer size; 18 – 27 base pairs
- Melting temperature; 55 – 62 °C
- Amplicon size 50 – 150 base pairs
- If possible, cross an exon boundary.

The primers were checked by analysing the melt curve, if a single amplicon was produced then the primers were used.

Primer sequences used were as follows:

Target Genes	FORWARD	REVERSE
Cyclophilin A	CCACCGTGTTCTTCGACATTG	TGTCTGCAAACAGCTCAAAGGA
MAP-2	CATGGGTCACAGGGCACCTATTC	GGTGGAGAAGGAGGCAGATTAGCTG
MAPT	AATAAAAAGATTGAAACCCACAAGCT	CGCCCCGTGGTCTGTCT
SOX2	TGCGAGCGCTGCACAT	TCATGAGCGTCTTGGTTTTCC
NeuroD1	TATCCCAACCCACCACCAA	AAGTCCGAGGATTGAGTTGCA
β-Actin	CTGGCACCCAGCACAATG	GCCGATCCACACGGAGTACT
Vimentin	TGGCACGTCTTGACCTTGAA	TGCAGCTCCTGGATTTCCTCT
NRXN1	GACTGCAGCCAAGAAGACAAC	ACTGCTGCTTTGAATGGGGT

2.3.2.6 Data Analysis

The data generated by the Light Cycler using an algorithm based on the PCR reaction was transferred to Microsoft Excel, where the data was corrected for cDNA quantified by Oligreen, and all the statistics were calculated in GraphPad PRISM.

2.3.3 Immunocytochemistry

SH-SY5Y cells were grown on collagen-coated 6-well plates. The treated cells were fixed with 4% paraformaldehyde for an hour and permeabilised with 0.1% Triton X-100 for 10 minutes. Cells were then blocked with 5% bovine serum albumin (BSA) for an hour before incubation overnight at 4°C with mouse anti-β3-Tubulin antibody (1/500) and goat anti-vimentin antibody (1/500) (Both Fischer Scientific, Loughborough). The coverslips were washed with PBS containing 0.05% Tween-20 twice for a total of 10 minutes before adding the

Alexa Fluor 488 anti-goat IgG and Alexa Fluor 594 anti-mouse IgG (Fischer Scientific, Loughborough). Those Alexa Flour conjugates were selected as they fit within the wavelengths measured by the Invitrogen™ EVOS™ FL Imaging System microscope (Loughborough, UK). The microscope contains light cubes able to detect 4',6-diamidino-2-phenylindole (DAPI), red fluorescent protein (RFP: 555 nm) and green fluorescent protein (GFP: 488 nm) wavelengths Images were collected at both x10 and x20 magnifications.

Table 2.3.2. Primary and Secondary Antibody Preparations

	Vimentin	**B-III-Tubulin**
Primary	1/500 Anti-Vimentin produced in goat.	1/500 Anti-B3-Tubulinproduced in rabbit
Secondary	Anti-goat Alexa Fluor 488 (green)	Anti-Rabbit Alexa Fluor 564 (red)

2.3.3.1 Fluorescence Quantification

The BMG FLUOstar Omega (BMG Labtech, Aylesbury) plate reader contains a matrix scan function, which allows the microplate reader to capture multiple measurements within each well (900 data points per well in a 30 x 30 data point matrix).

The excitation and emission settings in the BMG plate reader software were set to those in Table 2.3.3. The gain was adjusted to 30% on each plate.

Table 2.3.3. Excitation and Emission Measurement Values

	Excitation	**Emission**
DAPI	358-365 nm	461-470 nm
RFP	540-590 nm	590-650 nm
GFP	488 nm	509 nm

2.3.3.2 Data Analysis

The BMG software provided the average readings for the plate scan from the 900 data points within each well. For the RFP and GFP data, the values were corrected for the DAPI reading for that well, to correct for cell number. A blank plate with the same primary and secondary antibodies was applied as the treatment was used, and the fluorescence produced was deducted from the data obtained from the cells.

2.3.4 Statistical Methods

Data analysis was carried out using GraphPad PRISM software. The choice of statistical tests was informed by the experimental design and the nature of the data. The following tests were employed:

Two-way Analysis of Variance (ANOVA): Used to analyse statistical DNA assays. Also applied to gene expression data involving MAPT, SOX2, NRXN1, MAP2, and NeuroD1, and the protein expression of β3-Tubulin in GraphPad PRISM 10, to examine the effects of time and treatment, as well as their interaction.

One-way Analysis of Variance (ANOVA): Used to analyse data generated with the BrdU assay.

Tukey's Post Hoc Tests: Used to identify specific differences between groups upon detecting significant interactions in both the BrDU assay and gene/protein expression analyses.

2.4 Method Optimisation Process and Results

The methods and results are presented chronologically, aligning with the experimental progression. This approach was chosen to maintain clarity in tracking the evolution of the methodology over time. Following each experiment, several hypotheses were formulated to explain the lack of differentiation. Subsequently, additional experiments were conducted to test these hypotheses.

2.4.1 Determination of Basal SH-SY5Y Neuroblast Cell Proliferation Rate with Retinoic Acid (RA) Treatment

To accurately measure the proliferation rate of SH-SY5Y cells and the effect of RA on this process, we performed a DNA quantification time course. This assay provided an estimate of cell numbers at predetermined intervals. Establishing the proliferation baseline was critical; a reduction in proliferation upon RA treatment would suggest the onset of differentiation, as differentiation typically involves a transition from a proliferative, pluripotent state to a non-proliferative, specialised state. We also employed the BrdU Assay to measure SH-SY5Y cell proliferation.

2.4.1.1 Methods

As mentioned in section 2.3.1.1 for the DNA assay and 2.3.1.2 for the BrdU assay.

2.4.1.2 Results: RA Treatment Reduces Proliferation in SH-SY5Y cells.

There was a significant time-treatment interaction (*P<0.001*) due to significant decreases in DNA observed in 10 µM RA-treated cells on days 5, 7,

and 10 compared to control (ETH), as seen in *Figure 2.4.1*. There was significantly more BrDU incorporation in the ETH-treated cells than the RA-treated cells ($p<0.0001$) after 5 days of treatment, which suggests that proliferation started to decrease in those groups before day 5. SH-SY5Y cells are often differentiated using RA (10 μM) (231). Therefore, the results seen in *Figure 2.4.1* were expected regarding the proliferation of RA-treated cells being blocked, presumably because they were differentiating more than the control non-RA-treated cells.

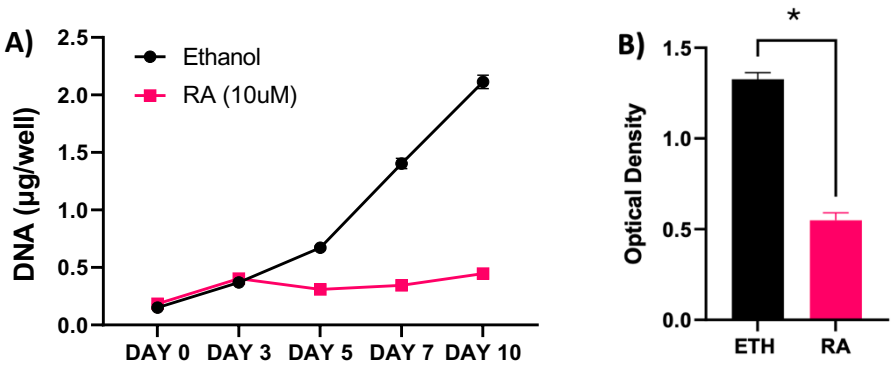


Figure 2.4.1 Effect of Retinoic Acid on SH-SY5Y Cell Growth

A) DNA concentration was measured after SH-SY5Y cells underwent 0, 3, 5, 7 and 10 days of incubation of negative control (ethanol (ETH)), positive control (10 µM retinoic acid, (RA). Values shown as means (±SEM). N=24. There was a significant interaction (*p<0.001*) between time and treatment. There was no significant effect of treatment on days 0, 3 and 5. On days 7 and 10 the RA had significantly less DNA (*p<0.05*) than the control. **B)** The BrdU incorporation was measured in SH-SY5Y cells after 5 days of incubation with the treatment groups. Values were shown as means (±SEM). There was significantly less BrdU incorporation in the RA treated cells * = (*p<0.0001*).

2.4.2 The effect of RA Treatment on Gene Expression in Neuroblastoma SH-SY5Y Cell Line.

To support the DNA and BrdU assays indicating reduced proliferation with RA treatment, we evaluated gene expression in SH-SY5Y cells at corresponding time points. Our goal was to identify gene expression trends over time, confirming that differentiation was occurring.

2.4.2.1 Methods

Cells were placed in 6-well collagen-coated plates, the protocol for which can be seen in section 2.3.1.3, and during the time course, the volume within the wells was always 2 ml. 250,000 cells were placed per well 24 hours before differentiation. SH-SY5Y cells were differentiated by changing the media to 2.5% FBS and adding 10 µM RA, and the control was 0.1% EtOH. The cells were scraped into 200 µl RNAse-free PBS, and RT-qPCR commenced as per the methods in section 2.3.2. Data was analysed by a Two-way ANOVA.

2.4.2.2 Results

2.4.2.2.1 Reference Genes and cDNA

We attempted to identify a suitable reference gene and tried Cyclophilin A (CypA) and Beta-Actin. There were significant changes in the expression of reference genes with a significant effect of time in Cyp-A ($p<0.05$) expression and a significant effect of treatment ($p=0.002$). There was also a significant time x treatment interaction in β-actin expression ($p \leq 0.05$), with post hoc testing also showing significant differences between RA treatment and control on day 3 *(Figure 2.4.2).* The cDNA was quantified to check for variation and error in the original cDNA dilutions, and there were no significant differences in the cDNA

over days, treatments, or interactions. However, the variation between biological replicates decreased after correcting the reference genes for the cDNA values. Therefore, all measured genes were corrected for cDNA, not reference genes.

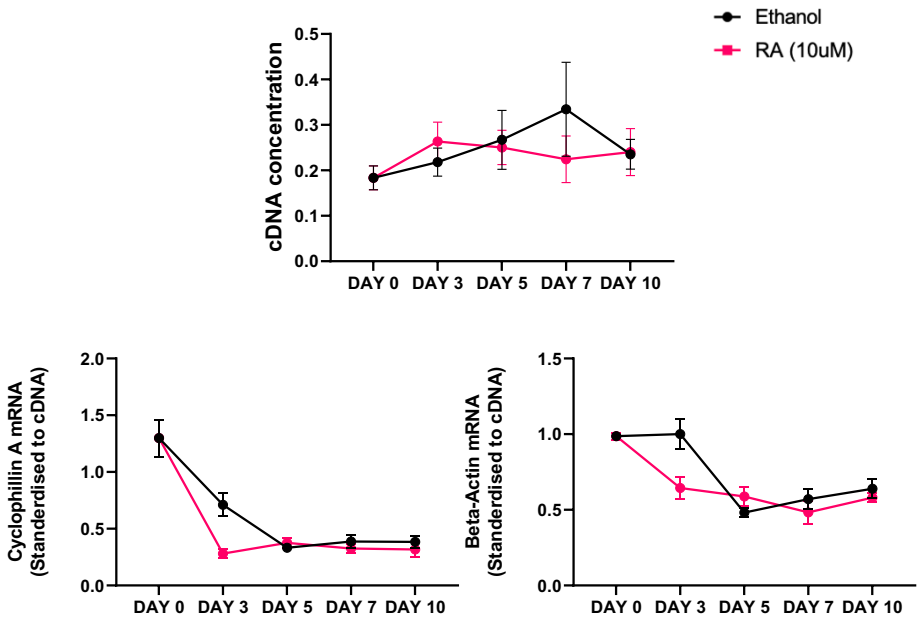


Figure 2.4.2 Reference Gene Expression

Each point has *n=4* biological replicates. The Cyp-A and B-Actin values were corrected for cDNA concentration. Data presented as mean ± SEM. There were no significant differences for the cDNA values. There was a significant effect of time in Cyp-A (*p≤0.05*) expression and a significant effect of treatment (*p=0.002*). There was a significant time x treatment interaction in β-actin expression (*p≤0.05*). Post hoc revealed a significant difference between Ethanol and RA-treated cells on day 3 in cyclophilin A and Beta-Actin expression (*p≤0.05*).

2.4.2.2.2 Genetic Markers for Neuronal Differentiation

RA treatment significantly reduced NeuroD1 and MAPT expression, where there was a significant effect of day and treatment ($p<0.05$). However, there were no significant changes between the control and RA-treated groups concerning SOX2, MAPT and MAP2. The significant changes observed do not indicate increased differentiation in the RA-treated groups, as seen in *Figure 2.4.3.*

It was hypothesised that in our SH-SY5Y cell culture, the gene expression characteristics of neuronal differentiation in N-type were being obscured due to dilution by S-type cells. This dilution effect was thought to occur because SH-SY5Y cells have the potential to differentiate into both N-type and S-type cells.

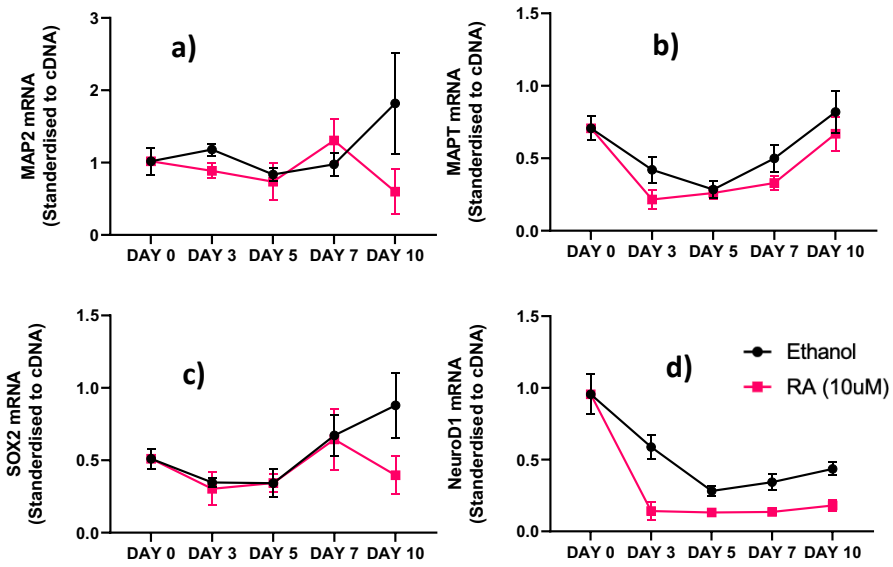


Figure 2.4.3 Gene Expression in SH-SY5Y cells treated with RA.

There were four replicates (*n=4*) per treatment per day data presented as mean ± SEM.

a) Expression of Microtubule Associated Protein 2 (MAP2). There was no significant effect of time or treatment on gene expression.

b) Expression of Microtubule-associated protein tau (MAPT). There was a significant effect of day and treatment but no interaction (*p<0.05*) on MAPT.

c) Expression of Sex determining region Y (SOX2). There was no significant effect of day on gene expression.

d) Expression of Neurogenic differentiation 1 (NeuroD1). There was a significant effect of day and treatment, but no interaction on NeuroD1 expression (*p<0.05*).

2.4.3 Investigation of the Influence of using Trypsin, Tryp-LE and EDTA Splitting Methods, on the subsequent Differentiation of SH-SY5Y cells

Due to the results from the previous experiment, we hypothesised that the presence of the S-type cells following differentiation treatment was affecting the expression of the neuronal-specific genes. We further hypothesised that this may be mitigated by altering the method of cell passage as Bell et al., (226) study showcased that simply changing the passage method allowed the reduction in S-type cells. The researchers successfully isolated N-type cells by gently agitating fewer adherent cells into PBS and moving them to a new flask. In contrast, S-type cells were harvested from those still adhering to the original flask. This method effectively produced a population rich in N-type cells with minimal S-type cells. Consequently, these findings and techniques were adapted to enhance the N-type and minimise the S-type cell populations in our SH-SY5Y cells (226).

In our experiments, we used the traditional method of trypsin-EDTA (0.025%) to dissociate the cells from the flask. However, due to our previous experiment results showing minimal gene expression associated with differentiation, we aimed to employ the method devised by Bell et al., (226) by gently agitating fewer adherent cells into PBS and moving them to a new flask as they successfully increased the N-type cells. Additionally, we investigated the effect of replacing trypsin with 0.5mM EDTA alone and the newer Gibco TrypLE enzyme to potentially preserve the surface proteins (245). This approach could

decrease the S-type cell population by selectively enabling the less firmly attached N-type cells to be passaged (226).

β3-Tubulin and Vimentin protein expression were quantified and visualised via immunocytochemistry, while proliferation and differentiation gene expression levels were evaluated to ascertain whether the passage method affected the gene expression in SH-SY5Y cells.

2.4.3.1 Methods

SH-SY5Y cells were re-suspended after cryopreservation in DMEM F-12, supplemented with 15% FBS. After 16 hours, the cells were passaged by tapping and without using Trypsin and retaining the old media to maintain the floating cells before centrifuging for 5 minutes at 1 rpm and resuspension in maintenance media (see section 2.3). 42 hours later, the process was repeated. Still, the cells were then split into 3 flasks for the 3 different passage methods (a summary can be seen in Figure 2.4.4); each was repeated four times before the cells were transferred onto plates and were differentiated using 10 µM RA for 3, 5, 7 and 10 days. We then measured the gene expression of MAPT and MAP2. A mixed-effect analysis was done to determine the significance of the effects.

2.4.3.1.1 Immunocytochemistry for Protein Quantification

Details on the Immunohistochemistry methods can be seen in Section 2.3.3. Two plates were used to measure β3-Tubulin and Vimentin protein expression in the SH-SY5Y cells after 7 days of differentiation with 10 µM RA. We chose the

7-day mark to test protein expression as evidence in our previous proliferation experiments, which show that the SH-SY5Y cells display a different morphology after 7 days of treatment. Previous immunocytochemical work by Bell et al., (226), who also quantified Vimentin and β3-Tubulin, also observed a clear difference between the S-type and N-type populations after 7 days of treatment.

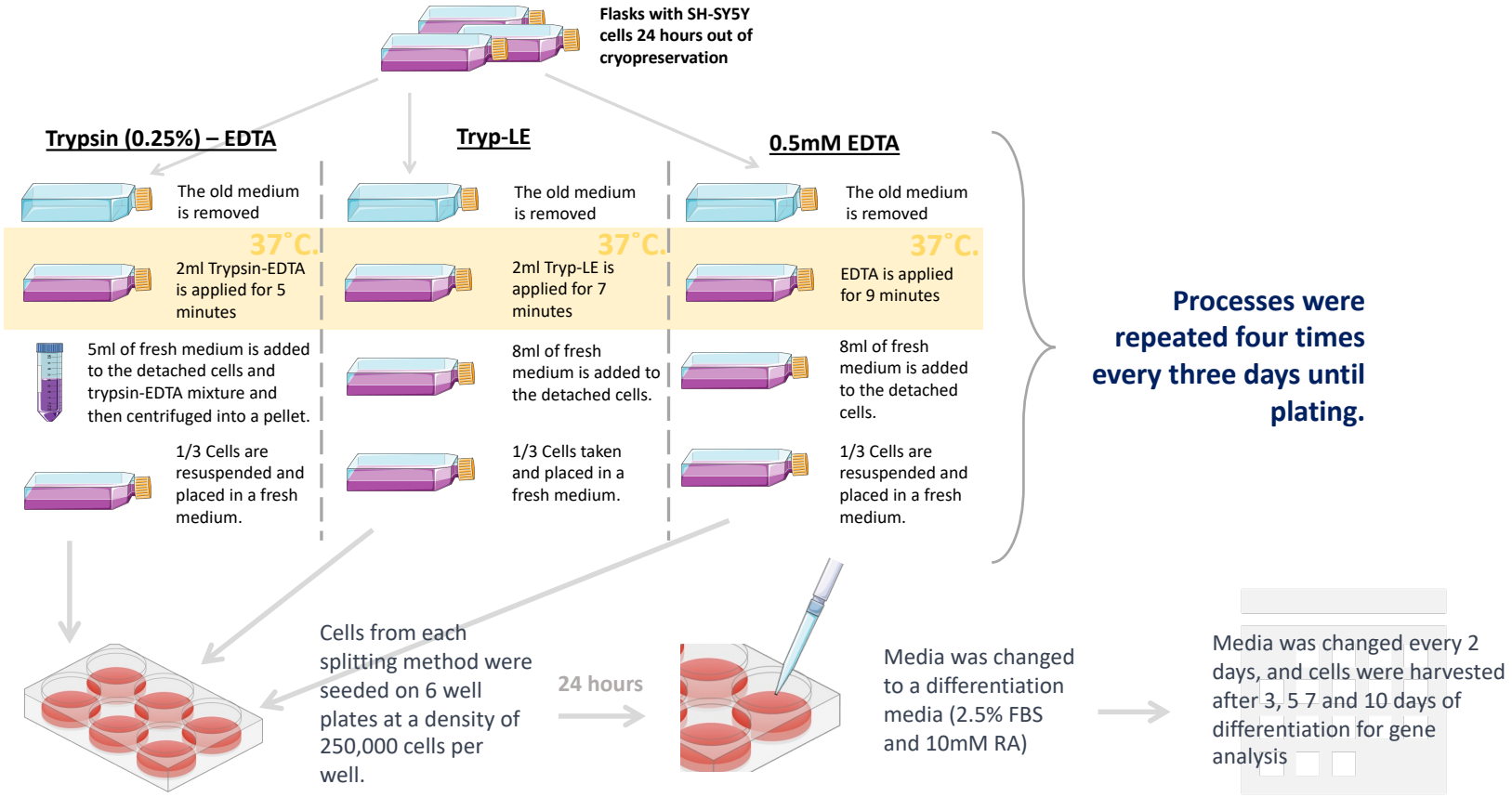


Figure 2.4.4. A Summary of the Passage Methods Utilised

2.4.3.2 Results

The expression of MAP2 did not significantly change with time or treatment. However, the trypsin treatment significantly reduced the expression of MAPT starting from day 3 when compared to the other two passage methods, suggesting that trypsinisation as a method of dissociation was reducing differentiation (Treatment X Time $p<0.001$) (*Figure 2.4.5*). Specifically, the Tukey post hoc revealed that expression of MAPT in Trypsin treated cells was significantly lower than the EDTA-treated cells on days 3, 5, 7 and 10 ($p<0.05$) and Tryp-LE treated cells on days 5, 7 and 10.

The EDTA splitting method enhanced SH-SY5Y cell differentiation, demonstrated by increased β3-Tubulin protein expression ($p<0.05$), decreased Vimentin levels ($p<0.05$), and increased MAPT gene expression from day 3, compared to the Trypsin and Tryp-LE methods. This improvement was visually evident, as EDTA-treated cells exhibited brighter red neuronal projections in greater numbers (refer to *Figure 2.4.6*). However, despite these advancements, S-type cells remained present in all cell cultures, irrespective of the splitting technique employed. Consequently, further refinement of the differentiation protocol is necessary to effectively diminish the S-type cell population and ensure an accurate reflection of SH-SY5Y cell differentiation in the positive control.

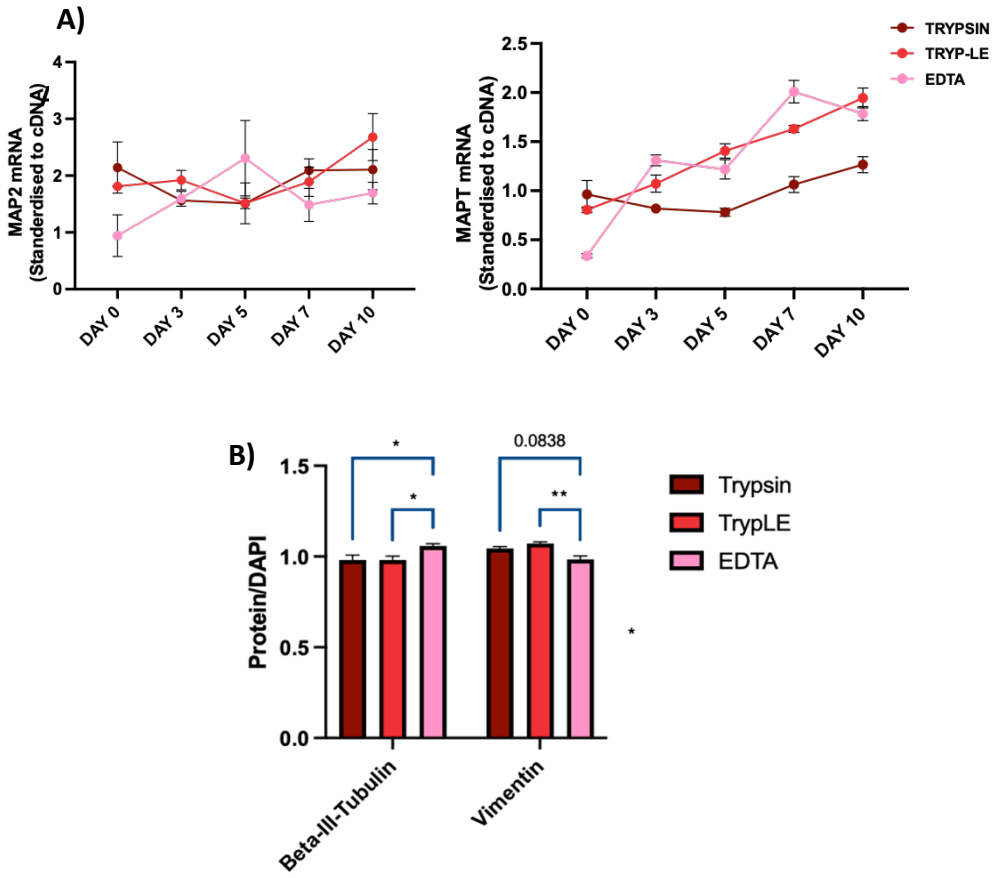


Figure 2.4.5. Time Course of Gene Expression of MAP2 and MAPT, and Vimentin and β3-Tubulin in SH-SY5Y cells

(A). *N=2* for all treatments on Day 0 and *N=4* for all treatments on days 3, 5, 7 and 10. Data is presented as mean ± SEM. Time and treatment had no significant effect on the expression of MAP2 (*p>0.05*). There was a significant treatment-time interaction on the gene expression of MAPT (*p<0.001*). The expression of MAPT in Trypsin treated cells was significantly lower than the EDTA-treated cells on days 3, 5, 7 and 10 (p<0.05) and Tryp-LE treated cells on days 5, 7 and 10. There was no significant difference between Tryp-LE and EDTA in any of the treatment groups. **(B)** N=4 for all treatments. Analysed by a one-way ANOVA. Measurements were taken using a well scan. Each protein was corrected for cell number by using DAPI readings and displayed as Mean ± SEM. * = *p<0.05* and ** = *p<0.001*.

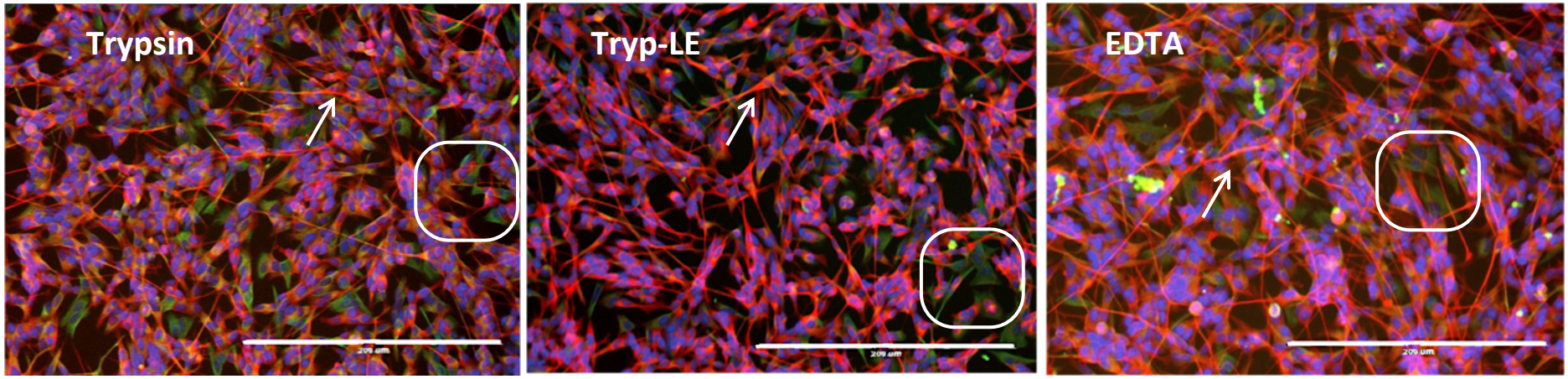


Figure 2.4.6. Images taken of SH-SY5Y cells after different splitting methods and 7 days of differentiation.

Immunofluorescence of β3-Tubulin and Vimentin in SH-SY5Y cells. B3-Tubulin was labelled in red; the Vimentin was in green, and the nuclei were labelled with DAPI in blue. Images were taken on a x 10 magnification. The circled area shows populations of S-type cells, and the arrows point at neuronal projections labelled with B3-Tubulin.

2.4.4 Influence of RA and NGF on Differentiation using the Updated Splitting Method.

Now that an optimum passage method was identified, we wanted to reduce the S-type populations further using NGF. In previous studies in SH-SY5Y cells, 50 ng/ml NGF was added after 5 days of differentiating the cells to maintain differentiation, which led to an increase in MAP2 expression (231). Extensive work by Encinas et al., (233) showed that the SH-SY5Y must reach a certain point in their differentiation cell cycle to respond to NGF's neurotrophic effects. Therefore, NGF was only added after 5 days of differentiation with RA. In addition to splitting the cells using EDTA instead of Trypsin and the addition of NGF on day 5, we also included a time point at 13 days of differentiation. This was because, in the previous experiment, there seemed to be a difference in SOX2 and MAP2 expression on day 10 between RA and ETH, so it was worth investigating if larger differences could be observed on day 13. The media was also changed to contain no FBS after 5 days as that has been reported to stop the proliferation of S-type cells, whereas if FBS was kept at 2.5%, the S-type cells continued to proliferate (233).

2.4.4.1 Methods

The methods remained the same as in Section 2.4.2.1, apart from adding 50 ng/ml NGF on day 5, and the media was changed to contain no FBS. The media was changed every two days, and the cells were collected after 0, 3, 5, 7, 10 and 13 days of differentiation. The RNA extraction and PCR methods were the same as described in Section 2.3.2. As previously, MAPT, NeuroD1, MAP2 and SOX2 gene expression were used as signs of neuronal differentiation. All data

was analysed by a Two-Way ANOVA, if there was a significant day-treatment interaction effect, then Tukeys multiple comparisons test was used.

2.4.4.2 Results

2.4.4.2.1 The effect of RA and NGF on the expression of SH-SY5Y differentiation genes.

NGF had no significant additional effect on gene expression in SH-SY5Y cells compared to RA alone, as seen in *Figure 2.4.7.* However, there were clear differences between the RA-treated cells and controls. MAPT expression was lower in the RA-treated groups on days 7 and 13, which resulted in a time x treatment interaction ($p<0.05$). Similarly, NeuroD1 expression was significantly higher in controls on days 5 and 10 ($p<0.05$), and expression was influenced by time and treatment ($p<0.05$). Additionally, SOX2 expression was significantly higher in the control group compared to RA-treated groups, a difference also attributed to the time x treatment interaction ($p<0.05$).

The presented data indicate that cells exposed to the control treatment (0.1% ethanol) exhibited gene expression patterns indicative of neurogenesis and differentiation. In contrast, cells treated with RA did not demonstrate a gene expression profile consistent with differentiation. However, visual examination of the RA-treated cells revealed evidence of differentiation, creating a discrepancy between the observed morphological changes (including increased neuronal projections) and the gene expression data. This incongruity suggests that mechanisms other than those detected at the gene expression level may be involved in the differentiation process induced by RA.

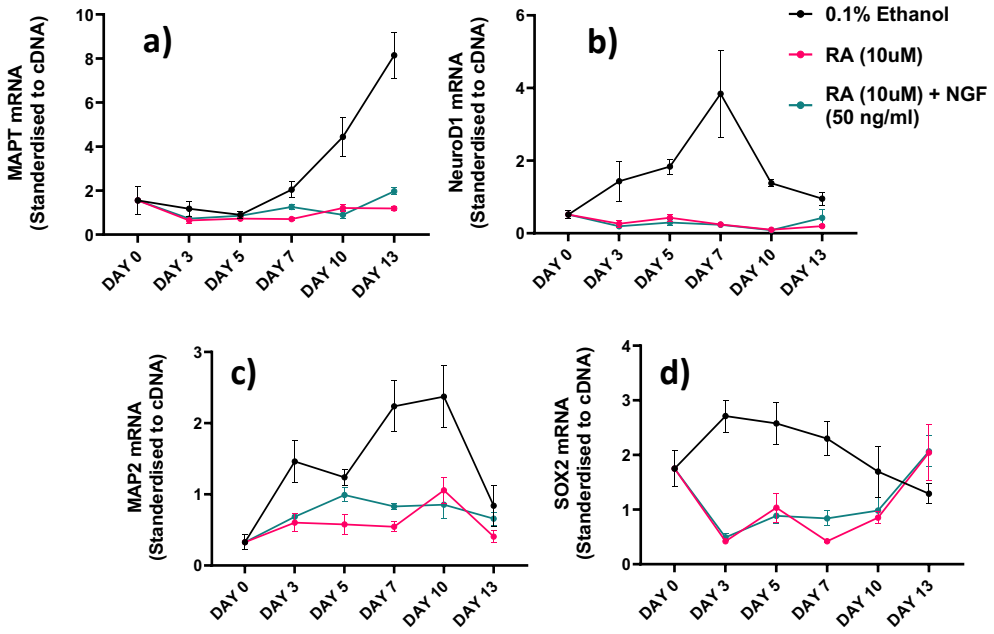


Figure 2.4.7. Gene Expression in SH-SY5Y cells treated with Retinoic Acid and Neuronal Growth Factor

Treatments were added on day 0 apart from NGF which was added from Day 5 onwards, and media changed to contain no FBS. There were four replicates (*n=4*) per treatment per day, data presented as mean ± SEM.

A) MAPT. There was a significant time x treatment interaction ($p \leq 0.05$). The NGF and RA-treated group significantly expressed more MAPT than RA on day 7 ($p \leq 0.05$). Both RA + NGF and RA had lower expression than control on day 13.

B) NeuroD1. There was a significant time x treatment interaction. On day 5 and day 10 the RA treatment significantly reduced NeuroD1 expression ($p \leq 0.05$).

C) MAP2. There was a significant time x treatment interaction ($p \leq 0.05$). On day 5 the RA treated group significantly expressed less MAP2 than the control treated group ($p \leq 0.05$). On day 7 the NGF and RA treated group expressed significantly more MAP2 than the RA treated group ($p \leq 0.05$).

D) SOX2. There was a significant time x treatment interaction ($p \leq 0.05$). On day 3, 5 and 7 the RA treated groups had less SOX2 expression than ETH treated groups ($p \leq 0.05$).

2.4.5 Immunocytochemistry to assess the influence of NGF and BDNF on SH-SY5Y morphology.

To better understand the gene expression data previously observed, the focus shifted to examining protein expression changes during differentiation, both in the presence and absence of NGF and BDNF. Considering NGF's negligible effect on gene expression, the research was directed towards evaluating BDNF as a potentially more effective neurotrophic factor. This evaluation involved qualitatively measuring β3-Tubulin and Vimentin protein levels to assess SH-SY5Y cell morphology and determine if these cells exhibited signs of differentiation. Additionally, the study sought to explore the variations in S-type cell population density across different treatments.

2.4.5.1 Methods

Treatment with NGF and BDNF and the change to 0% FBS was initiated from the fifth differentiation day. The effects were then measured at intervals - after 7, 10, and 13 days of differentiation, corresponding to 2, 5, and 8 days of neurotrophic factor treatment, respectively. Cells were seeded at a lower density of 100,000 cells per well to allow room for growth and space to examine the length of neuronal projections. Immunocytochemistry methods can be seen in Section 2.3.3.

2.4.5.3 Results

Through qualitative analysis, distinct differences in neuronal differentiation and interactions were observed across treatment groups. By day 13, cells without FBS, RA, BDNF, or NGF predominantly showed cell death, characterised by circular shapes and detachment from the plate well *(Figure 2.4.8*). Cells treated with 0% FBS and RA also experienced significant cell death but with fewer S-type cells than the 2.5% FBS and RA group. Notably, treatments with neurotrophic factors generally resulted in a reduced presence of S-type cells, with the 10 µM RA and 2.5% FBS group having the most.

BDNF increased the number and length of neuronal projections, forming distinct clusters, which were especially noticeable on day 10. In contrast, on day 7, NGF and BDNF-treated cells appeared similar. The RA + 2.5% FBS group had more S-type cells than the group with only 2.5% FBS, suggesting RA's role in promoting SH-SY5Y cells' transdifferentiation into S-type cells (233).

In summary, BDNF supplementation, serum starvation, and a 13-day differentiation period emerged as the most effective strategy for SH-SY5Y cell differentiation, making it the optimal positive control.

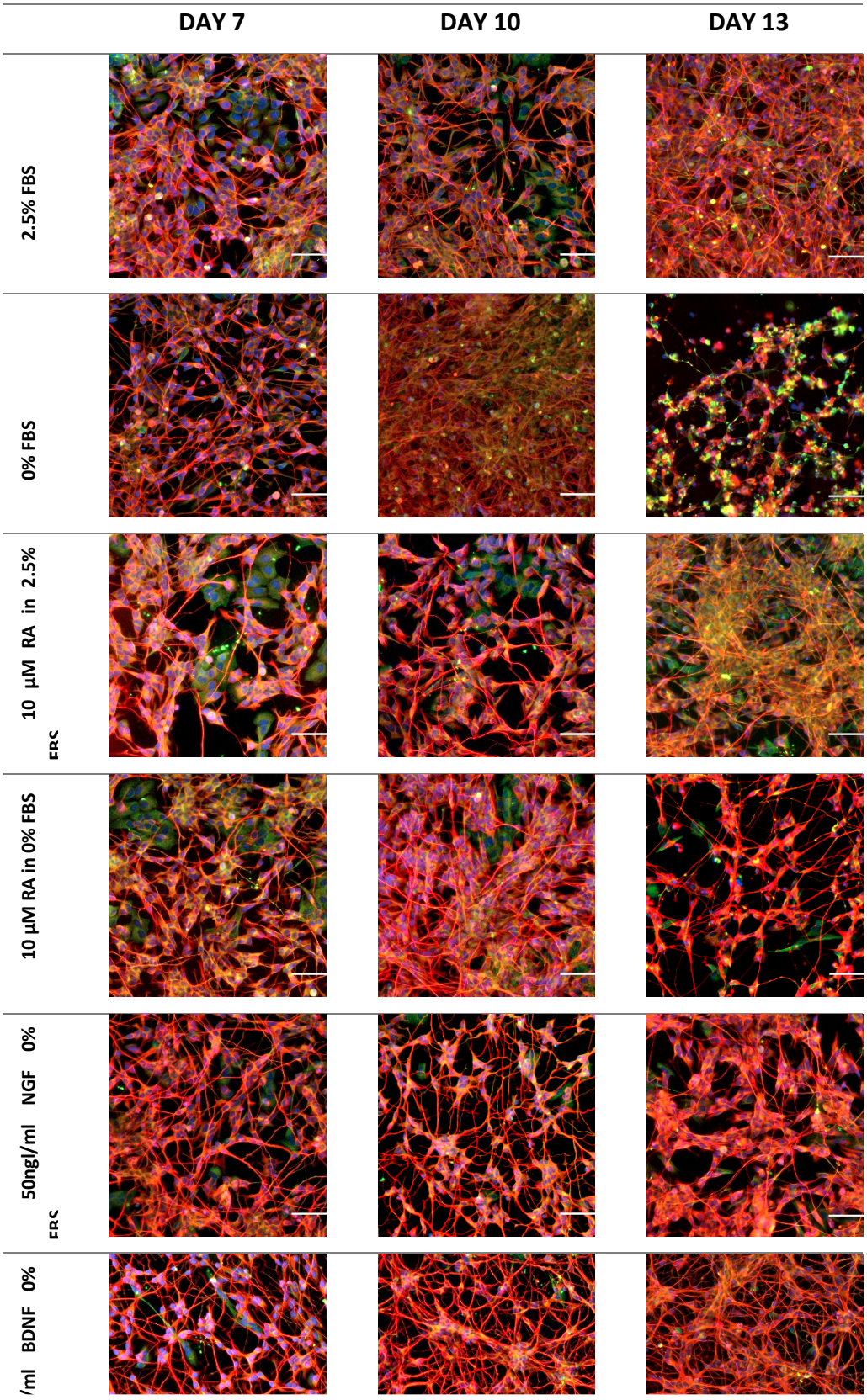


Figure 2.4.8 Immunocytochemistry of β3-Tubulin, Vimentin and Cell Nuclei

Immunofluorescence analysis of *β3*-Tubulin and Vimentin in SH-SY5Y cells. *B3*-tubulin was labelled in red; the Vimentin was in green, and the nuclei were labelled with DAPI in blue. Images were taken on a x20 magnification on an EVOS microscope.

2.4.6 The Effect of RA and BDNF on SH-SY5Y Differentiation Genes

The results from the previous study indicated that BDNF supplementation, serum starvation, and a differentiation period of 13 days as the most effective strategy for achieving differentiated SH-SY5Y cells containing mainly N-type cells, establishing it as the optimal positive control. Therefore, we investigated our original hypothesis that the S-type cells were affecting the expression of genes associated with differentiation. Alongside MAPT, NeuroD1, MAP2, and SOX2 genes we also included NRXN1 as a marker of synaptogenesis.

2.4.6.1 Methods

The media was changed every two days, and the cells were collected 0, 3, 5, 7, 10 and 13 days after initial treatment. The RNA extraction and PCR methods were the same as described in Section *2.3.2*. As previously, MAPT, NeuroD1, MAP2, and SOX2 gene expression were used as signs of neuronal differentiation, and an additional marker of synaptogenesis, NRXN1, was also measured.

2.4.6.3 Results

A significant time x treatment interaction was observed for MAPT, NeuroD1, and NRXN1 (*p≤0.05 for each*). Specifically, on day 3, both RA groups displayed elevated MAPT expression compared to the control, while by day 13, the RA+BDNF group exhibited reduced MAPT expression (*p≤0.05*). For NeuroD1, day 10 revealed a reduction in expression in the RA-treated groups compared to controls. As for NRXN1, on days 3 and 10, there was a heightened expression in both RA groups relative to the control, whereas, on days 7 and 13, only the RA+BDNF group demonstrated increased expression (*p≤0.05*). SOX2 and MAP2 had no significant interactions, although time had a significant effect (*p < 0.05*). There was a dip in markers of differentiation (MAP2, NRXN1 and MAPT) and an increase in markers of proliferation (SOX2) expression on day 5 (*p≤0.05*).

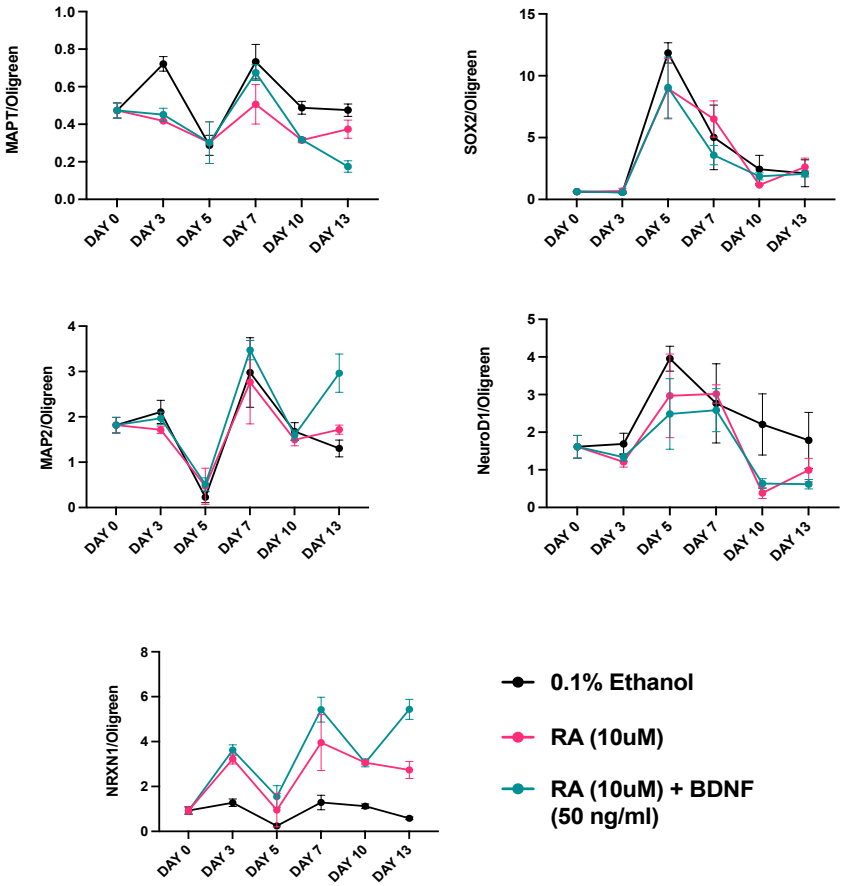


Figure 2.4.9. Gene Expression in SH-SY5Y cells treated with Retinoic Acid and Brain-derived Neurotrophic Factor

Treatments were added on day 0 apart from BDNF, which was added from Day 5 onwards. There were four replicates (*n=4*), and values are presented as Mean ±SEM**.** No significant difference was observed unless otherwise stated below.

A) MAPT. There was a significant time x treatment interaction (*p≤0.05*). Both the RA groups had significantly less MAPT expression than the control on day 3. On day 13 there was significantly less expression of MAPT in the RA+BDNF group than both other groups.

B) SOX2. There was no significant interaction, but time significantly increased then decreased SOX2 expression.

C) MAP2. There was no significant interaction, but time significantly affected MAP2 expression, as seen on the graph.

D) NeuroD1. There was a significant time x treatment interaction (*p≤0.05*). On day 10, the RA-treated groups had significantly less NeuroD1 expression than the control.

E) NRXN1 There was a significant time x treatment interaction (*p≤0.05*). On days 3 and 10, the RA groups had significantly more NRXN1 expression than the control group. On days 7 and 13, only the RA+BDNF group increased NRXN1 expression compared to just the control and RA treated cells.

2.5 Discussion

The overall aim of this chapter was to establish a good model of neurodevelopment and neurogenesis using the SH-SY5Y cells and to use the cell model to eventually study the effects of VD on differentiation. Overall, although the data suggests that RA treatment has a mixed effect on genes responsible for differentiation, there were clear differences between RA-treated and non-RA-treated cells, more specifically, in terms of their protein expression and morphology.

2.5.1 Proliferation.

Aligning with previous research, the DNA assay revealed that cells treated with RA significantly reduced proliferation rate from Day 5 of treatment onwards (246). This aligns with the literature, as RA was routinely used to differentiate cells (246). In these experiments, BDNF or NGF were not applied, as initially, the aim was to investigate the effects of RA alone. The observed decrease in proliferation rate either indicates that RA treatment impeded the cellular division process, or there was a reduction in cell numbers through cell death. In the nervous system, the transcription factor SOX2 plays a crucial role during early developmental stages and is a known regulator of proliferation in neurones (247-249). SOX2 expression within our experiments varied greatly between studies, with most of the data not showing clear differences between cells treated with RA, or controls. Cavallaro et al., (247) found that when NSC derived from SOX2 deficient mutants were grown in culture, the cells exhibited underdevelopment, limited branching (a crucial

neuron feature), and a lack of typical neuronal markers such as NeuN or MAP2. Their findings suggest that SOX2 expression is fundamental at early stages of differentiation, rather than its classic role in proliferation. Indeed, in our trial looking into the effects of NGF, we found that an early increase in SOX2 also corresponded with increased MAPT, MAP2 and NeuroD1 within the control group despite the RA treatment in the other treatment groups.

2.5.2 Differentiation

The results from this time trial have been perplexing; each experiment has provided differing gene expression profiles that were hard to interpret. It is unclear why the gene expression does not reflect the clear differences in neuronal morphology seen in both ordinary microscope images and the results from the immunocytochemistry experiments.

Within the first experiment, we investigated the effects of 10 µM RA alone on gene expression, with no neuronal growth factors. This was partly to see if RA could be a good positive control and if there were clear gene expression differences between the control and RA groups. No neuronal growth factor was applied within the first study, the same as in the DNA assay experiments.

The expression of MAP2 was not affected by RA treatment in this experiment. Although MAP2 typically increases with differentiation in SH-SY5Y cells, others have reported similar findings. Cheung et al., (250) found that RA treatment did not influence MAP2 protein expression, and Encinas et al., (233) also found similar

findings with both MAP2 and MAPT. The expression of NeuroD1 significantly decreased on days 3 and 10 in cells treated with RA. This finding is consistent with the findings of Wang et al. (2016), who demonstrated a decrease in NeuroD1 expression in rat cortical neurones following RA treatment. However, it contradicts the results of Zhang et al. (2014), who reported no impact of RA treatment on NeuroD1 expression in rat cortical neurones.

Since there is so much variation in culture and differentiation methods for SH-SY5Y cells, it was unclear what effects the RA treatment alone would have on the gene expression, particularly when most of the work has been done on protein expression rather than mRNA expression.

2.5.2.1 Effects of NGF and BDNF on Differentiation

Numerous studies highlight the importance of neurotrophic factors in maintaining differentiation and preventing variability in gene expression. In our observations, adding NGF following a 5-day treatment with RA did not alter the gene expression of the measured genes, contrary to previous findings (230-232). However, Dravid et al., (251) demonstrated that BDNF treatment could increase branch length compared to NGF. Consequently, we substituted NGF with BDNF in our experiment. After 13 days of this modified treatment, we noted significant morphology and gene expression changes. BDNF is particularly active within the brain in critical regions such as the hippocampus, cortex, and basal forebrain, which are vital for functions like learning, memory, and higher cognitive processes (252, 253). BDNF's role in neurogenesis contributes to long-term memory, where

certain adult brain regions maintain the ability to generate new neurones from NSC's (254). In SH-SY5Y cells, it is possible that BDNF can stimulate the formation of functional synapses, which are essential for communication between neurones (255). This might explain the significant reduction observed in the expression of Neurexin gene expression between cells treated with RA alone and those treated with RA and BDNF after 13 days, as it could be enhancing synaptogenesis.

We also removed FBS from the media when BDNF was added to the cell culture. This decision was based on previous findings by Encinas et al., (233) that cells responsive to BDNF survive, whereas unresponsive ones, like S-type SH-SY5Y cells, do not thrive. By eliminating FBS, we aimed to reduce the number of S-type cells, ensuring their presence would not overshadow any gene expression changes in the neurones. Our experiments confirmed this approach: cells treated with BDNF and without FBS developed more neuronal connections than those treated with RA and without FBS. This was visible in the physical connections and gene expression patterns. Particularly on day 13, the BDNF-treated cells showed significantly higher expression of MAP2 and NRXN1 genes than the RA-treated group, even though both groups were without FBS from the start of the experiment.

2.5.2.2 Cell morphology and passage techniques on Differentiation

N-type and S-type SH-SY5Y cells, two subtypes of the SH-SY5Y cell line, exhibit distinct morphological and functional differences. N-type cells display a neuronal-like morphology with extensive neurite outgrowth, while S-type cells have a

flattened epithelial-like shape. N-type cells have a higher potential for neuronal differentiation, expressing markers like MAP2 and β3-tubulin, whereas S-type cells remain undifferentiated. Indeed, immunofluorescence staining work by Bell et al., (256) and data using flow cytometry by Ferlemann et al., (227) found that there is a difference between the proteins expressed in the N-type and S-type cells, suggesting that there are also differences in gene expression. N-type cells are more responsive to differentiation-inducing factors, like RA and neurotrophic factors, whereas S-type cells cannot differentiate and therefore remain epithelial-like and round. These characteristics highlight the distinct nature of N-type and S-type SH-SY5Y cells, emphasising their differential potential and neurotransmitter profiles. The irregular gene expression within the gene expression experiments may be due to the overpopulation of the S-type cells and their precursors in culture. The S-type cells do not possess the nerve type (N-type) characteristics and are thought to be like Schwann cells, glial cells, and melanocyte precursors [25]. Their presence may dilute any gene expression changes in the N-type cells, which are the cells of interest.

To encourage greater N-type growth and reduce S-type growth, we used existing literature, such as work by Kovalevich et al., (257), Bell et al., (226) and Shipley et al., (223) to design our experiments to identify the best way to both growing and differentiating the cells.

Firstly, we wanted to find out if splitting the cells differently would result in a difference in differentiation later down the line. We found a significant reduction

in MAPT gene expression in cells split using the enzyme Trypsin. MAP2 and MAPT regulate and stabilise microtubule networks (240). The MAP2 expression in all the time points and treatment did not significantly differ, whereas the expression of MAPT both increased with time and in the EDTA and Tryp-LE treated cells, a similar finding to that of Encinas et al., (233) and Cheung et al., (250). The increased expression of MAPT but not MAP2 was suggested by Encinas et al., (233) due to MAP2 only being present in the proximal portions of the neurites, whereas MAPT was expressed in the longer distal portions. Hence, more MAPT expression is in the later stages of differentiation as the axons extend. In addition, the protein expression of ß3-Tubulin increased, and Vimentin decreased, suggesting that these differences in gene expression translated to the protein expression.

The reduced expression of MAPT in the trypsin-treated cells when compared with EDTA and Tryp-LE may be due to the animal-derived trypsin damaging the surface proteins of the cells (258). Since MAPT is expressed to a larger degree in axons rather than dendrites (as MAP2 does), it can be speculated that trypsinisation may disrupt the axonal projections, reducing optimal SH-SY5Y differentiation (240). Although Tryp-LE is also an enzyme, suppliers claim it is gentler on cells than trypsin in human stem cells and is a much purer enzyme (259, 260). EDTA contains no enzyme whatsoever and acts as a mineral chelator (261). This means that there is very little damage to surface proteins and, in theory, reduced damage to cell-cell connections. However, very little work has been done to compare EDTA alone with trypsin-EDTA. In the paper by Shipley et al., (223) the

authors suggest using trypsin only for a short period, so that the more “sticky” S-type cells remain stuck on the flask and not passaged. This may also be the case as to why EDTA and Tryp-LE contained cells with increased MAPT expression, as their gentle dissociation meant that there were fewer S-type cells with each passage and increased differentiation within the N-type cells.

2.6 Conclusion

The optimisation experiments designed to model neurodevelopment and neurogenesis using SH-SY5Y cells have elucidated the nuanced effects of RA and neurotrophic factors on cellular differentiation. RA was observed to induce morphological changes and mitigate proliferation, yet its influence on the gene expression of differentiation markers such as MAP2 and NeuroD1 was variable. In contrast, incorporating BDNF after 5 days of RA treatment and removing FBS from the differentiation medium post-day 5, significantly modified both the cells' morphological attributes and gene expression profiles, underscoring BDNF's critical role in promoting neural differentiation. This suggests that BDNF supplementation is vital for maximising neuronal differentiation. Moreover, the data showed that using trypsin when passaging the SH-SY5Y cells may hinder the expression of differentiation markers at both the genetic and protein levels, indicating the need for gentler dissociation methods. Utilising milder agents like EDTA or Tryp-LE instead of trypsin showed increased MAPT, minimising non-neuronal S-type cell proliferation and enhancing the population of neuron-like N-type cells.

In light of these findings, future experiments will implement these optimised methods to ensure a more consistent and reliable SH-SY5Y cell culture system. The introduction of BDNF following RA treatment, combined with refined passaging techniques and serum starvation, promises to significantly enhance the fidelity of SH-SY5Y cells as a model for studying neurogenesis.

3 Dose-Dependent and Temporal Effects of Vitamin D and Retinoic Acid on SH-SY5Y Differentiation:

3.1 Introduction

In neuronal development, proliferation and differentiation are tightly regulated. Neuroepithelial cells proliferate extensively in the early stages, later transitioning to radial glial cells that differentiate into neurones or glial cells (262, 263). This balance ensures proper brain development, shifting from a high proliferation phase to one where differentiation prevails to form mature neurones and establish functional neuronal networks (262).

A growing body of work has focused on examining the effects of maternal VDD on the neurodevelopmental outcomes in offspring (264). Accumulating evidence suggests that an optimal VD status in mothers is associated with enhanced brain development, reduced risk of neurodevelopmental disorders such as schizophrenia and autism, and higher cognitive development scores in children (264-266). Parallel observations have been made in animal models, where cognitive benefits and changes in brain structure have been documented, with prenatal VDD linked to altered locomotor activity in rat offspring and schizophrenia-like symptoms in adult rats (267, 268). The review by Cui et al., (263) summarises at great length the evidence so far linking VDD and schizophrenia. They conclude that animal studies in this field continuously find that VDD impacts the development and function of dopamine neurones in adult brains. The focus on

dopamine neurones stems from their critical role in brain function and the regulation of cognitive processes, making them a key area of interest in understanding how early-life VD levels could influence the later onset of schizophrenia (263). Specifically, studies investigating the impact of VD on dopaminergic neurones predominantly highlight VD's beneficial influence on the maturation of these neural circuits (269).

VD supplementation has been found to promote NGF and BDNF release in hippocampal neurones (270, 271). NGF and BDNF are crucial for neurite outgrowth and support differentiation, proliferation, and synaptic activity during brain development (270, 272-274). Synapses, the communication junctions between neurones, rely on synaptic plasticity to enable healthy brain development and function (275). VD has been shown to increase synaptic activity, indicating that VD directly affects differentiation processes associated with synapse formation (276, 277).

In-vitro studies investigating the effects of VD have revealed that it has beneficial effects on the health of neurones. NSCs express the VDR, and VD has been observed to promote both the proliferation and differentiation of NSCs into neurones and oligodendrocytes (278). NSCs are regulated by VDRE, which is activated by the heterodimer of VDR/RXR, influenced by VD status (279). However, the precise function of RA within the RXR-VDR heterodimer remains inconclusive (280). The work by Shirazi et al., (281) investigated the influence of calcitriol on NSC proliferation. They found that VD treatment significantly enhanced

differentiation in these NSCs, in addition to increased neurotrophic factor gene expression, including BDNF and Neurotrophin-3 (NT-3) (281). Similar findings were also found by other researchers (282-284). There is also evidence that VD influences ROS. For example, AlJohri et al., (285) used primary rat neurones to investigate the effect of 6 x 10^{-7} M calcitriol on the proliferation and oxidative stress response. They found that cell viability and levels of GSH were increased, and lipid peroxidation was increased with VD treatment (285).

As mentioned in Chapter 2, SH-SY5Y are neurone-like immortalised cells, which can be studied in non-differentiated and differentiated states. SH-SY5Y cells contain the VDR, suggesting they are VD-responsive (286). Indeed, multiple studies have found that VD influences proliferation in SH-SY5Y cells (286, 287). Several studies have utilised the SH-SY5Y cell model to investigate the effects of VD supplementation. For instance, the research by Pertile et al., (288) demonstrated that VD supplementation in SH-SY5Y cells led to an increase in dopamine release and enhanced neurite branching. Another study by Tetich et al., (289) revealed that VD offers protective effects to SH-SY5Y cells against hydrogen peroxide-induced damage.

3.2 Aims

Our research used the SH-SY5Y cell line as a model to simulate the developing brain. This cell line is particularly suitable because it undergoes proliferation and can be induced to differentiate, closely resembling NSCs. The SH-SY5Y cell line has been previously shown to express VDR, and treatment with VD causes slight differences in cell morphology (286). Despite these insights, there is a notable gap in research regarding the impact of physiologically relevant concentrations of VD on markers of proliferation and differentiation in SH-SY5Y cells.

Therefore, we aimed to investigate the following:

1. To determine if Calcitriol (VD) reduces SH-SY5Y neuroblastoma cell proliferation, measured through DNA quantity and BrdU incorporation.
2. To evaluate the time-dependent effect of VD on the expression of neuronal genes, including MAPT, SOX2, NRXN1, MAP2, and NeuroD1.
3. To investigate how VD concentrations affect β3-Tubulin expression and consequent morphological changes in SH-SY5Y cells, specifically neurite outgrowth.

3.3 Methods

3.3.1 Materials

The same materials were used in Section 2.4.

3.3.2 Vitamin D and its Influence on Proliferation

3.3.2.1 Cell Culture and Treatment Protocols

Cells were cultured on 6-well plates coated with collagen, as detailed in Section 2.3.1.3, and BDNF was added and FBS was removed in all groups from day 5 according to the protocol in Section 2.4.6 in all experiments bar proliferation assays. In the proliferation experiments, cells were grown in 96 well plates, no BDNF was added, and the FBS concentration remained at 2.5%. The cells were treated with a control (0.1% EtOH), 10 µM RA, and varying concentrations of VD (10^{-13}, 10^{-10}, and 10^{-7} M).

3.3.2.2 DNA Assay and BrdU Incorporation

Proliferation was assessed through DNA quantification and BrdU incorporation as outlined in Sections 2.4.1.1 and 2.3.1, respectively. These assays were conducted on cells treated with the aforementioned VD concentrations, including a group with a combined 10 µM RA and 10^{-10} M VD treatment.

3.3.3 Effects Of Vitamin D On the Expression of Genes Associated with Neuroblast Cell Differentiation

3.3.3.1 Gene Expression Analysis

The expression of proliferation marker SOX2 and differentiation markers (MAPT, MAP2, NeuroD1, NRXN1) was measured to understand the effects of RA, VD, and their combinations on proliferation and differentiation cellular processes.

Gene expression changes were quantified according to Section 1.3.1.3, and treatments were performed according to the cell culture and treatment protocols previously mentioned.

3.3.4 Protein Expression in SH-SY5Y cells treated with Vitamin D

3.3.4.1 Protein Quantification and Immunocytochemistry

Protein levels were quantified following the immunocytochemistry protocols in Section 2.3.3.1. Specifically, β3-Tubulin expression was measured after treatments with RA and VD at previously mentioned concentrations.

3.3.4.2 Visualisation of neurite Growth

Post-treatment, morphological changes in SH-SY5Y cells were evaluated at day 7 to complement protein expression data. Observations focused on neurite outgrowth and cellular morphology to assess differentiation, related to the treatment conditions established in the cell culture protocols.

3.3.5 Statistical Methods

Data analysis was carried out using GraphPad PRISM software. The choice of statistical tests was informed by the experimental design and the nature of the data. The following tests were employed:

Two-way Analysis of Variance (ANOVA): Used to analyse statistical DNA assays. Also applied to gene expression data involving MAPT, SOX2, NRXN1, MAP2, and NeuroD1, and the protein expression of β3-Tubulin in GraphPad PRISM 10, to examine the effects of time and treatment, as well as their interaction.

One-way Analysis of Variance (ANOVA): Used to analyse data generated with the BrdU assay.

Tukey's Post Hoc Tests: Used to identify specific differences between groups upon detecting significant interactions in both the BrDU assay and gene/protein expression analyses.

3.4 Results

3.4.1 Vitamin D and its Influence on Proliferation

Treatment of SH-SY5Y cells with RA alone, as well as in combination with 10^{-10} M VD and 10^{-7} M VD, resulted in a reduction in cell numbers at days 5, 7, and 10 post-treatment (*p<0.05*), relative to the control group (significant time-treatment interaction, *p<0.001*; refer to *Figure 3.4.1*). This effect was corroborated by a marked decrease in BrdU incorporation in cells treated with the VD at concentrations 10^{-7} M and the RA and the RA and 10^{-10} M VD mix compared to controls on day 5 (*p<0.0001*), indicating an onset of reduced cell proliferation on day 5 in these groups (as shown in *Figure 3.4.1*).

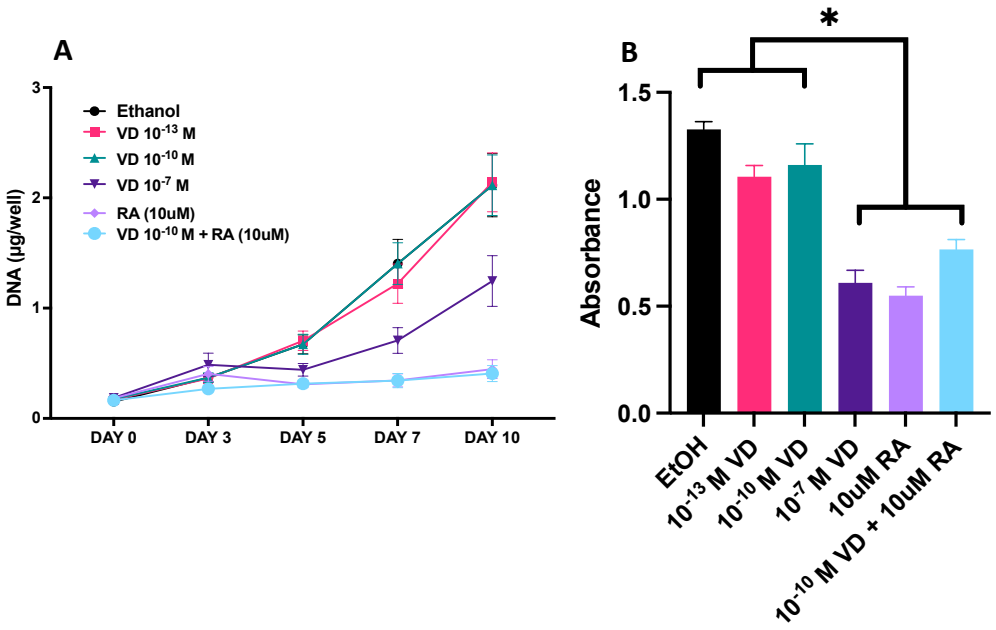


Figure 3.4.1. DNA and BrdU Expression in SH-SY5Y Cells Treated with VD and RA

A) DNA concentration was measured after SH-SY5Y cells underwent 0, 3, 5, 7 and 10 days of incubation of negative control (ethanol (ETH)), positive control (10 µM retinoic acid, (RA). Values shown as means (±SEM). N=3. There was a significant interaction (*p<0.001*) between time and treatment. Treatment of SH-SY5Y cells with RA alone, as well as in combination with 10^{-10} M VD and 10^{-7} M VD, resulted in a reduction in cell numbers at days 5, 7, and 10 post-treatments, relative to the control group (Tukey's: $p < 0.05$). **B)** The BrDU incorporation was measured in SH-SY5Y cells after 5 days of incubation with the treatment groups. Values are shown as means (±SEM). There was significantly less BrdU incorporation in the RA-treated cells and the cells treated with VD 10^{-7} M compared to the control (*p<0.0001*).

3.4.2 Effects Of Vitamin D On the Expression of Genes Associated with Neuroblast Cell Differentiation

Analysis of VD effects on the expression of MAPT, SOX2, NRXN1, and NeuroD1 found a time x treatment interaction *($p<0.05$*), suggesting that gene expression changes according to both time and treatment. Treatment was not an independent determinant of change in MAP2, but time was *($p<0.05$*). The summary of the results can be seen in *Figure 3.4.2.*

Contrary to expectations, RA treatment reduced the expression of MAPT, MAP2, and NeuroD1, suggesting an inhibitory effect on differentiation. High-dose VD induced these markers, supporting its differentiation role. The RA and low-dose VD combination yielded inconsistent expression, indicating complex interactions.

NRXN1 expression was significantly higher in cells treated with 10 µM RA; however, RA + 10^{-10} M VD treatment on day 5 *($p<0.05$*) significantly increased NRXN1 expression, indicating a specific synergetic effect on that day. The linear pattern in the DNA assay in Section 3.4.1 was not seen in the expression of the proliferation marker SOX2. Initially, SOX2 levels were low across all treatments. A notable rise occurred in the control group on day 5, followed by a peak in 10 µM RA + 10^{-10} VD treated cells on day 10. By day 13, SOX2 expression declined in all treatments, with the 10^{-10} M VD-treated cells showing significantly lower levels than those treated with RA. NeuroD1 was lowest with RA on day 3 (*$p<0.05$*) and peaked in controls on day 5. By day 13, 10^{-7} M VD treatments showed the highest levels (*$p<0.05$*). MAPT expression was suppressed by 10 µM RA at day 3 (*$p<0.05$*)

but increased with RA + 10^{-10} VD on days 5 and 13; notably, 10^{-7} M VD significantly elevated MAPT on day 5 when compared to control ($p<0.05$). MAP2 showed no treatment effect, but the time effect was significant ($p<0.05$); RA significantly reduced expression on day 3 ($p<0.05$), and controls and RA–only treated cells showed lower levels on day 5 ($p<0.05$).

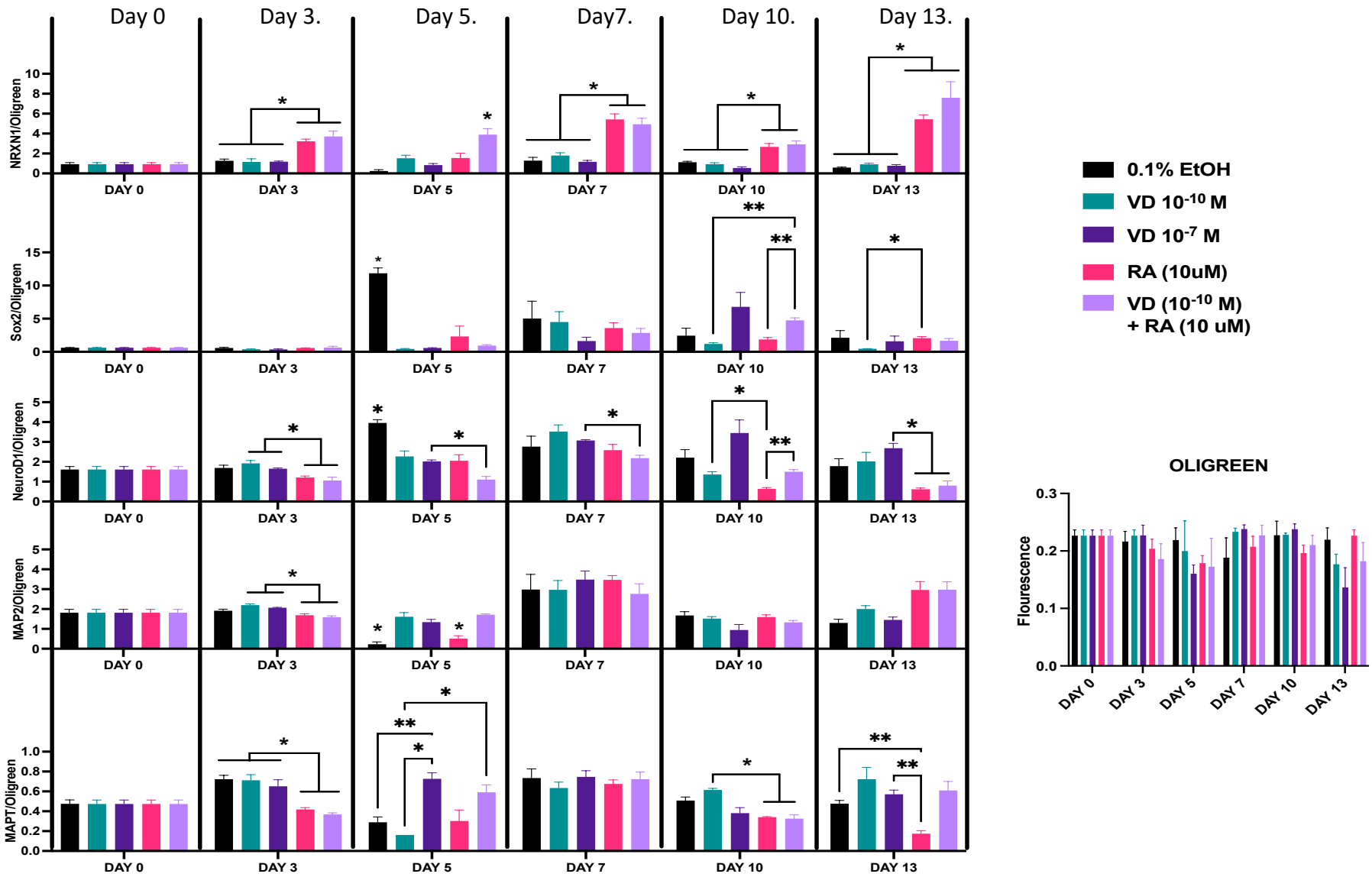


Figure 3.4.2. Effect of Vitamin D treatment on gene expression in SH-SY5Y cells.

Gene expression analysis of neural differentiation markers demonstrated treatment-specific effects. NRXN1 levels were markedly higher with 10 µM RA treatment, with a significant synergistic increase observed when combined with 10^{-10} M VD on day 5 ($p<0.05$). NeuroD1 expression was the lowest in RA-only treatments on day 3 ($p<0.05$), surged in control groups on day 5, and was most pronounced with 10^{-7} M VD treatment by day 13 ($p<0.05$). For MAPT, RA at 10 µM initially suppressed expression on day 3 ($p<0.05$), yet a combination of RA and 10^{-10} M VD resulted in heightened expression on days 5 and 13. Significantly, 10^{-7} M VD alone prompted a notable increase in MAPT expression on day 5 compared to control ($p<0.05$). MAP2 expression, displayed a significant time-dependent variation, with RA treatment reducing levels on day 3 ($p<0.05$) and the lowest expression observed in control and RA-only treatments on day 5 ($p<0.05$).

3.4.3 Beta-3-Tubulin expression in SH-SY5Y cells treated with Vitamin D.

3.4.3.1 Protein Quantification

The One-Way ANOVA revealed that treatment with RA treatment significantly upregulated β3-Tubulin expression in SH-SY5Y cells after 7 days, with no significant interaction with VD, as shown in *Figure 3.4.3.* Higher VD concentration (10^{-7} M) in combination with RA significantly increased β3-Tubulin to a greater extent than RA alone or 10^{-10} M RA treatments (*p<0.05*). In the absence of RA, 10^{-7} M VD showed a trend towards increased β3-Tubulin expression compared to both control (*p=0.0545*) and 10^{-10} M VD (*p=0.0593*), nearing significance. By day 10, however, VD's influence was not as pronounced, and its interaction with RA remained non-significant. This suggests RA primarily drives β3-Tubulin enhancement, with VD's effect being dose-dependent and transient and with little to no synergistic interaction.

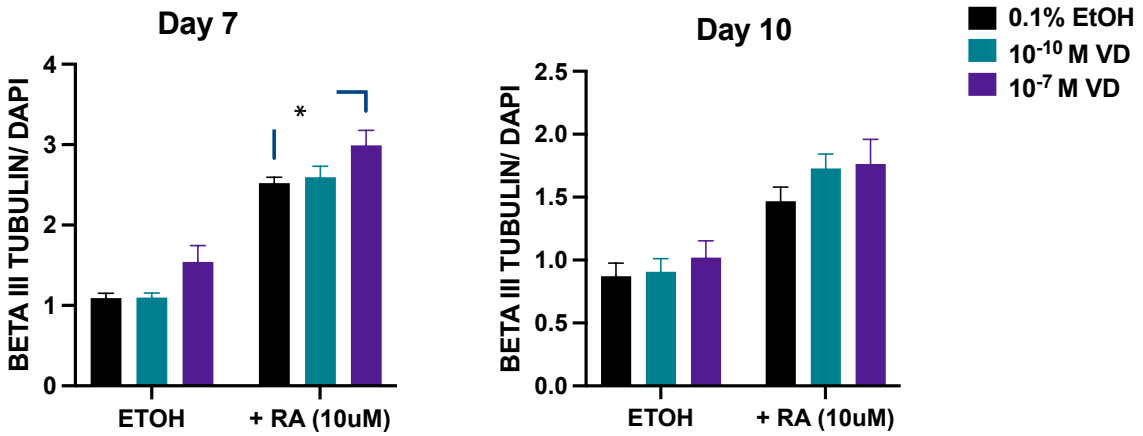


Figure 3.4.3. ß3-Tubulin expression in SH-SY5Y cells treated with VD and RA

The One-Way ANOVA revealed a significant RA-VD interaction was observed after 7 days of differentiation, indicating VD does not alter RA-driven differentiation. RA alone significantly upregulates ß3-Tubulin. Cells with 10^{-7} M VD and RA exhibit higher ß3-Tubulin than controls or 10^{-10} M VD. Non-RA cells at 10^{-7} M VD nearly reach significance in ß3-Tubulin expression compared to control (*p=0.0545*) and 10^{-10} M VD (*p=0.0593*). At 10 days (*N=4*), no RA-VD interaction is observed; RA maintains ß3-Tubulin upregulation, but VD's earlier effect is not detected.

3.4.3.2 Morphology of the SH-SY5Y Cell Change with VD and RA treatments.

Dose-dependent effects of RA and VD on cell morphology were evident, as indicated by increased neurite connections (*Figure 3.4.4*). Notably, cells treated with the highest VD dose (10^{-7} M) exhibited fewer neuritic branches than the 10^{-10} M VD group. However, when 10^{-7} M VD was combined with RA, there was a visually increased neurite outgrowth, surpassing that of control and VD-alone groups. These findings suggest a nuanced interaction where a high concentration of VD may enhance the differentiation effects of RA, particularly in terms of neuritic complexity.

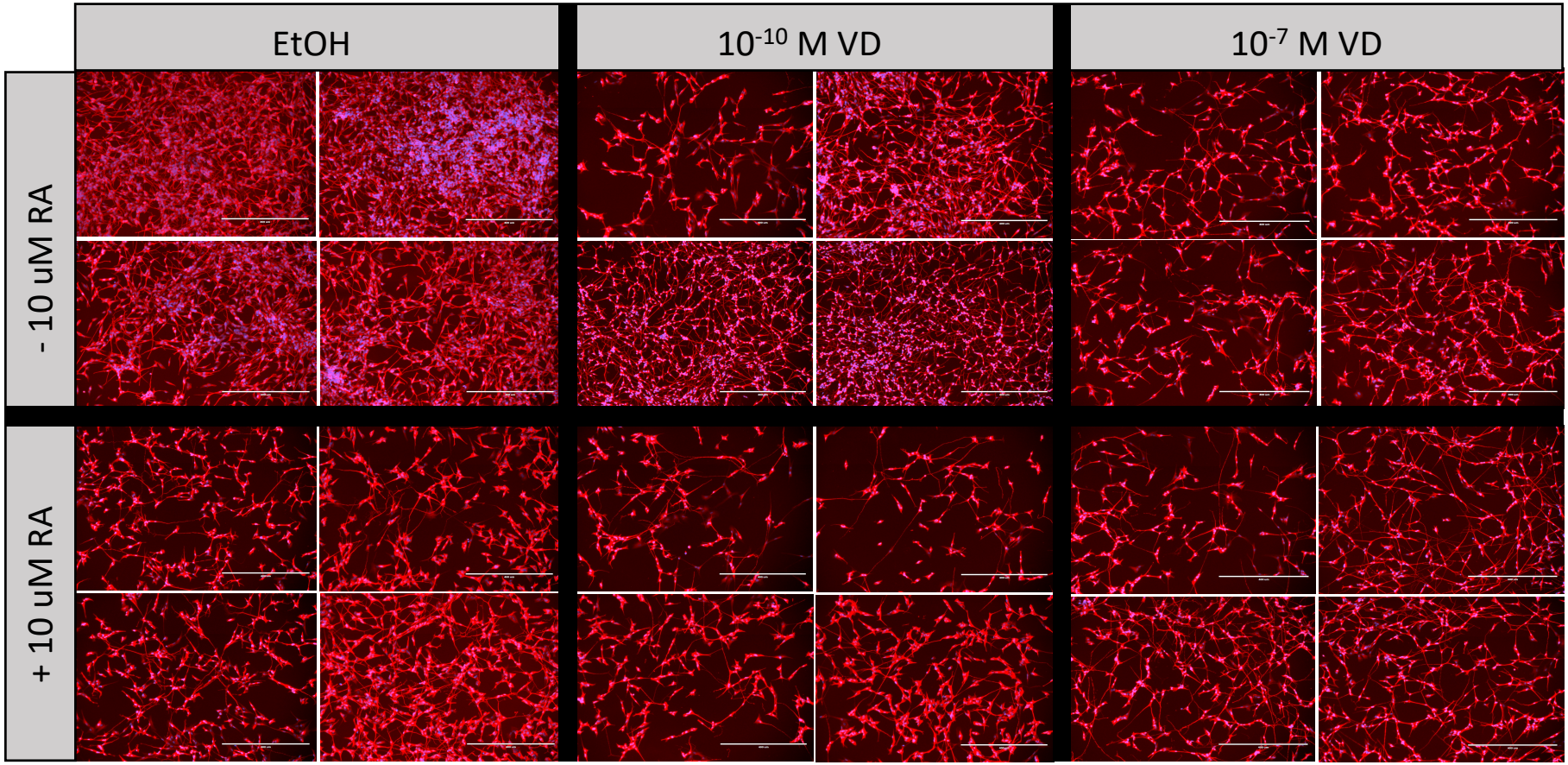


Figure 3.4.4. B3-Tubulin Expression in cells treated with VD and RA

Immunofluorescence of β3-Tubulin in SH-SY5Y cells. Each image represents a different plate (n=4). ß3-Tubulin is labelled in red, and the nuclei are labelled with DAPI in blue. Images were taken at x10 magnification on an EVOS microscope. Morphological changes show that with the RA treatment, the cells have a higher degree of interconnection, with the most being present with the 10^{-7} M VD and RA mix. Without RA, the 10^{-10} M VD had the highest number of interconnections between cells.

3.5 Discussion

VD demonstrated a complex, dose-dependent effect on the differentiation of SH-SY5Y cells. High-dose VD (10^{-7} M) transiently enhanced differentiation markers, including β3-Tubulin and neurite outgrowth, suggesting initial promotion of neuronal differentiation. However, this effect was not sustained, with reduced influence observed by day 10. Additionally, VD treatment modulated gene expression, with varying impacts on proliferation markers such as SOX2 and other neuroblast differentiation genes (MAPT, MAP2, NeuroD1, and NRXN1). Despite some results indicating VD enhances differentiation, VD's overall inconsistent influence indicates a delicate balance in its role as a differentiation agent, necessitating further investigation to optimise its therapeutic potential for neurodevelopmental diseases. This concern is particularly relevant for the gene expression results, given that the experiment was performed only once. These interactions' variable and inconsistent nature necessitates caution in drawing firm conclusions.

3.5.1 Examining the Varied Effects of Vitamin D on Cell Proliferation and Differentiation in SH-SY5Y cells

Starting with the impact on cellular proliferation, adding 10^{-7} M VD markedly decreased DNA content and BrdU incorporation, indicating a reduction in cell proliferation from day 7. This antiproliferative effect of VD, especially at higher concentrations, corroborates the hypothesis that VD may influence cellular growth dynamics. In contrast, lower concentrations of VD (10^{-}

^{13} M and 10^{-10} M) did not exhibit this antiproliferative effect when used without RA, suggesting a dose-dependent response of VD on cell proliferation. However, after 5 days of treatment, only the control group significantly increased SOX2 expression, with all the cells treated with VD and RA not displaying the same increase. An overexpression in SOX2 has been linked to reduced neuronal differentiation, and a reduction of SOX2 expression causes an increase in differentiation markers (290). However, we mostly do not see this inverse relationship in cells treated with VD or RA. On day 5, there was also a significant increase in NeuroD1 expression in the control group. This increase is likely related to the interaction between NeuroD1 and SOX2, given that SOX2 binds to the DNA of the NeuroD1 gene. This binding suggests that SOX2 plays a role in neurones' proliferation and differentiation (290). Interestingly, in studies where SOX2 is knocked out, there is a significant effect on the process of neuronal differentiation and a significant decrease in the expression of NeuroD1 (290).

It is also possible that proliferation and differentiation are independent of each other in regard to the influence of VD. VD, through its active form calcitriol, has been shown to control cell proliferation by inducing an arrest at the G0/G1 to S phase transition (291, 292). The progression from G1 to S phase is facilitated by D-type cyclins pairing with Cdk4 or Cdk6, forming an active kinase complex that partially phosphorylates the retinoblastoma (Rb) protein, leading to transcriptional activation of genes necessary for DNA replication and further cell cycle progression (291, 292).

VD induces the expression of p21waf1, a cyclin-dependent kinase inhibitor, which inhibits the formation of active cyclin-Cdk complexes, thereby maintaining Rb in a hypophosphorylated state that sequesters E2F transcription factor and suppresses the transcription of genes required for the S phase and subsequent cell cycle phases (291, 292). In a study using prostate cancer cell lines, the researchers found that cell lines which did not exhibit p21waf1 induction upon treatment with VD did not show the anti-proliferative response, whereas transfection with p21waf1 cDNA reinstated the growth inhibition, underlining the importance of p21waf1 (293).

The differential impact of VD on proliferation but not necessarily on genes related to differentiation in our experiment may be attributed to the selective modulation of cell cycle regulators such as p21waf1 that specifically inhibit progression through the cell cycle without directly affecting the differentiation pathways (291-293). While proliferation requires passage through specific cell cycle phases, differentiation is often a result of exiting the cell cycle and entering a differentiated state, which can occur independently of the proliferative control mechanisms affected by VD.

3.5.2 Exploring the Potential Role of Neurone-to-Neurone Interactions in SH-SY5Y Differentiation

The interaction between neurones, particularly in the control group, is another important factor. For our studies, we treated the cells at a lower

density than the suggested 70%, around 30-40% confluency at day 0, to allow sufficient growth space at later stages of interest. Our DNA and BrdU assay results indicate that cells treated with RA and 10^{-7} M VD showed less proliferation than lower concentrations of VD and control, reducing cell-to-cell contact. This low initial confluency might have affected both the growth and differentiation of the cells. By day 5, the control and 10^{-10} M VD treated cells, still proliferating, could have increased neurone-to-neurone interactions, possibly enhancing their neuronal connections (294). Immunocytochemical analysis revealed that cells at 10^{-10} M VD concentration seemingly had more interconnections than those at 10^{-7} M VD despite lower proliferation rates. This suggests that higher proliferation in the 10^{-10} M VD group could have facilitated the development of more neural connections. A study by Cullen et al., (294) supports this observation, showing that neurones seeded at higher densities had more neurite outgrowth than those at lower densities. The authors proposed that the distance an axon must extend to connect with a postsynaptic partner significantly influences synapse formation timing, with denser neuronal cultures maturing faster. However, in the developing brain, this process may differ. Unlike cancer cells, proliferation and differentiation in early brain development are strictly regulated (295). Neurones can form connections even over long distances. As the brain undergoes steps of proliferation, differentiation, and synapse pruning, the quality and quantity of neurones are important (295).

3.5.3 Synergistic Enhancement of NRXN1 Expression by Retinoic Acid and Vitamin D in SH-SY5Y cells

The administration of RA increased NRXN1 expression, a gene vital for synaptogenesis (242). Given that NRXN1 encodes Neurexins essential for synaptic transmission, this elevation indicates RA's role in promoting neuronal differentiation. (242). Conversely, VD did not significantly affect NRXN1 expression, indicating that VD alone, at the concentrations tested, does not influence NRXN1 expression. However, after 5 days of treatment, cells exposed to a 10^{-10} M VD and RA mix exhibited a significant increase in NRXN1 expression compared to other treatment groups. This finding suggests that VD might influence synapse formation, but this effect seems time-dependent and only in combination with RA. In the study by Pertile et al., (288) VD supplementation resulted in increased synapses in SH-SY5Y cells transfected with the VDR receptor. This finding suggests a possible explanation for the behaviour of standard SH-SY5Y cells: they may naturally express the VDR at insufficient levels to elicit a significant physiological response to VD.

Mutations in Neurexin genes have been associated with neurological disorders such as autism and schizophrenia (242). Since these disorders are also linked to VDD, exploring the potential relationship between VDD and Neurexin function could provide valuable insights (242). This line of research could not only enhance our understanding of the aetiology of autism and schizophrenia but also potentially lead to new therapeutic strategies targeting VD pathways or synaptic mechanisms in these disorders.

3.5.4 Evaluating the Efficacy of Retinoic Acid as a Positive Control in SH-SY5Y Cell Studies

As described in Chapter 2 there may be many reasons RA treatment did not influence gene expression in a typical pattern. Its role in encouraging differentiation was evident in the DNA, immunocytochemistry, and gene expression data relating to NRXN1 expression. The lack of positive control (RA) causing a sustained and significant increase in NeuroD1, MAPT, and MAP2 may be due to their function. While NRXN1 is crucial for synapse functionality, MAP2 and MAPT are integral to maintaining the physical structure of neurones. Therefore, it could be suggested that the SH-SY5Y cells do not express a higher degree of genes relating to structural differentiation but rather increase the expression of genes relating to synapse formation. The link between NeuroD1 and MAP2 protein expression highlights a crucial role NeuroD1 in neuronal differentiation. Wei et al., (296) demonstrated that introducing NeuroD1 significantly increased MAP2 expression, suggesting NeuroD1 directly or indirectly regulates genes involved in neuron maturation, including MAP2. The lack of increased MAP2 or MAPT expression in RA-treated cells might be due to insufficient overexpression of NeuroD1. Understanding this regulatory role of NeuroD1 is key to comprehending neuron development and addressing neurological disorders.

3.5.5 Neurite Outgrowth and Neuronal Differentiation: The Synergistic Effects of Vitamin D and Retinoic Acid

The morphological assessments on day 7 complement these findings by revealing subtle distinctions not evident in the quantitative protein data. Cells

treated with lower concentrations of VD (10^{-10} M) exhibited increased neurite connections, hinting at enhanced differentiation that might not have been captured at the gene or protein expression levels. Despite reduced cellular proliferation at the higher concentration of VD (10^{-7} M), there was noticeable neurite outgrowth, especially when used in conjunction with RA, suggesting that VD may potentiate the differentiation effects of RA. In our study, cells treated with VD and RA showed a notable increase in B3-Tubulin expression, suggesting enhanced neuronal differentiation. This finding is particularly significant in the context of neurodevelopmental disorders. In the study by Robicsek et al., (297) neurones from schizophrenia patients were reported to exhibit reduced B3-Tubulin when compared to controls. The increased expression in cells treated with VD and RA highlights a potential role for VD in improving neuronal differentiation, which could have therapeutic implications for conditions like schizophrenia.

3.5.6 Vitamin D's Multifaceted Role in Brain Development and Neuronal Health

Despite the results from these experiments, substantial evidence indicates that VD plays a crucial role in neuronal development; a simple visual summary can be seen in *Figure 3.5.1.* During mammalian brain development *in utero*, the foetus relies exclusively on maternal VD supply, making the mother's VD status pivotal for the developing brain (298). Adequate maternal VD levels may influence brain cells at various stages, including proliferating, differentiating,

and fully differentiated cells within the foetal brain (265, 269, 299). The active VD supplied by the mother interacts with the VDR, forming a heterodimer with the RXR. This complex can bind to the VDRE within the DNA, thereby modulating the transcription of thousands of genes (300).

VD's influence in MS has garnered interest, especially regarding its relationship with glial cells, particularly microglia (69, 301). A consistent finding is that optimal VD levels positively influence BDNF and Glial cell line-derived Neurotrophic Factor (GDNF) levels within glial cells (271, 302). These neurotrophins are crucial for neuronal health and function, supporting the differentiation and maintenance of both neurones and glial cells (303, 304). Conversely, VDD can lead to inadequate release of these factors, potentially disrupting neural and glial cell differentiation (271, 305). Given the speculated and evidenced links between imbalances in cell proliferation and differentiation with psychiatric disorders like schizophrenia, autism, and depression, VD's positive effects on these processes could be highly beneficial during brain development (297, 306). Furthermore, VD is known to steer glial cells towards an anti-inflammatory profile by increasing SOCS3 expression, which is advantageous for brain development (307).

In neuronal cells, VD exhibits a range of positive effects. For instance, adequate maternal VD levels during pregnancy have been associated with increased GAP-43 levels in offspring and in primary neurones (308-310). GAP-43 is crucial for the synapse plasticity (309). While our study did not observe VD influencing NRXN1 expression, investigating GAP-43 expression would have

been insightful. VD supplementation during pregnancy has also been linked to higher Tyrosine Hydroxylase (TH) levels in offspring neurones, which reduces OS (311). In psychiatric conditions like schizophrenia, where dopamine signalling is often dysregulated, studies have found that VD supplementation increases levels of Transforming Growth Factor β (TGF- β) and the transcription factor Nurr1, both key in dopamine signalling (311-314). Lastly, VD's role in neuronal myelination is noteworthy. Studies indicate that VDD correlates with reduced remyelination, whereas supplementation fosters this process (315, 316). The myelin sheath is critical for efficient nerve signal transmission, suggesting that VD's influence on myelination during brain development could have long-term impacts on neuronal function (316). What is important to note, though, is that most of the studies on the developmental influences of VD are from animal models, although there are few human trials.

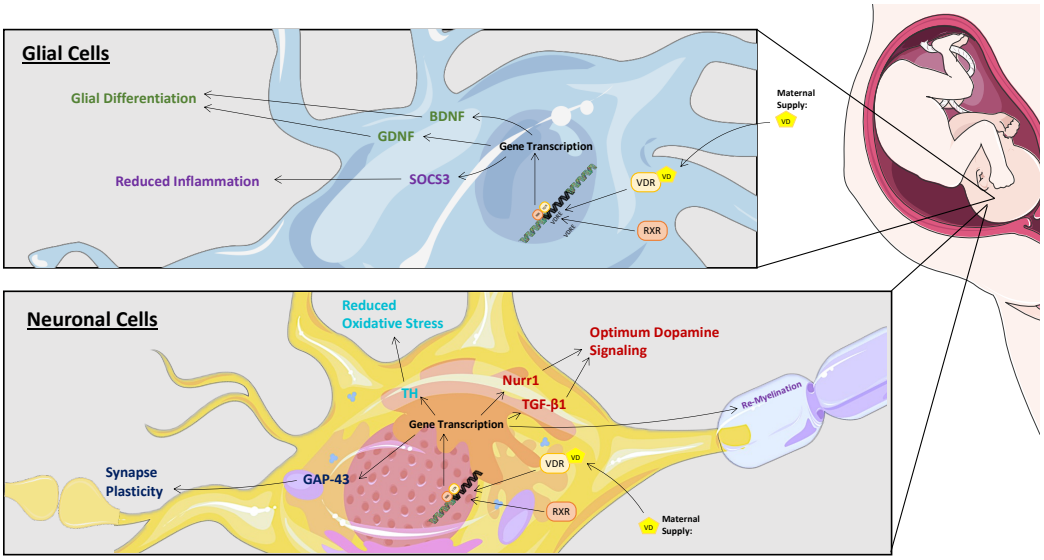


Figure 3.5.1. Summary of Hypothetical Mechanisms that VD Improves Brain Development.

Here, the pathway by which maternal VD affects the developing foetal brain is illustrated, from gene transcription influenced by the VDR-RXR complex to the differentiation and maintenance of neurones and glial cells. The diagram also highlights VD's role in upregulating neurotrophic factors like BDNF and GDNF, which are crucial for neural health, and its involvement in myelination processes. The potential therapeutic implications of VD for psychiatric disorders through modulation of neurotrophins and dopamine-related factors are noted.

3.5.7 Method limitations

Within our results, SH-SY5Y cells do not show sustained increased expression of NeuroD1, MAP2, or MAPT in response to RA and BDNF despite clear morphological changes and other protein expression alterations. This suggests these genes might not be the most relevant markers for studying differentiation in this context. Therefore, they may not accurately reflect the impact of VD on SH-SY5Y cell differentiation. This indicates the need to explore other genes or pathways to understand how VD influences these cells' differentiation process.

As ever, there are limitations to using immortalised cell lines as a model for the dynamic interactions that occur in the body. Studying the influence of VD on brain development accurately would require an in-vivo study.

Although we know the rough values of physiological calcitriol concentrations, we know very little of which are surrounding neurones in living bodies. Even though the highest concentration of VD used in our study is probably not present in human serum, the exact levels of VD within the brain remain unknown.

3.6 Future works

The study revealed that the highest concentration of VD (10^{-7} M), which surpasses typical physiological levels in humans, combined with RA, enhanced the β3-Tubulin expression (317). This finding suggests the need for further investigation into potential changes in gene expression caused by this combination. In addition, measuring the expression of VDR or the CYP24A1 or CYP24B1 enzymes in the SH-SY5Y cells would prove useful in validating that our SH-SY5Y stock is responsive to VD. Checking the VD levels in cell culture media post-removal would provide insights into the degradation and absorption dynamics of VD by cells, thereby further confirming that the cells are reactive to it.

In studies not included here, we found no significant impact on ROS formation when we treated SH-SY5Y cells with 25 mg/ml Lipopolysaccharide (LPS), which stimulates a bacterial infection. Therefore, future research should focus on effectively inducing OS in SH-SY5Y cells, allowing for a detailed investigation into VD's modulatory role. Including a mixture of differentiating microglia and SH-SY5Y cells in future studies could more accurately simulate the environment of a developing brain and provide a comprehensive model, as cells in the immune system induce the immune response.

4 A Scoping Review of In-Vivo Studies Investigating the Relationship Between Maternal VDD, Inflammation or Oxidative Stress, and Offspring Brain Development

4.1 Introduction

The Developmental Origins of Health and Disease (DOHaD) hypothesis theorises that environmental factors during embryonic and early postnatal development influence the health and risk of disease in later life (318, 319). This paradigm has gained growing traction, reshaping our understanding of the aetiology of chronic diseases. It emphasises the critical periods of growth *in utero* and early childhood, where epigenetic modifications can set the trajectory for long-term health outcomes (320). By identifying these early-life influences, there is an opportunity to intervene and modify disease risk, ultimately aiming to improve the lifelong health of individuals. Nutrition impacts epigenetic processes such as histone modification and DNA methylation (321). Within the DOHaD framework, there is a growing focus on VD's role, with studies suggesting that low VD levels during pregnancy may lead to negative neurological outcomes in offspring (322). The exact mechanisms behind this association remain unidentified. While the impact of VD on inflammation is well-studied, its specific connection to both inflammation and brain development requires further investigation.

4.1.1 Vitamin D and Pregnancy

VD is a fat-soluble vitamin obtained from synthesis in the skin upon sun exposure, or ingestion of dietary sources (323). VD status is determined by the

serum level of the inactive form, 25(OH)D (324-326), with vitamin D deficiency (VDD) defined as serum levels below either 30 nmol/L or 50 nmol/L, depending on the country (324-326). Low sun exposure, reduced dietary VD intake and older age are the most common factors associated with VDD, with older age associated with reduced synthetic capacity in the skin (323). Approximately 1 billion people worldwide are VDD, with up to 50% of the world's population having insufficient VD levels and pregnant women being at high-risk (323). In the UK, 63% of pregnant or lactating women are below adequate serum 25(OH)D levels (324-326), and this has been associated with increased risk of pre-eclampsia, gestational diabetes, obesity and bacterial infections, all of which are known to affect the health of the resulting offspring (74, 123, 327).

Maternal VDD is believed to significantly affect offspring health by limiting the availability of VD to the foetus (328, 329). This can result in lower birth weight, babies small for their gestational age, reduced bone growth, poor skeletal mineralisation, and lower bone mineral content (327, 330). There is also an increased risk of autoimmune diseases, insulin resistance, and obesity in later life (327, 330). During foetal development, the foetus depends entirely on the mother's VD supply, highlighting the importance of adequate maternal VD levels for foetal development (328, 329). VD also plays a critical role during embryo implantation, where immune suppression is necessary to prevent the embryo's rejection (14). The placenta, which displays placental and decidual tissue expression of enzymes crucial for VD metabolite synthesis, shows an upregulation of enzymes CYP27B1, CYP24A1, and VDR during this phase (27).

4.1.2 Inflammation and the Immune System

Initially, inflammation was defined as the response to infection or damaged tissue involves the mobilisation of cells and molecules of the host's defence system from the bloodstream to the site where they are needed (331). However, this definition encapsulates just one facet of inflammation. Building on this, Antonelli et al., (90), after reviewing the subject, proposed a more encompassing definition: inflammation is the innate immune response to potentially harmful stimuli such as pathogens, injury, and metabolic stress. Acute inflammation is a beneficial reaction to infection. In healthy individuals, once the infection is cleared, this inflammation should subside and return to normal (90). On the other hand, low-grade chronic inflammation, potentially resulting from factors like a sedentary lifestyle, smoking, or sleep deprivation, is widespread, and is even suggested to be linked to 50% of all deaths worldwide (332). This indicates a failure of our body to maintain inflammatory homeostasis, and such an imbalance can have numerous detrimental consequences in a pregnancy scenario.

The primary role of our body's immune system is to combat infections. Pathogen-Associated Molecular Patterns (PAMPs) are molecular structures on pathogens that trigger immune responses against infections (333). In contrast, Damage-Associated Molecular Patterns (DAMPs) are internal signals released from damaged cells, initiating repair and healing (333). Both are recognised by the immune system’s Pattern Recognition Receptors, but while PAMPs signal external microbial threats, DAMPs indicate internal cellular stress or damage (333, 334). When PAMPs and DAMPs are recognised during infection or injury,

immune cells like macrophages and T and B lymphocytes release cytokines (335). These cytokines are signalling molecules that the immune system utilises to regulate cell functions in our body. Among these cytokines, IL-1, IL-6, and TNF-α are notably pro-inflammatory (72). Pro-inflammatory cytokines can recruit immune cells to sites of inflammation, alter gene expression to a pro-inflammatory state, cause immune cell differentiation and promote further cytokine production (336). While these pro-inflammatory cytokines are crucial for battling infections, it is recognised that an excessive presence can result in autoimmune diseases, chronic inflammation, and even altered behaviour (335, 336). Anti-inflammatory cytokines, such as IL-1α, IL-4 and IL-13, reduce inflammation by inhibiting further cytokine release and promoting a T-Helper (Th) 2 cell response. In general terms, Th1 cells drive pro-inflammatory responses, while Th2 cells foster anti-inflammatory actions. Hence, during an infection, an elevated Th1 number helps fight the infection, and following an infection, an elevated presence of Th2 cells aids the body in recovering from the infection or injury (337).

Autoimmunity arises when the immune system targets the body's own tissues as foreign antigens, leading to over 80 different inflammatory autoimmune diseases (338). These diseases affect approximately 7-9% of the population, with Multiple Sclerosis (MS) being the leading cause of disability not caused by trauma in young adults (338-340). In the case of MS, an organ-specific autoimmune disease, the immune system attacks the central nervous system, damaging the myelin sheath and impairing nerve communication (338, 339). Autoimmune diseases are characterised by their clinical heterogeneity

and polygenic nature, involving a combination of genetic susceptibilities and environmental factors (338).

4.1.2.1 Oxidative Stress

Oxidative stress (OS), resulting from an imbalance between ROS production and the body's capacity for detoxification and repair can result in cellular damage and ensuing pathologies (341). Such an imbalance, often magnified during episodes of infection or chronic inflammation, can amplify cellular harm, resulting in chronic conditions including cardiovascular disease, cancer, and diabetes (341). At the cellular level, the body's defence against ROS includes antioxidant enzymes, primarily superoxide dismutase (SOD) and glutathione peroxidase (GP). Among the measurable markers indicating oxidative stress, malondialdehyde (MDA) is an indicator of lipid oxidative damage (342).

4.1.2.2 The Role of Inflammation During Pregnancy

Inflammation plays a crucial role in pregnancy, aiding in essential processes like embryo implantation, which helps the embryo attach to the uterus (343). It's also important in forming the placenta, the organ that provides nutrients to the foetus (344). At the start of the pregnancy, the macrophage profile shifts to an inflammatory M1 state, producing pro-inflammatory cytokines. During most of the pregnancy, the macrophages shift to an anti-inflammatory M2 profile, which is essential for maintaining the pregnancy and healthy development of the baby. As the pregnancy nears its end, inflammation is key in starting labour (345). It helps soften the cervix and triggers uterine contractions, leading to childbirth (345). However, the right balance of inflammation is critical; the

tightly regulated and sequential inflammation which occurs with pregnancy and gestation is very different to inflammation experienced by the mother due to infection or chronic conditions.

4.1.2.3 Inflammation and Brain Development

During brain development, the maternal environment significantly influences the quality of growth. However, immune cells within the developing foetus also contribute to the inflammatory environment. Microglia, a subset of macrophages residing in the CNS, are vital for brain development (346). They play roles in synapse formation, brain repair, immunomodulation, and maintaining neuronal networks. Yet, their dysregulation can harm neuronal cells, releasing inflammatory cytokines and free radicals within the brain (346). Indeed, maternal inflammation during pregnancy, either chronic or acute due to infection or trauma, is associated with many adverse effects in the offspring, specifically neurodevelopmental disorders such as autism spectrum disorder (ASD), Attention Deficit and Hyperactivity Disorder (ADHD) and Shicophrenia. In the review by Han et al., (347), the authors list factors such as pre-eclampsia, obesity, smoking, stress, inadequate sleep and physical inactivity as some risk factors for acute inflammation during pregnancy.

4.1.2.4 Causes of Inflammation During Pregnancy

4.1.2.4.1 Infection

Intrauterine infections during pregnancy, such as influenza, cytomegalovirus, rubella, and lymphocytic choriomeningitis, present significant risks to maternal and foetal health, often leading to serious abnormalities in

the developing brain (348-350). Most infections, like influenza, rarely cross the placenta and still adversely influence foetal brain development (351). This suggests that the maternal immune reaction, rather than the virus itself, may be the factor that disrupts brain development, possibly through an increase in inflammatory cytokines like TNFα and interferons via activation of NF-κB (352). The long-term consequences of maternal infections can be severe, including schizophrenia, autism, neurodevelopmental delays, and reduced scores on mental development indices (353-355).

4.1.2.4.2 Pre-Eclampsia

Preeclampsia is estimated to occur in 2.7–8.2% of all pregnancies and is characterised by proteinuria and hypertension (356, 357). It is the leading cause of maternal and foetal mortality due to its adverse effect on maternal kidneys and liver, as well as resulting in intrauterine growth restriction, premature birth, and low birth weight in the offspring (358-360). Reduced angiogenesis, increased OS and pro-inflammatory cytokines are a major part of pre-eclampsia and have been suggested to be the primary causes of its pathophysiology (361). Indeed, it is proposed that women with pre-eclampsia have an immune imbalance with an increase in pro-inflammatory cytokines, CD4+ T cells and ROS, but a decrease in the anti-inflammatory Tregs and IL-10 (361).

Many studies have shown that the offspring of pre-eclamptic pregnancies have irreversible alterations in neuroanatomy, possibly resulting in altered cognition, with offspring born to pre-eclamptic women being at higher risk of cognitive deficits (362, 363). Indeed, there is a relative risk of 2.77 of developing

ADHD in offspring born to mothers with pre-eclampsia (364). However, it is not known whether these changes in the brain are lifelong, although the Helsinki birth cohort study revealed a significant increase in the reporting of cognitive impairments in those 70 years and over when born to pre-eclamptic mothers (365), while others have shown that pre-eclampsia is associated with dementia in later life (366).

4.1.2.4.3 Obesity

The prevalence of obesity during pregnancy is increasing and around 22% of pregnant women in the UK are considered obese with a body mass index (BMI) ≥ 30 (367). Chronic inflammation during pregnancy is characterised by increased adipose tissue and systemic pro-inflammatory cytokines (NF-κB, IL-6, IL-8 and TNFα) and increased macrophage infiltration of adipose tissue (368, 369). These cytokines also reach the placenta, exposing the foetus to a pro-inflammatory environment. This can lead to placental remodelling and contribute to abnormal growth and poor neurodevelopmental outcomes in the offspring (370). Studies in rodents have shown that offspring born to obese dams have diminished proliferation and neuronal maturation in the cerebral cortex, associated with hippocampal learning deficits and impaired learning while epidemiological studies indicate an association between maternal obesity and risk of neurodevelopmental disorders (371-373). These include ADHD, cerebral palsy, anxiety, depression and autism (373). Undoubtedly, environmental factors such as socioeconomic status, quality of food, home environment and lack of stimulation also need to be considered (374-376).

4.1.2.4.4 Gestational Diabetes

Gestational diabetes mellitus (GDM) is a common pregnancy complication characterised by glucose intolerance, with some estimates approximating as high as 30% of pregnant women worldwide being affected by it (377). It shares common risk factors with type 2 diabetes, such as maternal weight gain, insulin resistance, increased inflammatory markers like high sensitivity C-reactive protein (hs-CRP), and a family history of diabetes, especially in certain ethnic groups (377, 378). The prevalence of GDM is increasing, presenting long-term health risks for mothers and their children, including higher rates of cardiovascular disease and type 2 diabetes, along with growth and neurodevelopmental challenges in offspring (379, 380). Early signs of GDM include an imbalance in pro- and anti-inflammatory mediators detectable in the first trimester (381). This inflammatory dysregulation results in heightened T-cell activation and decreased CTLA-4, suggesting compromised maternal-foetal immune tolerance (381). The role of Tregs in maintaining immune balance during pregnancy further highlights the importance of immune function in GDM development (382, 383). Moreover, the NRF2 pathway, a critical mediator in OS and inflammation, may contribute to GDM's pathogenesis (384, 385). NRF2 dysregulation could influence the metabolic and inflammatory states characteristic of GDM, offering a potential target for early intervention (384, 385). There is also evidence that NRF2 influences beta-cell formation *in utero* (386).

4.1.3 The Effect of Maternal Vitamin D Status on Brain Development.

Considering that both VDR and the enzyme responsible for active VD synthesis, CYP27B1, are found in tissues related to female reproduction, like the ovaries and the placenta, it is unclear at what exact stage VD status influences brain development (387). As previously described, VD can influence the inflammatory status during implantation and the inflammatory status and OS throughout pregnancy. At the same time, there is evidence that the VDR is present in the human brain while mouse studies have shown there is a distinct increase in VDR mRNA expression and protein observed between embryonic days (E) 15 and 23 in the mouse brain, a critical window for brain development (111, 388, 389). Therefore, it is likely that VD influences the foetus's brain development in more than one mechanism.

There is also broad expression throughout the brain of both the VDR and CYP27B1, which synthesises the active form of VD, which binds to the VDR, thereby regulating the transcription of hundreds of genes (390). Likewise, VD is metabolised in neurones, glial cells and astrocytes and is responsible for activating the transcription of numerous genes critical for brain development (391). In support of this, rodent studies have shown that embryos from VDD dams have enlarged brains, possibly due to decreased levels of apoptosis and/or differentiation, and increased proliferation (392, 393). Furthermore, two systematic reviews conducted in 2019 and 2020 (394, 395) concluded that maternal VD status may affect foetal brain morphology, physiology, and behavioural outcomes, including language and motor development, in young children.

The mechanisms by which maternal VD status impacts brain development and function are still unknown. However, in the reviews by Cui et al., (396), and Eyles et al., (397), it was suggested that it might relate to VD's anti-inflammatory and antioxidant activity. Evidence suggests that VD exerts anti-inflammatory effects through non-genomic actions, where activated VDR acts as an anti-inflammatory mediator, and through genomic action, where VD binding to the VDR leads to the transcription of genes associated with anti-inflammatory processes within the brain (398). There is also evidence that VD influences DNA methylation (399). As most cells within the immune system contain the VDR, it is not surprising that there has been extensive study into VD's immunomodulatory effects (400). So, even though VD can reduce inflammation, there is also evidence that a healthy VD status can reduce infection. A case-control study conducted by Gao et al., (401) showed that pregnant women infected by the Hepatitis B Virus had significantly lower serum VD levels when compared with non-infected women. On the other hand, a recent study showed that a higher VD status had protective effects against the transmission of cytomegalovirus in pregnant women (349). However, very few studies link those effects with brain development in utero.

4.2 Aims

As our grasp of inflammation deepens, it's evident that the body's inflammatory regulation significantly impacts health outcomes. VD is now recognised as an essential immune system modulator, traditionally associated with bone health, possessing anti-inflammatory and antioxidant traits. Its role in the maternal immune environment and presence in brain structures indicate the potential pathways through which maternal VD levels might influence foetal brain development. Understanding this interplay is essential, given early-life brain development's lasting effects on cognition and behaviour. The prevalent issue of VDD among pregnant women highlights the importance of proper nutrition for foetal brain health.

This scoping review aims to systematically scope and evaluate the literature on the relationship between maternal VD deficiency, inflammation, and foetal brain development. The primary goal is to identify studies examining the impact of maternal VD deficiency on inflammatory processes and foetal brain outcomes. The methodologies and analytical techniques used in these studies were also assessed to gauge the consistency and strength of existing findings. Through this review, we intend to identify gaps in the current research and highlight areas needing further investigation. We hope to inform and guide future research by providing a clear overview of the existing literature.

4.3 Methods

Unlike a systematic review, a scoping review maps key concepts underpinning a research area. This scoping review aimed to identify primary research into the relationship between maternal VD status, inflammation or oxidative stress and brain development. We followed the Arksey and O'Malley (2005) (402) methodological framework as described, and the PRISMA-ScR Checklist (403) was also used to ensure the repeatability and clarity of the methods.

One limitation of scoping reviews is the challenge of comparing studies due to their broad selection criteria, and in some cases, it may be impossible to establish quality rating criteria. In our review, we refrained from conducting quality assessments on these studies because of their considerable heterogeneity.

4.3.1 Research Question

The research questions were.

1. *Does maternal Vitamin D status during pregnancy influence either inflammation or oxidative stress in both mother or foetus and*
2. *Does that have any influence on brain health, neurones, or behaviour?*

For any study to be included, it had to include data that answered this question.

4.3.2 Identifying Relevant Studies

To maximise the number of studies while simultaneously narrowing down the studies in terms of relevancy, time was taken to create a list of search terms

specific to the three aspects of the question: gestational VD status, inflammation or reactive oxygen species and brain development. This process then resulted in the following search terms:

("Vitamin D" OR "cholecalciferol" OR "calcitriol" OR "ergocalciferol" OR "calcifediol") AND (("brain development" OR "neurodevelopment" OR "neuroprotective" OR "neuroprotection" OR "neurodevelopmental disorders" OR "neurodevelopmental diseases") OR ("neuroinflammation" OR "neuroinflammatory response")) AND (("inflammation" OR "inflammatory response" OR "neuroinflammation") OR ("reactive oxygen species" OR "ROS" OR "oxidative stress" OR "oxidative damage" OR "oxidative injury" OR "oxidative imbalance") OR ("anti-inflammatory" OR "anti-inflammatory effects") OR ("antioxidant" OR "antioxidant properties" OR "antioxidant activity") OR ("inflammatory signalling" OR "inflammatory pathways")) AND ("pregnancy" OR "gestation" OR "maternal" OR "prenatal" OR "placenta" OR "foetal" OR "embryonic" OR "in utero")

Searches were conducted using three databases (Scopus, Web of Science and Pubmed) Between August and October 2023. Following the title screen and abstract selection, the reference list of relevant articles was searched to identify any additional studies.

4.3.2.1 Inclusion and Exclusion Criteria and Study Screening

Any studies which did not fit within the conceptual framework were excluded. This review included studies which measured:

The influence of maternal VD status on antioxidant activity, immune response or ROS in the mother or offspring, and the subsequent influence on brain or neurone health/behaviour/ neurodevelopment in the offspring.

We included studies on both animals and humans and cell culture studies if primary cells were used from animals or humans, and they included VD manipulation. Studies which speculated on the effects of VD on either inflammation or the brain rather than directly manipulating or measuring it were excluded. We also excluded any studies which involved confounding factors such as the use of steroids (e.g., progesterone), other vitamins, and/or minerals. We did not limit the location or date of publication.

After the database searches, duplicates were removed, and then the title and abstract were screened using the defined inclusion criteria stated above.

4.3.2.2 Data Extraction

This review had three parameters: maternal VD status, inflammation, and offspring behaviour/neurology/brain outcomes, so the data extraction was split into those categories. For each study, the first bit of data extracted was the form of VD used, as well as how VD was manipulated, i.e., whether VD was injected, VD administered orally, or VDD induced. The studies were only selected if VD was manipulated in the mothers, so it was also important to extract data on the length of treatment and the stage of pre-natal/pregnancy that VD was manipulated. In some studies, VDD was induced, but the offspring were then subject to VD administration, so this information was also extracted.

In terms of inflammation, data was extracted which either directly measured inflammation/ oxidative stress or induced it. Finally, brain outcomes included any influence on neurodevelopment, neurology, behaviour, defects, or neurotransmitter concentrations. All the data was compiled in two tables to form a summary of all the studies, and to make for easier comparison. We also included if the studies were done only on male mice or rats.

4.3.2.3 Collating results

Study characteristics and data available were tabulated using Microsoft Excel. A diagram was created to summarise the findings. Parts of the diagram were developed by using pictures from the website: Servier Medical Art by Sevier is licensed under a Creative Commons Attribution 3.0 Unported License (https://creativecommons.org/licenses/by/3.0/).

4.4 Results

These studies exhibited substantial heterogeneity in both methods and results, as seen in *Table 4.4.1* and *Table 4.4.2,* encompassing diverse measures of inflammation and their consequences on brain development. There was a notable degree of inconsistency among the studies. Inconsistencies in the methodology are apparent in *Table 4.4.1.* These inconsistencies include but are not limited to, variations in the forms of VD used, diverse approaches to testing brain development, different methods employed to assess inflammatory responses, a predominant focus on male offspring in most studies, variations in the age of the offspring studied, and differences in how VD was manipulated (such as through supplementation, deficiency, or sufficiency diets). Consequently, combining their findings into a definitive conclusion was a challenge. Most of the studies only investigated the effects on the brain in male offspring and excluded females from the studies, which is a clear limitation.

4.4.1 Eligibility of studies

The outcome of the searches and screening is summarised in *Figure 4.4.1.* After the deletion of duplicates and screening of title and abstract using the defined inclusion and exclusion criteria, 28 articles were included in the scoping review. Of these, 20 were subsequently excluded, as more detailed screening indicated that they did not investigate inflammation and/or oxidative stress on offspring brain development. Therefore, eight studies were included in the final scoping review.

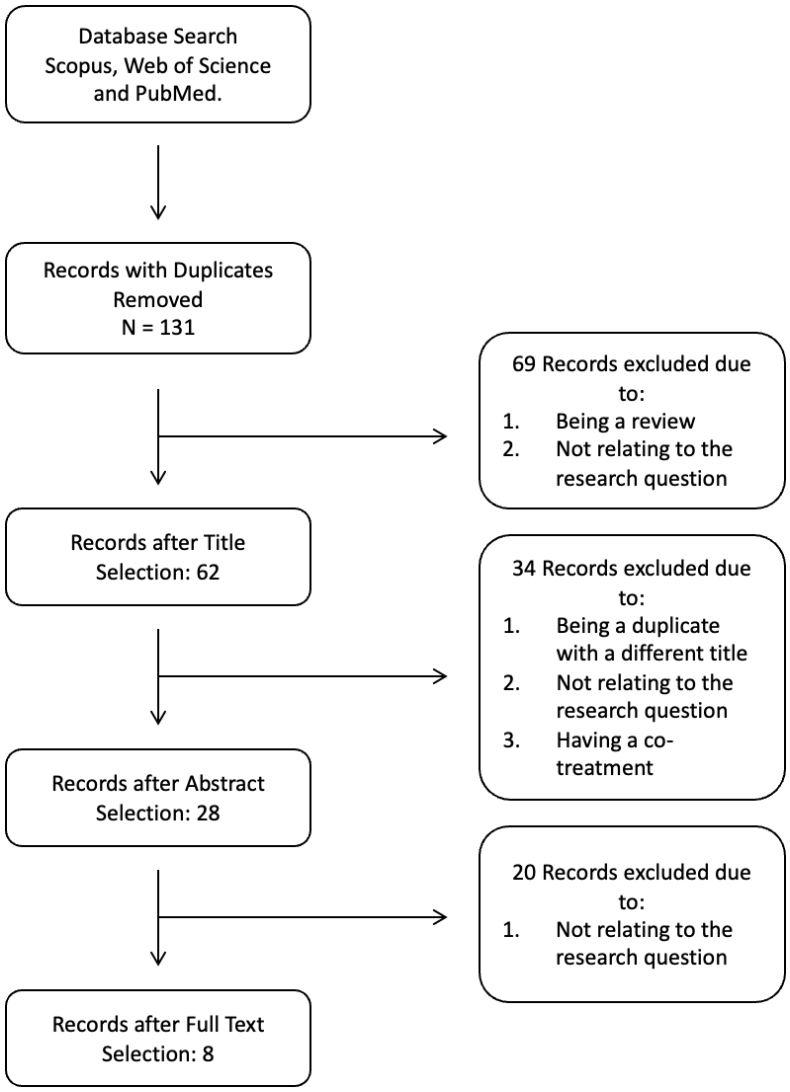


Figure 4.4.1. Flow Diagram for Study Selection

4.4.2 Study Characteristics

All the papers, were published between 2008 and 2023. Six of the studies were *in vivo* studies in rodents (404-409) and two were human prospective birth cohort studies (410, 411). The *in vivo* rodent studies were carried out in Sweden (404), Italy (405), China (406, 408), Iran (407) and Switzerland (409). The two studies in humans were on Turkish (410) and Chinese (411) cohorts.

4.4.3 Methods used by the Studies.

Four animal studies used mice (405, 406, 408, 409) and two used rats (404, 407). Two were human observational studies (410, 411). These methods are summarised in *Table 4.4.1*.

Study	Animal/Population	Intervention (Vitamin D)	Inflammation/ROS Outcome	Brain/Neurones/ Behaviour Outcome
Alessio et al., (405)	C57Bl/6J mice	<5 IU/kg or 1500 IU/kg cholecalciferol 3 weeks before breeding and maintained until culling.	Reactive oxygen species were measured by DCF-DA and d-ROM assays of microglia collected from pups of treated and non-treated mothers.	Primary microglia were collected from pups of treated and non-treated mothers. Tactile threshold was measured
Liang et al., (408)	Cyp27b1 mice or null Cyp27b1$^{-/-}$ or shCyp27b1	Mothers treated with 2200/kg or 0 IU/kg cholecalciferol. Then the offspring would either be placed on 2200/kg or 0 IU/kg diet. Extracted neurones from the offspring were also infected by a VDR knockdown or VDR overexpression lentivirus.	Hypoglycaemia was induced by 35 mg/kg streptozocin. Cytokines were measured in the offspring. Oxidative stress was measured in ***male offspring***	Neurones were extracted from amygdala for ex-vivo experiments. Social interaction and ultrasonic vocalization were measured in ***male offspring,***
Kazemi et al., (407)	Wistar rats	1000 IU/kg cholecalciferol vs 0 IU/kg for six weeks and only rats with cholecalciferol levels < 10 ng/ml were mated	Serum pro-inflammatory cytokines were measured in the ***male offspring***	Elevated plus maze, forced swimming test and open field test. BDNF was measured ***in male offspring***
Chen et al., (406)	ICR mice	All animals were given standard chow but the mothers were given two injections of 1000IU/kg of cholecalciferol	Inflammation was induced by LPS, and inflammatory cytokines measured in maternal sera and amniotic fluid.	Neural Tube defects and supraoccipital bone scores.
Vuillermot et al., (409)	C57BL6/N mice	Pregnant dams on GD9 were injected subcutaneously with 400 ng/kg/2ml 1,25OHD (Calcitriol) or vehicle (VEH). All mice were fed a chow containing 1000 IU/kg.	Injected with poly(I:C) and inflammatory cytokines were measured in ***male offspring***	Elevated plus maze test, modified Y-maze, Marble burying test, cued Pavlovian fear conditioning in ***male offspring***
Adzemovic et al., (404)	Inbred Dark Agouti (DA) rats	1000 IU/kg, 200 IU/kg cholecalciferol and deplete diet 6 weeks before breeding and maintained until weaning, after which offspring were put on regular diet.	Induced autoimmune encephalomyelitis, measured lysosomes from macrophages, t-lymphocytes, and cytokines.	Measured inflammation within the spinal cord and measured the demyelination score and axonal damage.
Mutlu et al., (410)	Human Neonates with HIE and controls	Cord Blood 25(OH)D Levels	Cord Blood Antioxidant Enzymes (GP and SOD), MDA, AOPP	HIE incidence.
Wang et al., (411)	Human MIH-Hefei Birth Cohort	Cord Blood 25(OH)D Levels	Cord Blood Metabolic Markers (C-peptide, HDL-C, LDL-C, TG, hsCRP)	Neurodevelopmental Delay (DDST-II and GDS)

Table 4.4.1. Summary of Study Methods in The Animal and Human Papers Used in This Review.

Data was extracted which corresponded to the subjects of species or human cohort population (***Animal/Population***), the type of Vitamin D, and manipulation technique (***Vitamin D***), the inflammation or reactive oxygen species (ROS) which was measured or manipulated (***Inflammation/ROS***) and the measured effects on the brain or neurones or behaviour (***Brain/Neurones/Behaviour***). HIE: Hypoxic-Ischemic Encephalopathy.

4.4.3.1 Methods of Vitamin D Manipulation

Both human observational studies measured cord blood cholecalciferol levels (410, 411). The animal studies also primarily investigated the impact of cholecalciferol supplementation/deficiency (404-408), with only the study by Vuillermot et al., (409) injecting calcitriol. Doses of VD ranged from 0 IU/kg - 2200IU/kg and were either administered via injection or diet, however the treatments were administered at different time points from pre-pregnancy, during pregnancy and post pregnancy. Furthermore, diets of the offspring often remained as that of the dam except for Liang et al., (408), who weaned pups on either 2200IU/kg or 0IU/kg regardless of the mother's diet. The food was the primary way in which VD was controlled, with Alessio et al., (405), Liang et al., (408), Kazemi et al., (407) and Adzemovic et al., (404) all using either VDD feed or control containing between 1000 IU and 2200 IU. Kazemi et al., (407) only bred rats which had cholecalciferol levels below 10 ng/ml. In the studies conducted by Chen et al. (50) and Vuillermot et al. (53), mothers were fed a standard chow diet. Chen et al. administered 1000 IU/kg of cholecalciferol or a control solution to the mothers via gavage between gestational days 14 and 17. On the other hand, Vuillermot et al. provided pregnant dams with subcutaneous injections of 400 ng/kg/2ml of calcitriol (1,25OHD) on the ninth day of gestation.

4.4.3.2 Methods of Measuring Inflammation, Oxidative stress, or antioxidant levels after VD manipulation.

4.4.3.2.1 Oxidative Stress

Four studies assessed OS or antioxidant enzymes (405, 408, 410, 411). Alessio et al., (405), Liang et al., (408), and Wang et al., (411) conducted various studies to measure OS and related markers in offspring. Alessio et al., (405) used 2',7' –dichlorofluorescin diacetate (DCF-DA) and reactive oxygen metabolites (d-ROM) assays to assess reactive oxygen species (ROS) in microglia from offspring. Liang et al., (408) induced hypoglycaemia in male offspring and measured OS by examining O2- release, 3-nitrotyrosine (3-NT), and γH2AX. Wang et al., (411) evaluated cord blood markers, including C-peptide, HDL-C, LDL-C, TG, and C-reactive Protein (hs-CRP), which are also metabolic markers, some linked to OS. Mutlu et al., (410) focused on cord blood, measuring antioxidant enzymes like glutathione peroxidase (GP) and superoxide dismutase (SOD), along with malondialdehyde (MDA) and advanced oxidation protein products (AOPP).

(404, 406-409)

4.4.3.2.2 Inflammation

Liang et al., (408), Kazemi et al., (407), Chen et al., (406), Vuillermot et al., (409) and Adzemovic et al., (404) measured cytokine levels (IL-1β, IL-6, MCP-1, TNF-α, and MCP-2), including in various samples, including, maternal sera (406), amniotic fluid (406) and offspring serum (407, 408). Adzemovic et al., (404) examined lysosomes from macrophages and T-lymphocytes after injection with myelin oligodendrocyte protein to induce experimental autoimmune

encephalomyelitis (EAE), an animal model for MS. Chen et al., (406) and Vuillermot et al., (409) both aimed to induce maternal immune activation (MIA) by either administering LPS (406) or Polyinosinic: polycytidylic acid (poly (I: C)) (409) to pregnant mice.

4.4.3.3 Methods of Measurements of Brain Outcomes

Two studies extracted cells from male and female offspring of VDD and control mothers at E18 (408) and P2-4 (405) to measure the influence of VDD on the brain. In the study conducted by Alessio et al., (405), primary microglia were collected for analysis from the offspring of both treated and non-treated mothers. Research by Liang et al., (408) involved extracting neurons from the amygdala for *ex-vivo* experiments and measuring social interaction and ultrasonic vocalisation in male offspring. Adzemovic et al., (404) similarly measured gene expression of inflammatory markers, demyelination scores and axonal damage within the spinal cord. Both Kazemi et al., (407) and Vuillermot et al., (409) measured behaviour using an elevated plus maze, however Kazemi et al., (407) additionally used the forced swimming test, and open field test, as well as measuring BDNF levels, while Vuillermot et al., (409) additionally used the modified Y-maze, marble burying test, and cued Pavlovian fear conditioning. Chen et al., (406) evaluated the incidence of neural tube defects and supraoccipital bone scores. Mutlu et al., (410) measured Hypoxic-Ischaemic Encephalopathy incidence at birth, and Wang et al., (411) measured neurodevelopmental progress by using the Gesell Developmental Schedules

(GDS) and Denver Developmental Screening Test II (DDSTII) in the offspring between 6-36 months of age.

4.4.4 Findings from the Studies

The main findings from this scoping review are noted in *Table 4.4.2.* Although the methods were varied, the studies in this review provided some interesting insights into VD's role in inflammation and neuronal outcomes in offspring. In this review, all studies showed that good maternal VD status positively influenced inflammatory status and/or behavioural outcomes. In most studies, apart from Vuillermot et al., (409), VD led to a decrease in inflammatory markers, a reduction in OS, and a beneficial antioxidant profile.

	Vitamin D Control/Measurement	*Inflammation/ROS*	*Behaviour*	*Neuro*
Alessio et al., (26)	*1500 IU/kg cholecalciferol 3 weeks before breeding and maintained until culling.*	↓ beta-gal, ↓ ROS,	↓ Pain Behaviour	↓ Ki-67
Liang et al., (29)	*Mothers treated with 2200/kg. Then the offspring continued the same diet.*	↓ IL-1b, ↓ IL-6, ↓ MCP-1, ↑NRF2, ↑SOD2, ↑ GSG/GSSG	↓ Autistic Traits, ↑ Social Behaviour	
Kazemi et al., (28)	*1000 IU/kg cholecalciferol for 6 weeks before mating.*	↓ IL-1b, ↓ TNF-a	↓ Depression and anxiety like behaviour	
Chen et al., (27)	*Standard chow and two injections of 1000IU/kg of cholecalciferol on GD4 to GD6.*	* ↓ IL-1b, ↓ IL-6, ↓MCP-1, ↓MCP-2, ↓TNF-a	N/A	↑BDNF, ↓NTD
Vuillermot et al.,(412)	*Standard chow containing 1000 IU/kg cholecalciferol. Pregnant dams on GD9 were injected subcutaneously with 400 ng/kg/2ml calcitriol.*	No change.	↑Social interaction ↑Stereotyped behaviour ↑Memory	–
Adzemovic et al., (25)	*1000 IU/kg cholecalciferol 6 weeks before breeding and maintained until weaning, after which offspring were put on regular diet.*	↑ CD4+ ↓ CD45RA+ B, ↓MOG-specific T cells	–	↓ EAE ↓ myelin loss
Mutlu et al., (31)	*Cord Blood 25(OH)D Levels measured. Lower groups compared with highest groups.*	↑GP, ↑SOD2, ↑CRP, ↑ MDA	–	↓ HIE
Wang et al., (32)	*Cord Blood 25(OH)D Levels measured. Lowest groups compared with highest.*	↓ hsCRP	↓ Neurodevelopmental Delay	–

Table 4.4.2. Summary of the Findings from the Studies in this Review.

Results presented are limited to only the results within the studies that are significant, and relevant to the research question. * is indicative of a measurement done within the mother, and all the rest of the results were measured within the offspring. decrease (↓), increase (↑), beta-galactosidase (β-gal), reactive oxygen species (ROS), interleukin-1 beta (IL-1β), interleukin-6 (IL-6), monocyte chemoattractant protein-1 (MCP-1), nuclear factor erythroid 2-related factor 2 (NRF2), superoxide dismutase 2 (SOD2), glutathione (reduced form) / glutathione (oxidized form) (GSG/GSSG), tumor necrosis factor alpha (TNF-α), brain-derived neurotrophic factor (BDNF), neural tube defects (NTD), cluster of differentiation 4-positive T cells (CD4+), cluster of differentiation 45RA-positive B cells (CD45RA+), myelin oligodendrocyte glycoprotein-specific T cells (MOG-specific T cells), experimental autoimmune encephalomyelitis (EAE), glycoprotein (GP), C-reactive protein (CRP), malondialdehyde (MDA), hypoxic-ischemic encephalopathy (HIE), and high-sensitivity C-reactive protein (hsCRP).

4.4.5 Influence of Vitamin D on Inflammation and Reactive Oxygen Species.

4.4.5.1 Immune System and Inflammation

All of the relevant studies found that maternal supplementation with cholecalciferol significantly reduced inflammatory markers (404, 406-409). There was a consistent decrease in inflammatory cytokines after supplementation, particularly IL-1β observed across multiple studies (406-408), while IL-6 and MCP-1 were also found to be diminished in the works of Liang et al. (408) and Chen et al. (406). Despite investigating similar biological avenues, Vuillermot et al. (409) reported no significant changes in cytokines after calcitriol supplementation.

Adzemovic et al. (404) found that VD supplementation changed immune cell population numbers, namely, increased CD4+ cells and decreased myelin oligodendrocyte glycoprotein (MOG) specific T cells. Wang et al. (411) found a decrease in hs-CR levels in neonates' cord blood with higher serum VD concentrations. Hs-CRP is known to be elevated in individuals with systemic inflammation (413).

4.4.5.2 Maternal Vitamin D Status Influences Antioxidant and Reactive Oxygen Species Profiles in Offspring

Mutlu et al., (410) found that GP and SOD levels were reduced in newborns with lower VD status. However, the study investigated HIE; therefore, the difference may be due to the high risk of HIE in the comparison group. In

contrast, in Liang et al. (29), VD supplementation led to an upregulation of the antioxidant markers NRF2, SOD2, and the GSH/GSSG ratio with VD supplementation, suggesting a possible compensatory anti-oxidative response. Alessio et al. (405) observed a reduction in beta-galactosidase (β-gal) and ROS in the offspring of mice maintained on a sufficient VD diet. Given that β-gal is an indicator of cell senescence, which is linked to elevated low-grade inflammation, this decrease in β-gal and ROS suggests a more favourable anti-inflammatory state in the offspring.

4.4.6 Vitamin D Influences Brain Development of Offspring from Mothers with Maternal Immune Activation.

Two studies in this review (406, 409) induced maternal immune activation (MIA). The purpose of activating the mother's immune system (often via administration of substances like LPS or poly (I:C)) is to mimic bacterial or viral infections and then study the impact on the offspring's brain and behaviour. Chen et al., (406) found that continuous exposure to LPS led to a high incidence of NTDs accompanied by disruption of folate transport among foetuses. Remarkably, VD supplementation alleviated the LPS-induced inflammation, increased the amount of folate in the foetuses, and reduced the occurrence of NTDs. Similarly, Vuillermot et al., (409) showed that while VD administration did not alter the pro-inflammatory cytokine levels in maternal plasma or foetal brains, it mitigated the behavioural deficits observed in the poly(I: C) exposed offspring. The finding by Vuillermot et al., (409) suggests that the influence of

VD on brain development is not only through reducing inflammation but directly impacts brain development and behaviour.

4.4.7 Maternal Vitamin D Supplementation Influences Offspring Neurological Health.

Maternal VD supplementation or higher VD status multifacetedly impacted cellular and neurological health. Alessio et al., (405) found that it led to a decrease in Ki-67, indicative of lower cell proliferation rates. Additionally, there is a noted reduction in BDNF and Neurotrophin-3, which are critical to neuronal development and survival in the study by Chen et al., (406). These changes coincide with the study by Adzemovic et al., (404), who found a diminished progression of EAE and a decrease in myelin loss, suggesting a protective role of VD against the mechanisms of demyelination. Furthermore, a higher level of VD in the cord blood of neonates was associated with decreased incidence or severity of HIE, a condition characterised by brain injury due to insufficient blood flow and oxygenation (410).

Maternal VD supplementation or a higher VD status was associated with decreased pain and autistic traits in the offspring and, at the same time, social behaviour was enhanced in the studies by Alessio et al., (405) and Liang et al., (408). Furthermore, Kazemi et al., (407) found an observed reduction in depression and anxiety traits in the offspring. Interestingly, social interaction, stereotyped behaviour, and memory increase in Vuillermot et al., (409) study. There is also a noted decrease in neurodevelopmental delay in the human

cohort study by Wang et al., (411), suggesting an overall beneficial impact of maternal VD supplementation on the developmental outcomes in offspring.

4.4.8 Post-natal Vitamin D Supplementation does not always Reverse pre-natal and Gestational VD deficiency.

In some studies, VD was applied to primary cultures or given postprandially to VDD and control animals. The findings of the various supplementation methods were varied. Alessio et al., (405) showed that VDD led to increased β-Gal expression (a marker of cell senescence and cellular stress (414)) and elevated levels of reactive oxygen species (ROS) in primary microglia cultures when compared to the cultures from control mice. These adverse effects were mitigated by incubating the cells with 100 nM cholecalciferol. The offspring of VDD dams also had reduced tactile thresholds, meaning these mice were more pain-sensitive.

In support, Liang et al., (408) found that while prenatal VDD on its own did not lead to autism spectrum disorder (ASD)-like characteristics, it substantially enhanced the autism-related effects when combined with gestational diabetes. Postnatal supplementation failed to significantly modulate the autism-like behaviour induced by gestational diabetes, which suggests that the effects of prenatal VDD can influence behaviour and are unlikely to be changed postnatally. Furthermore, it was suggested that the offspring of VDD dams exhibited impaired pancreatic development and islet structure with reduced insulin secretion, pointing to structural and metabolic alterations in the pancreas being responsible for this (408).

In the study by Kazemi et al., (407) it was revealed that VDD in mothers led to increased anxiety and depression-like behaviours in the offspring. Additionally, elevated levels of TNF-α and IL-1β were observed in the serum and decreased protein levels of BDNF and VDR in the prefrontal cortex. Nonetheless, exercise or maternal VD supplementation (and combination) reversed these alterations. Importantly, the protein expression was only measured in male offspring, and female offspring were not included in this study.

4.5 Discussion

The predominant consensus among the studies in this review is that a higher VD during pregnancy was beneficial in brain development or function and the immune profile in animal and human offspring. This is particularly relevant given the global prevalence of VDD. It is crucial, therefore, that women of childbearing age maintain adequate VD levels. The precise timing of VD's critical influence during pregnancy remains undetermined, yet the evidence points to its importance throughout gestation.

Research on this subject is sparse, and our scoping review indicates wide variability in the methods. Nonetheless, the data predominantly suggest VD's role in modulating inflammation and supporting neurodevelopment and function. However, the link between the two remains to be elucidated. The evidence points to there being multiple mechanisms in which VD has neuroprotective effects during pregnancy.

4.5.1 A Healthy Maternal Vitamin D Status Reduces the Damage of Maternal Immune Activation.

Chen et al., (406) research demonstrated that VD significantly decreased NTDs in the offspring of mothers experiencing MIA. Vuillermot et al., (409) study, found that VD treatment in mothers with MIA led to offspring with social behaviours comparable to those from mothers without MIA, highlighting the potential normalising effect of VD on behaviour. A similar study by Luan et al., (415) also found that VD supplementation reduced the alteration of DA neurones dopaminergic cells in offspring of mothers who experienced MIA; however, no markers of inflammation were measured in this study. Abnormal

function in DA neurons, a key feature of schizophrenia and ASD, suggests that VD may contribute to healthy brain development by influencing this pathway (416). These studies indicate that VD supplementation in pregnant mothers experiencing MIA is associated with a reduction in neurodevelopmental issues in offspring, including structural, behavioural, and cellular abnormalities. This underscores VD's potential to mitigate maternal immune challenges' impact on foetal brain development. MIA in humans typically occurs when a pregnant woman is exposed to an infection. Such infections, including intrauterine ones, pose serious risks to maternal and foetal health (348). Research, including a review by Han et al., (417), indicates that MIA can independently induce neurodevelopmental disorders (NDDs) in offspring, regardless of the specific infecting pathogen.

The outcomes of MIA are influenced by the timing, intensity, and nature of the immune challenge and the severity of the MIA response. The role of VD in this context is complex, as demonstrated by contrasting findings in studies by Vuillermot et al., (409) and Chen et al., (406). While both studies found VD supplementation improved neuronal outcomes, Vuillermot et al., (409) did not find that VD reduced inflammatory markers in offspring. This suggests that VD's influence on behaviour was not due to the changes in the inflammatory markers measured in the offspring. However, it does not rule out changes in other inflammatory markers not measured. The differences between the studies might be attributed to the type of VD used and the timing of its administration. Vuillermot et al., (409) used calcitriol, the active form of VD, whereas Chen et al., (406) used cholecalciferol, a precursor to the inactive form.

The chemical properties of calcitriol, including its instability and shorter half-life, might affect its efficacy compared to cholecalciferol (418). Moreover, the timing of VD administration and cytokine measurement varied in these studies. Chen et al., (406) measured cytokines 50 hours post-VD, while Vuillermot et al., (409) did so just four hours after VD and poly(I:C) treatment. The earlier time of VD supplementation reduced pro-inflammatory cytokines, implying that gaining VD sufficiency earlier in pregnancy could benefit the developing foetus most. These differences highlight the drawbacks of having so much variation between studies.

4.5.2 VD Status Relates to Inflammatory and Antioxidant Markers.

Mutlu et al., (410) human cohort study showed a positive correlation between increased VD levels and heightened antioxidant enzyme activity in neonates' cord blood, consistent with other studies (39). This underscores the vital role of VD in strengthening the body's defence against oxidative damage (419). Antioxidant enzymes are key in counteracting the potentially harmful effects of ROS, which are necessary in controlled quantities for vital cellular functions, including activating redox-sensitive transcription factors and protein kinases (420). In the context of pregnancy, the placenta's production of ROS is a normal and necessary physiological process (157). However, the balance between ROS and antioxidant capacity is crucial. If not adequately offset by antioxidant defences, an excess of ROS can result in OS potentially damaging the placenta and affecting distant tissues, such as the developing foetal brain (157). Therefore, maintaining an equilibrium between ROS and antioxidants is

essential for foetal development. Further elaborating on this dynamic, Wang et al. (411) study highlights that lower neonatal 25(OH)D levels correlate with increased high-sensitivity C-reactive protein (hs-CRP) levels. Elevated hs-CRP, an inflammation marker, is associated with higher risks of cardiovascular mortality and, according to some studies, cancer (421). Moreover, high hs-CRP levels have been linked to Alzheimer's Disease in certain research, though the exact mechanisms of this association remain to be fully clarified (422).

4.5.3 Vitamin D status can influence Multiple Sclerosis Metabolites.

The studies reviewed suggest that VD plays a multifaceted role in controlling ROS and inflammation during infection, underlining the need to examine the effects of various forms and timing of VD administration on foetal development in MIA scenarios. VD's role in immune regulation is evidenced by its correlation with reduced incidence of specific viral infections. However, its potential influence extends to other immune-mediated conditions, including MS (423). The exact mechanism through which VD influences MS is not definitively established. However, it is observed that MS is associated with an increased activity of Th1 cells (338, 339). Conversely, VD can decrease Th1 levels while enhancing Th2 levels (339). This alteration in the balance of Th1 and Th2 by VD could offer insights into its mechanism of action in MS (339). Notably, the review by Carlberg et al., (339) provides a detailed examination of studies focusing on the epigenetic effects of VD, which might be responsible for this anti-inflammatory shift. Research by Adzemovic et al., (404) has shed light on the effects of VD supplementation in the context of gestational VDD. Their

study found that VD supplementation in young juvenile rats after gestational VDD reduced MOG-Specific T-cells. This effect, however, was not observed in those treated during their early development stages. MOG-specific T cells specifically target cells with myelin and are known to be elevated in animals with EAE (424). Therefore, reducing these immune markers with VD supplementation suggests a shift toward an anti-inflammatory state. The reduction in MOG-specific T cells suggests VD can modulate T-cells in the myelinated neurone domain.

Human cohort studies have shown similar trends. Lower maternal VD concentrations have been linked to a higher incidence of MS in offspring (404, 425-428). These findings in animal and human studies highlight the potential impact of maternal VD levels on the development of MS in offspring. Despite these insights, research in this area remains limited. There is a significant need for more comprehensive studies to fully understand the role of maternal VD in the development of MS in offspring. This is especially important given the implications of VD supplementation during different stages of development and its potential long-term effects on immune-mediated neurological conditions like MS.

4.5.4 Vitamin D Status Can Influence the Severity of Adverse Effects Associated with Non-Communicable Diseases.

Maternal VD levels may influence offspring immunity and impact neurological health through metabolic pathways involved in glucose control. Altered glucose control can precipitate inflammatory responses (15, 16), which,

if unchecked, might risk neural structures and contribute to neurodevelopmental issues (16). The progression from VDD to disrupted glucose metabolism, inflammation, and possible neural impairment presents a vital research frontier, with the findings from Liang et al., (408) opening a promising avenue for further exploration. In a recent systematic review and network meta-analysis, VD effectively improved glycaemic control in adults with T2D (429). There is also evidence that poor glycaemic control leads to a pro-inflammatory state and that VD can potentially reduce this inflammation (430, 431). However, no study apart from by Liang et al., (408) has investigated the impact of that on brain development in utero. In the context of maternal health, the interplay between VDD and the rising prevalence of maternal obesity adds complexity to offspring health outcomes, as obesity can also result in poorer glucose control and VD status. There is evidence that maternal obesity impacts neurodevelopment, and VD levels negatively correlate with obesity due to altered metabolism and distribution of VD in the body (432, 433). Interestingly, VD supplementation has been noted to assist in weight loss (434, 435).

4.5.5 Vitamin D may Influence the Inflammatory Cascade.

Many of the studies in this review found a significant reduction in inflammatory cytokines in mothers with adequate VD (404, 406-408, 415). There may be many possible mechanisms by which VD exerts its effects due to VDR not only affecting the transcription of thousands of genes but also through non-genomic actions through the VDR (436).

Inflammatory signals, such as TNF-α, IL-1β, IL-6, IL-10, and PAMPS are elevated during infection or in individuals with chronic inflammation (437). These signals are recognised by brain cell receptors that, upon activation, initiate IKK, leading to the release of NF-κB into the cell (352, 437). VD's binding to the VDR inhibits the IKK complex, reducing NF-κB activity (438), and enhances IκBα stability, preventing NF-κB binding at transcription sites (439, 440). This reduction in NF-κB activity is beneficial, as NF-κB is a factor linked to neurodegeneration through cell death and damage (441).

Additionally, inflammatory signals trigger the p38-MAPK pathway, which can be inhibited by MKP-1 and upregulated by VD (326, 442). This intricate cascade reveals the potential for maternal VD to modulate inflammatory pathways and protect against neurodevelopmental changes in offspring, a summary of which can be seen in *Figure 4.5.1*.

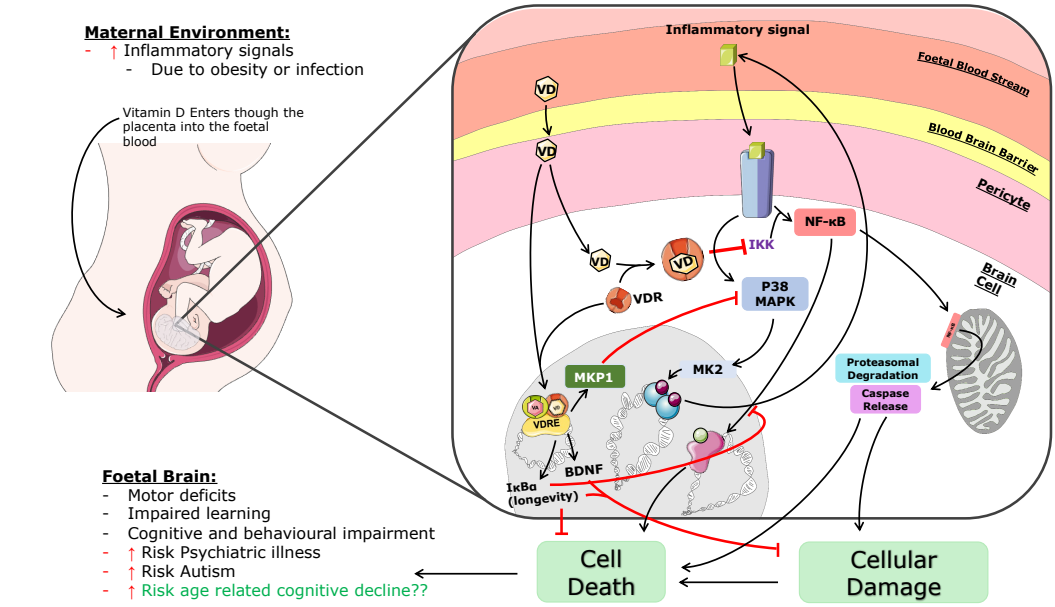


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Figure 4.5.1. Potential cascade of Vitamin D's influence on Inflammatory mediators in the developing brain, genomic and non-genomic effects.

Vitamin D can enter brain cells by crossing the blood-brain barrier. It then modulates inflammatory cytokines (TNF-α, IL-1β, IL-6, IL-10) and inhibits the IKK complex through VDR interaction, thereby reducing neurodegeneration-related NF-κB activity. This also includes reducing caspase activity through reducing NF-κB. stabilising IκBα, hindering NF-κB transcriptional activity, and suppressing the p38-MAPK pathway via MKP-1 upregulation.

4.5.6 Cell Senescence is influenced by Vitamin D status.

Alessio et al., (405) revealed that VDD had profound influences on the function and morphology of microglia. Microglia extracted from the brains of VDD offspring showed increased ß-gal and ROS; interestingly, these effects were reduced when VD was applied.

Increased levels of ROS and ß-gal in microglia are indicators of cell senescence (443). Elevated ROS can damage cellular components, leading to dysfunction. At the same time, increased ß-Gal activity is a hallmark of senescent cells, which have ceased to divide and can disrupt tissue homeostasis (443, 444). In the context of brain development, senescent microglia can have detrimental effects. They release inflammatory factors that can impair the proliferation and differentiation of neural progenitor cells, leading to altered brain development (445). Since VD can also influence proliferation and differentiation, this finding suggests it does so through multiple mechanisms (281). Furthermore, microglia under OS are less efficient at clearing debris and dead cells, which is crucial for normal brain development and preventing neurodevelopmental disorders (446, 447). The balance of microglial activity is essential; too much senescence can disrupt brain development, potentially leading to long-term cognitive and functional deficits (448, 449).

4.5.7 Maternal Vitamin D Status influences BDNF.

BDNF is a vital protein in the brain that supports neuron survival, fosters the growth and differentiation of new neurons and synapses, and plays a key role in learning, memory, and overall brain health (450). Kazemi et al., (407) research, while not the first to propose a link between VDD and an increased risk of depressive and anxiety disorders, contributes to a growing body of evidence, including several meta-analyses, supporting this association (451, 452). VD deficiency may impact brain function via multiple pathways in the central nervous system, potentially involving BDNF. Kazemi et al., (407) also reported significantly lower levels of VDR and BDNF in the offspring of VD-deficient mothers. VDR is crucial for regulating genes associated with cognition, neurogenesis, and behavioural functions, and its dysfunction has been connected to abnormal social, emotional, and motor behaviours, though the specific effects on the brain remain a subject of ongoing research (398, 453). The connection between VDD, inflammation, and the rise in depressive and anxiety behaviours remains ambiguous, with questions about whether these effects are interconnected or independent. Moreover, BDNF is upregulated by VD, which has been shown to mitigate brain dysfunction related to ageing (454). It can be inferred that the protective effects of VD, such as minimising neuronal death and damage, may contribute to mitigating the detrimental consequences of inflammation in the brain.

4.6 Conclusion

In conclusion, the existing evidence, encompassing two human and six rodent studies, isn't ample enough to draw definitive conclusions about the influence of maternal VDD on neurodevelopment via inflammation. However, the prevailing data consistently underscores the benefits of maintaining optimal VD levels for enhanced brain development, possibly through modulating inflammation, managing ROS, or other routes. Significantly, no studies indicate any detrimental effects of VD. This highlights the potential for fostering cognitive development and possibly shielding against various diseases and infections. As research in this area progresses, a deeper understanding of these complex interactions and their implications for paediatric health is expected.

Notably, the number of fitting studies for this review is limited, with high observed variability. Much of the reviewed literature resonates with other studies, suggesting VD's positive effects on immune processes, such as modulating autoimmune diseases like multiple sclerosis and bolstering immune defences during infections. One clear takeaway from this review is the significant variability across studies, especially regarding inflammation. Yet, it's evident that optimal maternal VD levels create an environment favourable for healthy foetal brain development. Emerging data suggest potential mechanisms through which VD imparts its protective influence, including its antioxidant, and anti-inflammatory attributes, and direct impact on neurogenesis markers.

There is a significant body of evidence that VD directly influences neurodevelopment. However, with the studies in this review, there is emerging evidence that VD can also influence inflammation, which can contribute to the formation of an optimal environment for brain development in utero. For example, researchers observed notable improvements in offspring with higher VD levels (406-411). Specifically, there was an increase in antioxidant enzymes and a decrease in pro-inflammatory cytokines and metabolites of OS in offspring. These changes collectively contribute to a reduction in cellular stress. This is particularly beneficial in the developing brain, where cellular stress can lead to various adverse effects (455, 456). Indeed, all studies which measured inflammatory, or OS stress markers found that VD supplementation resulted in better behavioural or neurological markers, suggesting a possible link (406-411).

Offspring of mothers with MIA but with sufficient VD levels showed a substantial reduction in NTDs compared to those with VD deficiency, which was associated with improved folate transport mechanisms (406). Although public health measures like folate fortification are in place, NTDs remain a global health concern due to their association with serious neurological damage (457). Hence, achieving any substantial decrease in the incidence of NTDs is crucial for preventing these debilitating conditions.

Within the brain, maternal VD status significantly influences the microglia, the amygdala and the pre-frontal cortex regarding inflammation and ROS. Maternal VD supplementation resulted in microglia with reduced ROS and

beta-Gal formation (405). These two factors decrease cell senescence, and maintaining a balanced senescence homeostasis is critical for optimal neurodevelopment and healthy ageing (443). With maternal VD supplementation, there was also a rise in VDR and BDNF (406). A higher level of BDNF is beneficial in neurodevelopment and neuroplasticity (458). Within the mouse amygdala, maternal VD supplementation also resulted in reduced oxygen-free radicals and increased GSG/GSSH ratio, Nrf2, and Heme oxygenase 2 (HO-2), all of which also contribute to healthy cellular homeostasis, reducing cellular stress (459-461).

We hypothesise that these effects on the inflammatory profile of the developing brain are partly the reason for the beneficial effects seen on the developing brain. The outcomes of the studies included reduced cellular proliferation, indicated by lower Ki-67 levels, and an increase in neuronal development factors like BDNF, aligning with a decrease in myelin loss and slowed progression of demyelination conditions, such as EAE. Additionally, higher VD levels in neonates correlated with a lower incidence of brain injury from HIE. Offspring also benefited from maternal VD with fewer pain and autistic traits, improved social behaviours, and reduced neurodevelopmental delays. A schematic overview, *Figure 4.6.1*, visually represents our hypothesis.

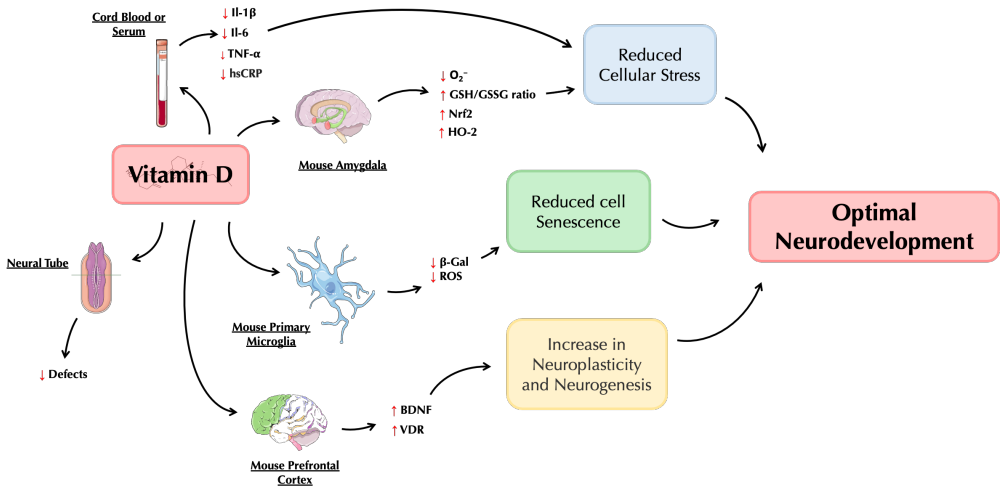

Figure 4.6.1 A Schematic Summary of the Markers Measured within this Scoping Review Relating to the Results and their Link to Improved Neurodevelopment

In the context of this review, with a higher maternal VD status, there is a notable reduction in cellular stress, signifying VD's potential as a protective agent against cellular damage. Concurrently, VD is instrumental in diminishing cell senescence, promoting cellular longevity and ensuring sustained functionality. Further elucidating its neuroprotective capabilities, VD augments neuroplasticity and accelerates the process of neurogenesis, highlighting its influence in enhancing the adaptability and regenerative capacities of the brain.

4.7 Limitations and Future Considerations

This review cannot compile data for direct comparison as there is so much heterogeneity. We did not attempt to review the quality of the studies in this review, as there was very little to go off to establish a good quality criterion. The methods used to manipulate VD status in animal and human studies vary widely. This variability includes dosage, timing, and mode of administration, which can lead to inconsistent results and complicate data synthesis. Studies assessed a broad range of outcomes related to inflammation, brain function, and behaviour, making it difficult to create a unified framework for analysis. The variability in outcome measures complicates the synthesis of findings. However, this was unavoidable due to the limited number of studies in this area.

Good quality studies are needed to study this intricate relationship. Future animal studies must include both sexes, as some uncovered relationships may exist within this topic.

5 The Effect of Maternal Vitamin D Deficiency on Offspring Body Composition, Brain Morphology and Behaviour in Mice

5.1 Introduction

During pregnancy, the foetus depends on maternal support to provide an optimal nutritional environment for growth and development, including brain development and function (462, 463). Current research provides substantial evidence indicating that undernutrition, specific health conditions, stress, and drug misuse during pregnancy can negatively impact brain development and subsequent behaviour in offspring. Consequently, it is recommended that pregnant women avoid exposure to these detrimental factors (464-466).

5.1.1 Vitamin D and Brain Development

Due to the difficulty in both human neuroscience research and the fact that VD is not exclusively obtained through diet (as the skin also synthesises it), it has been difficult to study the influences of VD on human brain health, let alone development (467). Therefore, most systematic reviews on this topic have concluded that the evidence for VD's beneficial effect on brain development is inconclusive. For example, the review by Pet et al., (468) found that although there were "subtle" effects of maternal VDD on offspring brains, the evidence is limited by findings which depend on ethnicity, species of animal used and the age of the offspring being studied. A more recent review focussing on gestational VD levels and offspring psychiatric outcomes found some evidence that VDD during pregnancy is associated with an increased risk of developing

ADHD, schizophrenia and ASD (469). Even more recently, a meta-analysis investigating the influence of prenatal VD on offspring neurodevelopmental outcomes concluded that increased prenatal VD exposure is linked with enhanced cognitive development and reduced risk of developing ASD and ADHD (207). However, all the reviews concluded that the evidence is limited, and studies with larger sample sizes and longitudinal designs are required to draw conclusions (207, 468, 469).

5.1.2 Insulin Sensitivity, Vitamin D and Brain

Insulin, a key hormone in glucose metabolism, facilitates glucose uptake into muscle and fat by stimulating the exocytosis of GLUT4 transporters (470). Disruptions in insulin regulation due to modern lifestyles characterised by over-nutrition and sedentariness can lead to metabolic syndrome, obesity, type 2 diabetes mellitus (T2DM), and cardiovascular diseases (471). Insulin resistance, where cells become less responsive to insulin, often precedes the development of T2DM (472, 473). Moreover, insulin resistance in children has been linked to reduced glucose metabolism in brain regions related to autism, suggesting a connection between metabolic health and neurodevelopmental disorders (474, 475). Additionally, there is growing evidence that VDD is an independent predictor of insulin resistance in children, which may implicate VDD in the development of such brain-related conditions (476, 477). The complexity between VD levels and insulin sensitivity was touched on in the review by Tai et al., (478), who concluded that although there is an association between low

plasma VD and higher glucose concentrations, most of the evidence is conflicting and requires more study.

The role of insulin extends beyond metabolic regulation, reaching into neurodevelopmental and neurodegenerative diseases like dementia and Alzheimer's (479). These conditions have been associated with altered insulin signalling in the brain, affecting cognitive functions and suggesting a broader implication of insulin resistance on brain health (479). Evidence shows that VD curbs inflammation and protects the epigenome by averting gene hypermethylation linked to diabetes (480). VDD can compromise these protective mechanisms, elevating the risk of developing diabetes and potentially exacerbating the damage hyperglycaemia can cause (481).

5.1.3 Differences and Similarities between VD Metabolism and Signalling in Mice and Humans

In humans and mice, VD synthesis occurs upon sunlight exposure, despite mice's nocturnal habits and fur coverage limiting their sun exposure relative to humans and dietary sources also contribute to VD levels in both species (482). The VDR gene in humans consists of six exons (1a-1f), with exon 1a being non-coding but responsible for transcription initiation. VDR gene transcription is pivotal in calcium-phosphate homeostasis, immune function, and hair growth regulation in both species, suggesting similar functions (483, 484). The *in-silico* work by Halsall et al., (483) compared VDR gene conservation between mice, rats and humans and concluded that the VDR gene is well conserved between humans, mice and rats, with the 1a, 1d and 1c exons being strongly conserved,

but with exons 1b, 1f and 1e being poorly conserved, although the promoter region for exon 1f was highly conserved between species. The complexity of the VDR gene is greater in humans as the VDR gene in rats does not have the 1f and 1c exons, and with mice not containing 1f (483). This additional complexity in humans leads to the generation of diverse VDR protein isoforms through alternative splicing, influencing VD's role in various physiological processes differently than in mice (483). Thus, while murine models provide critical insights into VD biology, they represent one piece of the larger puzzle in understanding its comprehensive role in human health and disease. Additionally, mutations in the VDR and CYP27B1 genes in humans lead to specific diseases like hereditary Vitamin D-resistant rickets (HVDRR) and Vitamin D Dependent Rickets type 1 (VDDR-I), with phenotypic manifestations differing from those seen in murine models (482).

Despite these differences, utilising mouse models in VD research is beneficial due to their genetic and physiological similarities to humans, which allow for exploring VD's roles in various biological processes under controlled conditions (485). Mouse models are invaluable in dissecting the molecular pathways regulated by VD and VDR, contributing to our understanding of the impact of Vitamin D Deficiency (VDD) (483-485). Moreover, they offer a platform for testing therapeutic interventions that are cost-effective and ethically more feasible than human studies (486).

5.1.4 Neurodevelopmental Disorders Associated with Vitamin D Deficiency

5.1.4.1 Autism Spectrum Disorder

ASD is a neurodevelopmental disorder characterised by impairments in social communication and interaction, sensory anomalies, repetitive behaviours, and often varying levels of intellectual disability (207). Globally, the prevalence of autism is estimated at 1 in 132 individuals, equating to approximately 52 million people worldwide (487). This prevalence is consistent across different regions, ethnicities, and socioeconomic backgrounds, although the most recent data suggests prevalence is increasing in high socio-economic status countries (487, 488). Autism is caused by both genetic and environmental factors, with over 1,000 associated gene polymorphisms identified, yet 70% of individuals with ASD lack these polymorphisms, indicating that the environment has a greater influence (489). A link between VD status and ASD was identified when children with ASD were observed to have lower VD levels (490). This discovery prompted further studies to assess VD levels in pregnant mothers and the subsequent ASD risk in their children. Meta-analyses' conducted by Garcia-Serna et al., (491) and Wang et al., (492) substantiated these observations, confirming an inverse relationship with lower VD levels in mothers correlating with a higher risk of ASD in their offspring.

The potential mechanisms through which VD may influence ASD are multifaceted. Genetically, VD influences gene transcription, and certain

genotypes related to VDBP and significantly lower 25(OH)D concentrations are more prevalent in individuals with ASD (493).

VD has been shown to reduce the proliferation of neuronal cells; deficits in VD may contribute to the characteristic brain overgrowth in early ASD development (204, 205, 494-496). Additionally, VD modulates neurotransmitter systems and imbalances implicated in ASD by affecting the synthesis and metabolism of key neurotransmitters such as serotonin and dopamine and is involved in cognition and behaviour (497, 498). Immune dysregulation, a noted feature in ASD (499), may also be influenced by VD through its immunomodulatory properties, details of which are discussed in Chapter 4. VDD can alter the immune system, potentially exacerbating autoimmune responses and inflammation observed in ASD (500).

5.1.4.2 Schizophrenia

Schizophrenia is a complex mental disorder and has a global lifetime prevalence of about 0.4% (501). Characterised by a spectrum of symptoms divided into positive (such as delusions and hallucinations) and negative (including social withdrawal and anhedonia) traits (501). Despite the availability of antipsychotic medications designed to alleviate these symptoms, treatment outcomes are often suboptimal due to low response rates, delayed efficacy, and the potential for severe side effects (502). The disorder's aetiology remains elusive, with traditional biochemical research focusing on dopamine and glutamate pathways (503). The neurodevelopmental hypothesis of schizophrenia has a large evidence base, with a lot of consensus being that

environmental triggers such as infection, stress or drug misuse in the late first or early second trimester during pregnancy can result in neural circuit pathology (504). There is also evidence that the brains of those suffering from schizophrenia have abnormal features, such as larger ventricles and differences in the hippocampus and cingulate cortex, as well as abnormal distribution of white matter in the frontal and temporoparietal regions of the brain (505).

VD was first linked to schizophrenia when researchers noticed that those born in the winter months were around 10 – 15% more likely to develop schizophrenia; in addition, neonates with VDD also had a higher risk of developing schizophrenia in adulthood (263, 501). This suggests that sufficient VD levels may be required for optimal brain development. Effectively studying the influences of gestational VDD to the point of conclusion in humans is unlikely, as schizophrenia symptoms start to emerge usually after early adulthood (506). Therefore, studies would have to take place for at least two decades, and due to the low prevalence of schizophrenia, would require many participants (506). Although there have been a few epidemiological longitudinal studies, most of the work has been done in animals (506). In animal models, gestational VDD often results in abnormal brain structures linked to upregulated proliferation and reduced neurite outgrowth (263). VD has also been shown to regulate dopaminergic (DA) cell maturation during brain development in rodents (206, 507). Therefore, further study in humans and animals is warranted to gain a stronger evidence base to encourage supplementation.

5.1.4.3 Anxiety and Depression

Those with anxiety often deal with intense fear and nervousness and tend to avoid situations they believe to be threatening (508). Depression is a mental health disorder characterised by persistently low mood, loss of interest in activities, and significant impairment in daily functioning (509). These disorders are the most prevalent worldwide and often co-occur (510). Impaired serotonin release, function and uptake are associated with anxiety and depression (511, 512). Currently, antidepressants and anti-anxiety medications are prescribed to treat these conditions; however, there is evidence that these drugs have adverse long-term effects, such as weight gain or loss, dry mouth and sexual dysfunction (513).

5.2 Aims

The primary aim of this study was to elucidate the effects of maternal and subsequent VD status on neurodevelopmental outcomes in mice. Specifically, we sought to understand how VDD, during gestation and throughout the offspring's life, impacts the offspring's brain structure and function, as indicated by measurements of ventricle size and markers of neural cell differentiation and proliferation. Additionally, we aimed to investigate the consequences of VD levels on body composition, insulin sensitivity and survival rates in offspring. The study also focused on behavioural implications, particularly how prenatal VD status may influence anxiety-like behaviours and memory functions in the progeny. A further objective was to assess the sex-specific effects of VD on these developmental outcomes.

5.3 Methods

5.3.1 Materials.

Materials were purchased from Sigma-Aldrich, (Dorset) unless stated. Phosphate Buffered Saline (PBS) concentration was always at 0.1M and prepared by dissolving a PBS tablet in 200 mL of dH2O and then autoclaving. The anti-freeze stock comprised 600 ml Ethylene glycol, 600 ml Glycerol and 800 ml PBS.

5.3.2 Power Calculations

Power calculations were conducted using GenStat 21st Edition, based on standard deviations from previous pilot data. The probability of a type I error (α) was set at 0.05, and that of a type II error (β) was capped at 0.20, ensuring a power of over 0.80. To ensure the detection of significant differences, a power calculation was based on our pilot data assessing the influence of maternal vitamin D on anxiety-like behaviours. The mean % spent in open arms was 25.8 and 19.9% for control and VDD, respectively, with a Standard Deviation (SD) of 3.27%, indicating the necessity of 8 mice per sex to yield sufficient and meaningful data for all of the experimental groups. To explore the effects of diet on offspring health throughout their lifespan, a minimum of two parental groups (control and treatment) were required, each expected to produce approximately four offspring per litter, with an equal sex distribution (two males and two females). Consequently, to generate a total of 136 offspring, 34 female mice per treatment group were needed. However, accounting for natural variations in litter sizes and potential losses during

gestation or at birth, the study utilised 42 female mice per treatment group to ensure adequate sample size and robustness of the data.

5.3.3 Animal Husbandry

Forty-two virgin C57BL/6 mice, approximately 3 weeks old and weighing 14-16 grams, and sixteen stud males aged 12 weeks were sourced from Charles River, UK. Both sexes were maintained under controlled conditions (temperature: 21-23°C; light: 12:12 dark cycle, lights on at 0700h). Virgin females were pair-housed and had ad libitum access to either a control diet (2.2IU/g vitamin D, TD.89123) or a vitamin D deficient diet (0.0IU/g vitamin D, TD.89124), both from Inotiv, Leicestershire, UK. In contrast, stud males were singly housed and given *ad libitum* access to the control diet. Food intake and body weight were monitored biweekly. All experimental procedures complied with the Animals (Scientific Procedures) Act of 1986, under project license PP6562343.

5.3.4 Breeding Procedure and Maternal Vitamin D Analysis

After a week of acclimation, virgin female mice were fed either a control (n=20) or VDD diet (n = 22) for 7 weeks before mating with stud males for a week. Pregnancy was confirmed upon visualisation of a mucus plug. Pregnant mouse dams were maintained on their pre-pregnancy control or VDD diet throughout pregnancy and lactation. At weaning, the offspring were again maintained on the same diet as their mothers, either control or VDD diet, until euthanasia at 3, 6 or 12 weeks of age. All mouse dams were euthanised by gaseous terminal anaesthesia at weaning, as described in Section 5.3.5.

5.3.5 Euthanising Procedure

All animals were euthanised in a chamber containing 5% isoflurane gas. Blood was collected by cardiac puncture into 1.3ml plastic lithium heparin tubes (TekLab, County Durham). Plasma was separated by centrifugation (30,000rpm, 10 minutes), before being snap frozen and stored at -80°C for the measurement of 25-hydroxyl vitamin D by enzyme-immunoassay (Immunodiagnostic System, Boldon, UK) according to the manufacturer's instructions.

5.3.6 General Methods for Offspring Analysis

The body weight of all offspring was measured from postnatal day (PD) 4 twice a week throughout the study, as well as food intake from weaning. At weaning, the sex of the offspring was assessed, and mice were paired with the same sex from a different litter unless it was impossible to do so. In this study, pairs of subjects were assigned randomly to one of four experimental groups. The first group was culled without undergoing any tests. The second group, consisting of 8 to 33 subjects per group, was evaluated for muscle function at 3, 6, and 12 weeks of age, using a grip strength test (this specific test will not be covered in detail in this chapter). The third group, comprising 16 subjects per group, was subjected to behavioural assessments, including the Y maze and elevated plus maze tests, conducted at 3 weeks (corresponding with weaning), 6 weeks, and 12 weeks. Finally, insulin tolerance tests (ITT), with 14 to 15 subjects per group, were performed at 6 and 12 weeks of age. A summary of the procedures can be seen in *Figure 5.3.1.* Any deaths were recorded. However, the cause of death was not recorded before 3 weeks. Survival was

determined by dividing the number of animals that survived until analysis by the total number of offspring born in each diet group.

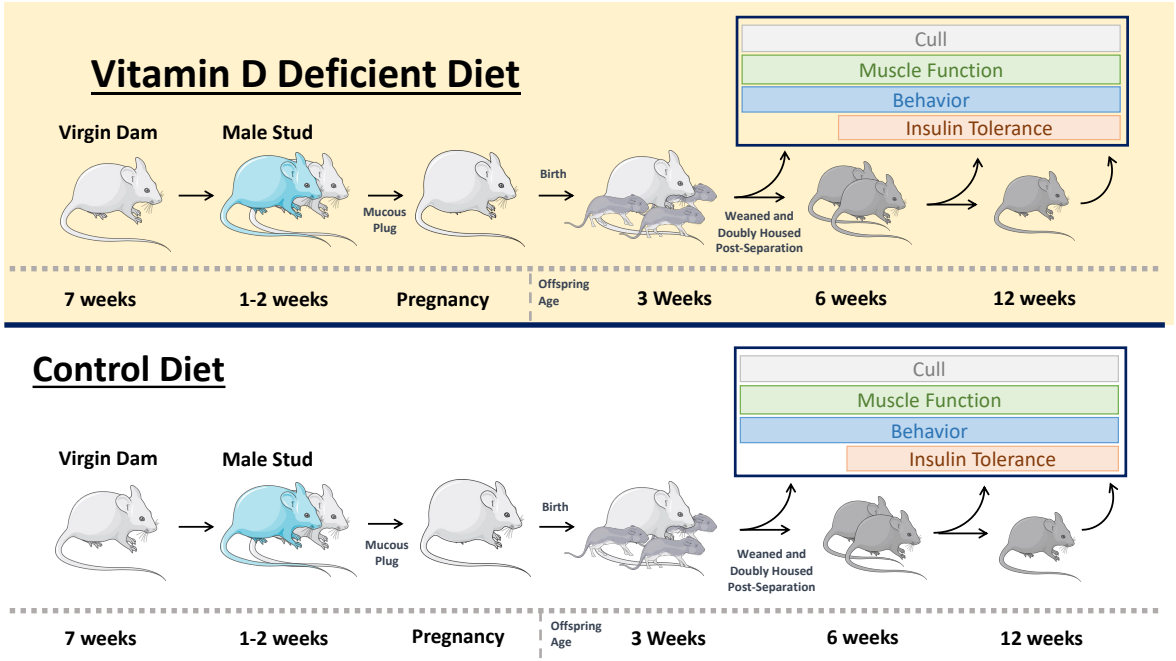

Figure 5.3.1. Visual Summary of the Animal Trial Timings and Procedure.

This figure provides an overview of the experimental procedures and measurements conducted. Body weight was monitored bi-weekly, and food intake was recorded post-weaning. Offspring were sexed at weaning and paired with same-sex individuals from different litters for random assignment into four experimental groups. Group 1 was culled without testing. Group 2 (8-33 subjects/group) underwent muscle function evaluations at 3, 6, and 12 weeks via grip strength tests. Group 3 (16 subjects/group) participated in behavioural assessments (Y maze and elevated plus maze) at 3, 6, and 12 weeks. Group 4 underwent insulin tolerance tests (ITT) with 14-15 subjects/group at 6 and 12 weeks.

5.3.7 Elevated Plus Maze

The elevated plus maze test was used to evaluate innate anxiety-like behaviours in mice (514). The maze was made of black 6 mm Perspex with four arms of 45 cm, radiating from a central square with dimensions 15 x 15 cm. This maze was raised 70 cm off the ground and featured two opposite arms enclosed with black walls of height 10 cm, while the other two were open and unenclosed.

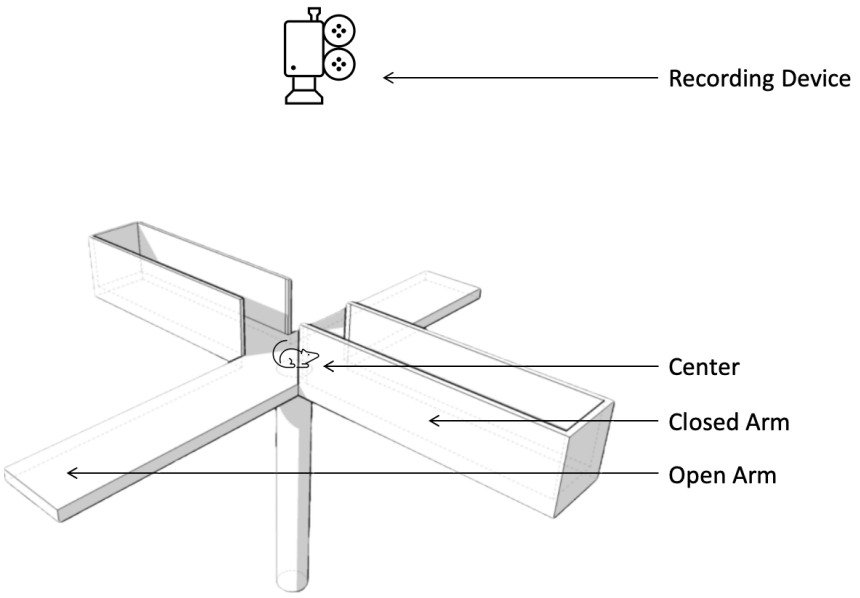

Figure 5.3.2. The Elevated Plus Maze.

Illustrated is an elevated plus maze with a Perspex of 6 mm, consisting of a central square measuring 15 x 15 cm, from which four 45 cm arms extend. Elevated to a height of 70 cm, the structure includes two opposite arms with 10 cm high walls and two arms that remain open without any enclosing barriers. A recording device is placed above, tracking the mice.

Before testing, mice were placed in the testing room containing the maze for 30 minutes to acclimatise and reduce stress. Each mouse was placed at the entrance of one arm and allowed to move freely for a 5-minute session. After each mouse, the maze was cleaned with 75% EtOH. The testing was carried out

at 3, 6, and 12-week intervals, ensuring that no mouse underwent more than two behavioural tests at each time point to prevent confounding interactions.

A digital camera positioned above the maze captured the activity. Behavioural parameters were measured with EthoVision video tracking software (Noldus Ethovision XT ver 7.1). Software determined the latency to enter an open arm, the duration spent in open versus closed arms, and the total distance moved within the open arms compared to the closed arms.

The percentage time and entries in the open arms (open/(open + closed) were calculated from the raw data. All data was analysed by a 2-Way ANOVA using GraphPad PRISM.

5.3.8 Spontaneous Alternation: Y – Maze

The Y-maze test evaluated mice's exploratory behaviour and cognitive function based on their natural tendency to alternate arm entries when placed in a novel environment. The Y-shaped maze had three white Plexiglas® arms at a 120° angle from each other (41.5 cm in length and 6 cm in width with 15 cm walls). The start point (6 cm x 7.5 cm) was in the maze's centre.

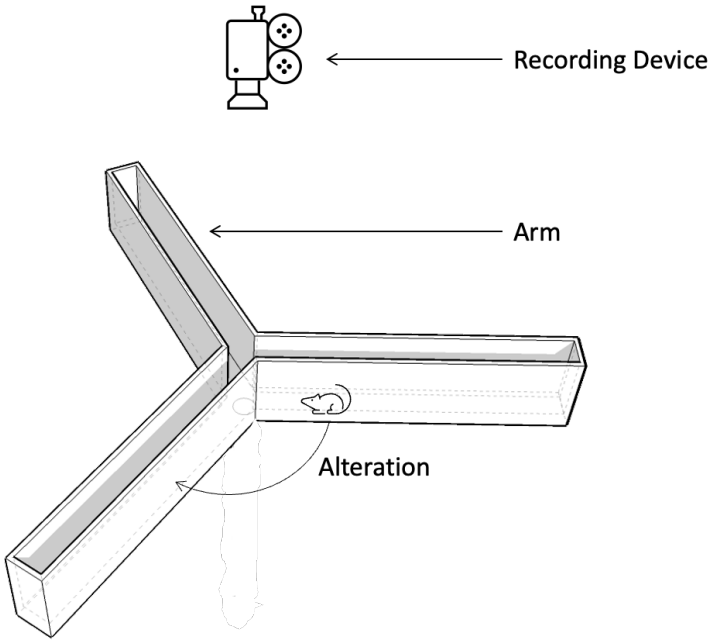

Figure 5.3.3. The Y-Maze

Depicted is a Y-shaped maze constructed from white Plexiglas®, with three arms set 120° apart, each 41.5 cm long and 6 cm wide, enclosed by 15 cm high walls. The maze's central starting point measures 6 cm by 7.5 cm. A recording device is placed above, tracking the mice.

On the day of testing, the animals were habituated to the testing room for 30 minutes before the behavioural assessment. Each mouse was placed at the entrance of one arm and allowed to move freely for a 5-minute session. After each mouse, the maze was cleaned with 75% EtOH. The testing was carried out at intervals of 3, 6, and 12 weeks, with no mouse subjected to more than two

behavioural tests at each interval to prevent test-test interactions. Whenever possible.

A video camera recorded the session (Noldus Ethovision XT ver 7.1). The software determined the alternation rate, which measures arm entries without immediate repetition. It indicates maintained cognitive abilities, requiring the mice to remember the previously entered arm. Therefore, the latency to enter an arm and the total time spent were measured. It also recorded the total distance moved and average velocity. The distance moved, and the software produced average velocity values, and raw values were used for statistical analysis. The percentage alternation (spontaneous alternation divided by total alternation x 100) was calculated from the raw data.

5.3.9 Insulin Tolerance Testing in Offspring

An insulin tolerance test was conducted at 6 and 12 weeks in another group of offspring mice *(n=6-8* per diet group per sex) as described by Benedé-Ubieto et al., (515). Local anaesthesia agent EMLA® (AstraZeneca) was applied before the tail vein puncture to eliminate pain and stress. Following a 6-hour fast, mice received an intraperitoneal (IP) injection of insulin (0.75IU insulin/kg body weight, Actrapid 100IU/ml, Human insulin, Novo Nordisk) and tail vein blood was obtained before the injection (time 0) and after 15, 45 and 90 minutes post-injection for measurement of blood glucose using an Alpha Trak blood glucose monitor (Zoetis, Surry, UK) (516).

If glucose levels fell below 1mmol/L or signs of hypoglycaemia like tremors or hunched posture appeared, mice received an IP injection of 10% glucose solution and were removed from the analysis. Following the final glucose measurement at 90 minutes, food and a mash of crushed food pellets and water were returned to the cage. The animals were closely observed for the first six hours post-injection and again after 24 hours for food intake, body weight, and any adverse behaviours such as lack of grooming or changes in faeces. Mice at 12 weeks old were culled after the 90-minute mark or earlier if they exhibited symptoms of hypoglycaemia.

5.3.10 Tissue Weight and Blood Collection in Offspring.

Offspring at 3 (weaning), 6 and 12 weeks of age were euthanised, as described in Section 5.3.5. Various tissues, including the brain, liver, kidneys, brown fat (BAT), abdominal fat (WAT (AB)), intrascapular fat (WAT (IS)), along with gastrocnemius and soleus muscles, were dissected at room temperature, weighed, and immediately snap frozen on dry ice. Brain samples from half the mice were placed on a metal block on dry ice to maintain the structure, and brains from the other half were placed in a 4% paraformaldehyde solution. All snap-frozen samples were stored at -80°C for further analysis.

5.3.11 Assessment of Plasma Vitamin D Levels in Offspring

Plasma vitamin D (25-hydroxyvitamin D) was determined using a commercial enzyme immunoassay (EIA) kit (immunodiagnostic Systems). Calibrators, buffers, and reference preparations were supplied in liquid form and ready to use, and all incubations were conducted at room temperature. Calibrators, buffer, reference preparations and samples were diluted with 25-hydroxyvitamin D Biotin solution (1:40) and vortexed for 10 seconds. 200 µl was added to appropriate wells in the antibody-coated 96 well plate provided, sealed, and incubated for 2 hours. Incubated plates were washed thrice with wash solution before adding 200 µl of enzyme conjugate to all wells for a further 30-minute incubation. Three washes followed this before 200 µl TMB substrate was added for 30 minutes. All reaction was stopped by adding 100 µl of stop solution to all wells. Absorbance was measured at 450nm with a microplate reader (Omega, FlUOstar, BMG LabTech, Ortenberg), and a calibration curve was evaluated for linearity to obtain vitamin D levels. The

results from the EIA were analysed using a t-test for the maternal plasma and a three-way ANOVA for the offspring plasma (measuring the effect and interaction between diet, time, and sex).

5.3.12 Preparation of Brain Tissue for Ventricle Size Assessment and Immunocytochemistry

Half of the collected brain samples were immersed in 4% paraformaldehyde for fixation, roughly 24 hours. Post-fixation, these samples were dehydrated in a 30% sucrose solution until they settled at the vial's bottom. Subsequently, the brains were transferred to a PBS solution and stored at 4 °C.

5.3.12.1 Sample Preparation using the Cryostat.

Only brains of offspring culled at 6 weeks were analysed (n=3 for Controls and n=4 for VDD). To prepare the brain sample for sectioning, it was first embedded in Optimal Cutting Compound (OCT) (VWR, Lutterworth), first left to reach -80°C for 24 hours and left in the cryostat machine at between -15 °C and -17 °C for an hour to reach machine temperature. The sample was then mounted on the cryostat stage with the olfactory bulbs facing forward and the cortex upwards. The brains were consistently maintained at a minimum of -15 °C. Initially, the OCT block containing the brain was trimmed to a 50 µm thickness, and sectioning commenced from the point where the optic nerve/chiasm appears, continuing through to the substantia nigra, with the sections being 40 µm thick. Each tissue section was methodically arranged in a 12-well plate, with each subsequent section placed in the following well, thereby organising them into twelve sequential series. The series was kept at -

10 °C in anti-freeze. When ready to stain, samples were selected based on morphological assessment and rinsed with PBS 3 times for 5 minutes each time. Brain slices were placed on gelatinised slides for Cresyl violet staining and ventricle size comparison and allowed to dry overnight. For immunohistochemistry, blocking commenced after the wash.

5.3.12.2 Cresyl Violet staining

A 0.1% Cresyl violet solution was prepared by mixing 200 mg of Cresyl Violet Acetate with 200 ml of dH2O, adding 600 µl of acetic acid (0.3% acetic acid). The Cresyl Violet solution was placed in a Wheaton dish and pre-warmed to 37°C 10 minutes before use. Acidic alcohol (0.25%) was prepared by adding 500 µl of acetic acid to 200 ml of 95% denatured ethanol.

The steps for staining:

1. The sections were rehydrated in dH2O for one minute.
2. The sections were placed into the pre-warmed Cresyl violet solution for 15 minutes.
3. The sections were washed in dH2O to remove excess stain.
4. The rack was dipped onto the tissue to remove excess fluid.
5. The rack was placed in 70% (v/v) ethanol in H2O for 5 minutes.
6. The rack was placed in acidic alcohol for 10 minutes
7. The rack was then placed into 100% ethanol for 10 minutes.
8. The rack was removed, dabbed at the base to the tissue, and immediately placed into a Wheaton dish containing Histo-Clear II (SLS, Ireland) for 10 minutes.
9. The tissue sections were mounted with DPX onto a coverslip and left to dry overnight. Once dry, the slides were placed in a slide storage box for imaging and stored at room temperature.

Images were captured by positioning the slides on a light box, where they were photographed using a SONY RX100 camera. Using the ImageJ software, the right and left ventricular areas were measured and divided by the total brain slice area to calculate a percentage.

5.3.12.3 Immunohistochemistry to Assess Differentiation and Proliferation

Buffer 1: A mixture of 1% BSA (100 mg) and 0.3% Triton X-100 (30 µl) was made in 0.1 M PBS (10 ml), ensuring thorough mixing.

Buffer 2: This buffer was composed of 0.3% BSA (30 mg) and 0.1% Triton X-100 (10 µl) in 0.1 M PBS (10 ml), mixed well for uniformity.

Blocking Solution (2% BSA): The solution was prepared by adding 100 mg of BSA to 10 ml of Buffer 1, followed by thorough mixing.

Primary Antibodies: The ki-67 conjugated with ALEXA FLOUR 555 (Bioss Antibodies, Statech, Ely) and the β3-Tubulin conjugated with ALEXA-FLOUR 488 (Sigma-Aldrich, Dorset) concentration was prepared and diluted in Buffer 1 at a 1:100 dilution.

Two brain sections closely resembling the area where the dentate gyrus emerges were selected for the immunohistochemical analysis of ki-67 and β3-Tubulin to assess the effects of VDD on neuronal proliferation and differentiation in offspring *(Figure 5.3.4).*

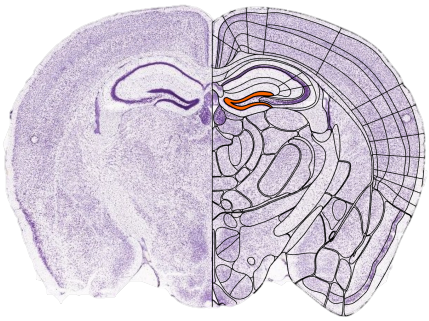

Figure 5.3.4. The reference point for section IHC.

The image was taken from The online Mouse Atlas, Science (517). The section in orange is the Dentate gyrus.

The following steps were taken for immunohistochemistry using the reagents described in section 5.3.12 above:

1. To reduce non-specific background staining, sections were incubated for 20 minutes at room temperature with the blocking solution.
2. 1 ml of primary antibody was diluted in Buffer 1, applied via a Pasteur pipette to each section, and incubated for 2 hours at room temperature.
3. After the incubation, the primary antibody solutions were removed, and the slides were washed in the washing buffer thrice for 5 minutes each.
4. Sections were briefly transferred into DAPI solutions for 5 seconds for counterstaining.
5. The sections were washed again in PBS and then mounted onto gelatinised slides and allowed to air dry.
6. Once air-dried, the slides were dipped into dH2O to remove salt residues.
7. An aqueous mounting medium, 1,4-dizabicyclo [2.2.2] octane (DABCO), was applied, and a coverslip was carefully placed to avoid air bubbles.

Images were taken at a 4x magnification around the dentate gyrus area using an EVOS FL Microscope (Invitrogen, Monmoth) at 458 nm (blue), 518 nm (green), and 585 nm (red) emissions, retaining the same parameters for each image. The quantification of fluorescence at each emission was carried out on ImageJ as previously described by Jensen (518). An additional step was incorporated into the method to account for inconsistencies in tissue quality. For each image, the background was also measured, and this value was subtracted from the fluorescence value at each wavelength. To produce a ratio between proliferating/differentiating cells, the value for Ki-67 (red) was divided by the value for β3-Tubulin (green). This ratio was then analysed by a 2-way

ANOVA, on GraphPad PRISM. Images were also compiled and presented in a tabular view.

5.3.13 Statistical Analysis

Data analysis was carried out using GraphPad PRISM software. The choice of statistical tests was informed by the experimental design and the nature of the data. The following tests were employed:

- Unpaired T-test: To compare serum VD concentrations between mothers on the VDD diet and those on the control diet.
- Two-way Analysis of Variance (ANOVA): Used to compare VDD na dcontrol maternal food intake and body weight prior conception. Also is applied to examine the interactions between time and diet on the offspring's VD levels.
- Three-way Analysis of Variance (ANOVA): Used to investigate the combined effects of diet, sex, and time on body weight and composition, insulin tolerance, brain weight, and behavioural assessments in Y-maze and elevated plus maze tests.
- Chi-square: used to analyse the birth rates and survival between treatment groups.

All tests were two-tailed, with significance set at $p<0.05$. Data were presented as mean ± SEM. The respective figure legends detailed the number of samples (n) for each analysis.

5.4 Results

5.4.1 Maternal Food Intake and Body Weight Prior Conception

The body weight and food intake of dams were documented before pregnancy, as seen in *Figure 5.4.1*. Analysis via a two-way ANOVA highlighted a significant effect of both time and VD treatment on body weight. Control and VDD dams showed an overall increase in body weight as time progressed ($p<0.001$ for both factors), although the VDD dams had an overall higher body weight ($p<0.001$). However, despite these general trends, Bonferroni post hoc testing showed no specific weeks where there was a significant difference in body weight between the two groups, implying that the variations in body weight were quite subtle.

Regarding cumulative food intake, it escalated over time ($p<0.001$). Yet, there was no difference between the food consumption of the control and VDD dams, as determined by a two-way ANOVA. This suggests that the lack of VD in the diet did not affect the overall food intake of the dams.

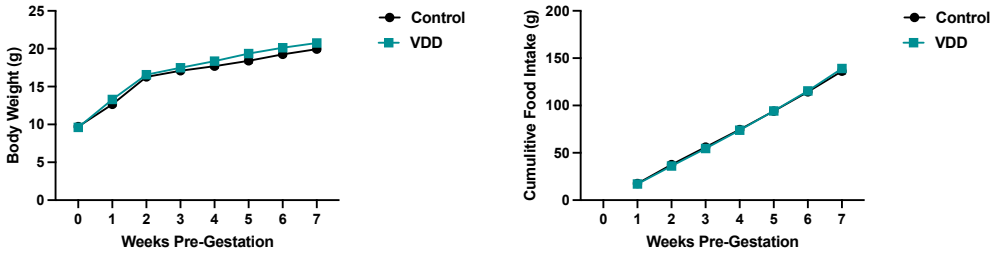

Figure 5.4.1. Maternal Pre-Pregnancy Body Weight and Food Intake

All values are presented as mean ± SEM, analysed using two-way ANOVA with Bonferroni post hoc tests. **A)** Body Weight of Dams: Analysis revealed a significant effect of both time and VDD treatment on body weight, with VDD dams having a higher overall body weight (both *p<0.001*). **B)** Cumulative Food Intake: While food intake significantly increased over time (p<0.001), there were no significant differences in consumption between control and VDD dams, indicating that VD status did not influence overall food intake.

5.4.2 Maternal and Offspring Vitamin D Status Is Influenced by Diet

The effect of maternal or offspring exposure to a VDD on 25-hydroxyvitamin D (VD) status is presented in *Figure 5.4.2.A.* Mothers exposed to the VDD diet had significantly lower VD concentrations in their serum than those on the control diet (*p<0.0001 Figure 5.4.2*.A). Offspring exposed to VDD had significantly less serum VD (*p<0.01*) Figure 5.4.1.B). They also had a significant interaction between time and diet (*p<0.01*, *Figure 5.4.2.B*), indicating that the diet's impact on VD levels varied over time. This can be observed in Figure 5.4.1, where the difference in serum VD was significantly more pronounced between the control and VDD group in the 12-week-old offspring compared to those euthanised at 6 and 3 weeks of age. Sex had no significant influence on the VD status.

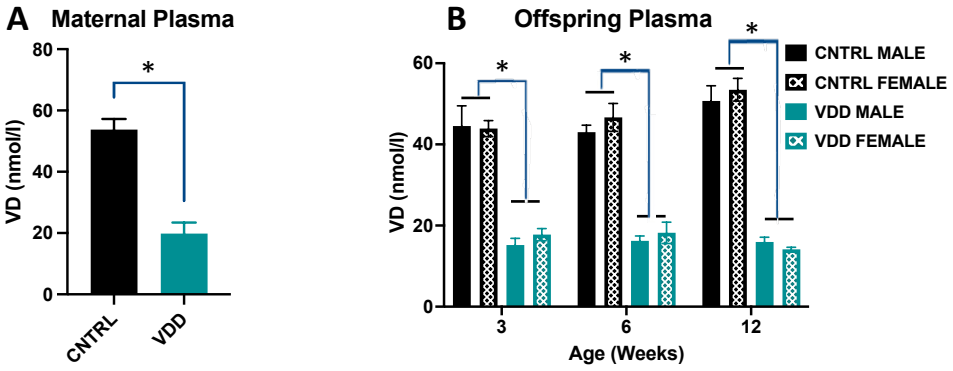

Figure 5.4.2. Serum 25-hydroxyvitamin D Concentrations of Mothers and Offspring on either Deficient or Control Diets.

A) Maternal Serum 25-hydroxyvitamin D (25(OH)D) Concentrations: Presented as mean ± SEM. Using an unpaired t-test, maternal serum levels post-weaning showed a significant reduction in the VDD group (n=15) compared to the control group (n=13), with a highly significant difference (* = *p<0.001*). **B)** Offspring Serum 25-hydroxyvitamin D Concentrations: Displayed as mean ± SEM for groups ranging from 6 to 27 offspring. A three-way ANOVA indicated significant interactions between time and treatment, with offspring from VDD dams exhibiting notably lower serum 25(OH)D levels when compared to controls (*= *p < 0.01*).

5.4.3 Offspring Survival and Numbers

Females on the VDD diet had a higher pregnancy rate, resulting in a greater number of offspring compared to the control group. The average litter size was also greater, but not significantly so, in the VDD group (6.8 pups per mother) compared with the control (5.4 pups per mother) with Chi-squared analysis. The litter range was marginally significantly different between the two groups (*p=0.077*) of 1-9 in the control group and 3-9 in the VDD group. However, the survival rate of offspring differed significantly (*p<0.0001*), with only 70.6% of VDD offspring surviving for analysis, compared to 97.2% in the control group. There were no significant differences between sex ratios. A summary can be seen in *Table 5.4.1.*

Table 5.4.1. Offspring Birth Rates and Survival.

	Control	Deficient	P
Number of Mothers	12	20	*P<0.001*
Total Number Born	72	143	*P<0.0001*
Total Number Survived	69	101	*P<0.0001*
% Survival	97.2	70.6	*P<0.0001*
Average Number of Offspring	5.4	6.8	*NS*
Range of Litter Size	1 - 9	3 - 9	*P=0.077*
Males	36 (52.2%)	52 (51.5%)	*NS*
Females	33 (47.8%)	49 (48.5%)	*NS*

The numbers were analysed using GraphPad PRISM. The Chi-square test analysed offspring number, survival, and sex differences. An unpaired t-test assessed average offspring numbers and litter size range.

5.4.4 Offspring Food Intake and Body Weight

When food intake was statistically analysed, there was a significant Time X Sex X Diet interaction (three-way ANOVA: *p<0.001*), suggesting the combined effects of these variables on feeding behaviour. Specifically, the post hoc revealed that the control females and VDD males had a significantly lower food intake when compared with the control males and VDD females (*p<0.05*)

Body weight also varied significantly among the treatment groups (Figure 5.4.3*. C)*. There is a significant Time X Sex X Diet interaction (three-way ANOVA: p<0.0001), indicating differential growth patterns influenced by these factors. Specifically, control males consistently exhibited higher body weights compared to all other groups (*p<0.05*). A notable increase in body weight across all groups was observed between weeks 3 and 5, aligning with the weaning period. However, post-weaning, gender differences in the control group became more pronounced, whereby the body weight of the control males was significantly higher than the control females. However, there was no significant difference between VDD males and VDD females in terms of body weight, suggesting VDD reduces gender-specific body weight changes.

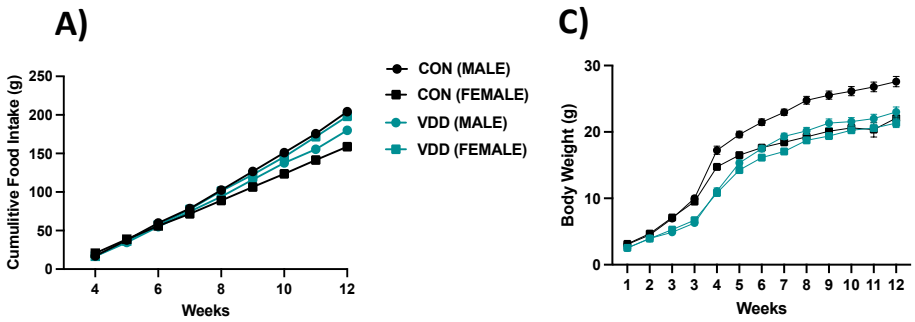

Figure 5.4.3. Food Intake and Body Weight

A) Cumulative Food Intake: Weekly cumulative food intake per mouse, presented as Mean ± SEM (n=19-10). Analysed using three-way ANOVA, which indicated a significant Time X Sex X Diet interaction (*p<0.0001*). **B)** Body Weight Tracking: Body weight of mice measured from postnatal day 4 (PD4) until demise, with values displayed as Mean ± SEM (n=49-18). A three-way ANOVA demonstrated a significant Time X Sex X Diet interaction (*p<0.0001*).

5.4.5 Maternal VDD Influences Body Composition of Offspring.

The animals were weighed before their cull, after which their respective tissues, including WAT and brain, were also weighed. The composition of those tissues can be seen in *Figure 5.4.4*. At weeks 3 and 12, males in the VDD groups had higher body weights than the other diet groups. However, at week 6, there were no significant weight differences between sexes in the VDD group, indicating a significant Diet x Sex interaction ($p<0.05$). Control group males weighed significantly more than their VDD counterparts and had a higher fat mass ($p<0.05$). When examining Abdominal white adipose tissue (WAT AB), significant interactions were noted across Time x Diet, Time x Sex, and Diet x Sex (all $p<0.05$ *Figure 5.4.4.B*), with the most pronounced increase in adipose tissue weight occurring in control males, starting from 6 weeks. The total white adipose tissue analysis relative to body weight revealed significant Time x Diet and a Diet x Sex interaction ($p<0.05$, *Figure 5.4.4.C*), but post hoc analysis revealed that significant differences between the diet groups were most profound on 12 weeks of treatment. This highlighted a general increase in fat mass per gram of body weight over time.

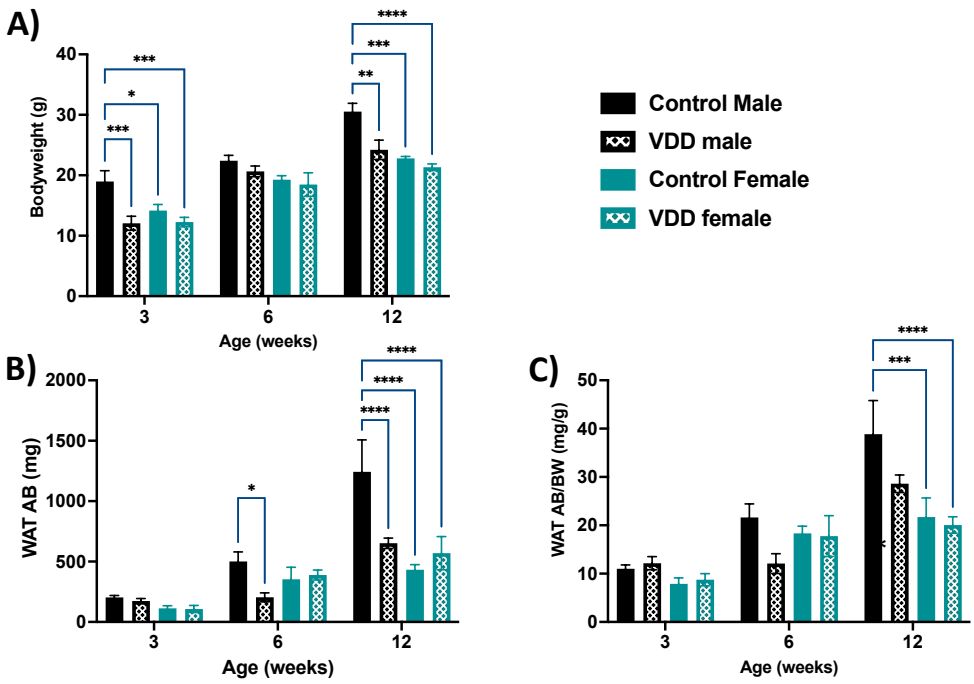

Figure 5.4.4. Body Composition of Offspring.

All values were displayed as Mean ± SEM (*n = 6 – 17*) and analysed by a 3-way ANOVA on GraphPad PRISM. For post hoc analysis: **=p<0.05, **=P<0.01, ***=p<0.001, ****=p<0.0001.* **A)** Body weight of offspring used for analysis. There was a significant Diet x Sex effect (*p<0.05*). There was also a significant effect of time (*p<0.0001*). Tukey post hoc revealed that control males had a higher body weight on weeks 3 and 12 than the other groups. **B)** Total weight of white adipose tissue. Significant Time X Diet, Time x Sex and Diet x Sex interactions (*p<0.05*). **C)** Total weight of white adipose tissue/body weight. There was a Time x Diet and Sex and Diet interaction (*p<0.05*).

5.4.6 VDD influences Insulin Tolerance Testing (ITT)

The number of animals used to analyse the ITT data varied due to hypoglycaemia (*Table 5.4.2*). This introduces a potential bias in our results because we excluded those animals from our analysis. Therefore, the results represent animals that did not develop hypoglycaemia, potentially skewing the data towards higher glucose levels, especially in the female mice in the 6-week ITT and male mice in the 12-week ITT.

In the Three-way ANOVA analysis, there was a marginally significant Time X Diet X Sex interaction in 6-week-old mice ($p<0.07$, *Figure 5.4.5*.A) and significant interactions for Time x Diet ($p<0.005$, *Figure 5.4.5*.A) and Sex x Diet ($p<0.05$, *Figure 5.4.5*.A), suggesting the influence of these combined factors on glucose regulation in 6-week mice. However, the 12-week-old mice independently demonstrated only significant effects of Time and Diet ($p<0.05$, *Figure 5.4.5*.B)) with sex not showing a significant effect. This was evident as both sexes exhibited a similar downward trend in glucose levels post-insulin injection, yet the VDD animals consistently maintained higher levels than the controls. This response pattern was underpinned by the differences in plasma glucose concentrations after ITT in 6-week-old and 12-week-old offspring, depicted in *Figure 5.4.5*, with distinct insulin responses observed between 6-week and 12-week-old mice. Most 6-week-old mice responded to insulin injection with decreased glucose levels, except for the female VDD mice, who showed a negligible response according to the post-hoc analysis ($p<0.05$, *Figure 5.4.5*.A). Furthermore, control males exhibited a more pronounced decrease in glucose levels than VDD males, indicating a faster insulin response ($p<0.05$).

Table 5.4.2. Number of Animals with Hypoglycaemia

6 weeks				
	CON male	CON female	VDD male	VDD female
Start N number	8	6	8	8
N of Animals with hypoglycaemia	1	2	1	4
% hypoglycaemia	12.50%	33%	12.50%	50%
12 weeks				
	CON male	CON female	VDD male	VDD female
Start N number	7	6	7	8
N of Animals with hypoglycaemia	3	1	1	0
% hypoglycaemia	43%	17%	14%	0%

Animals which suffered from hypoglycaemia were taken out of the full analysis.

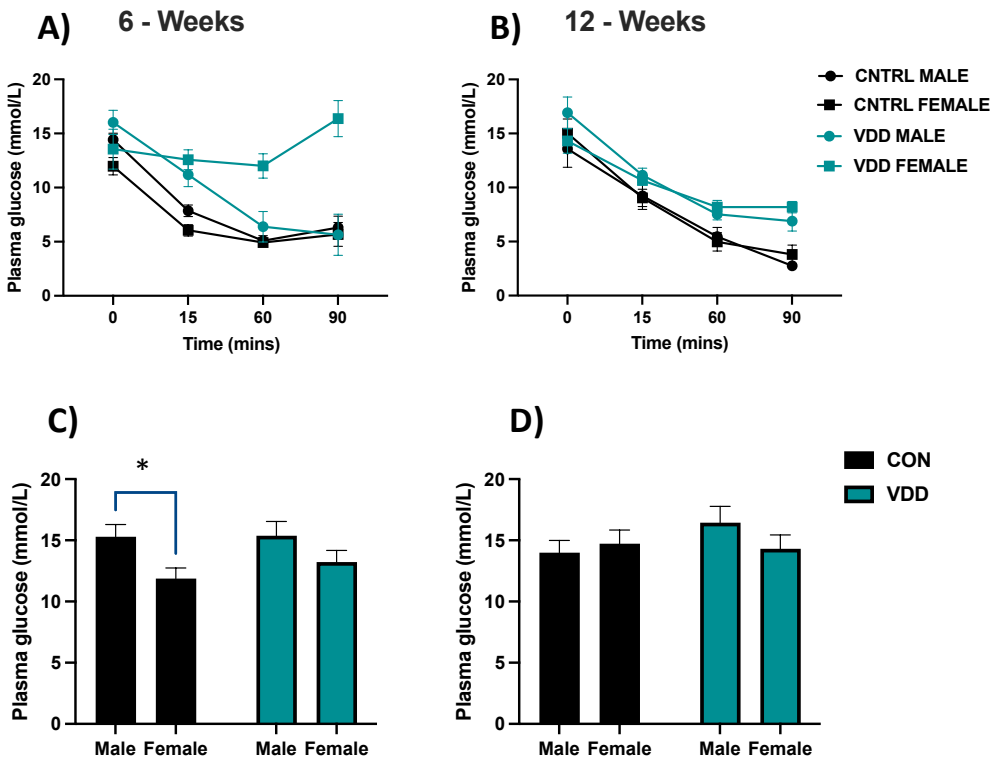

Figure 5.4.5 The Effect of Insulin on Plasma Glucose Concentrations

A) & **B)** are displayed as Mean ± SEM and analysed by a 3-way ANOVA**. A)** Plasma glucose concentrations in 6-week-old mice post-insulin injection showed a marginally significant Time X Diet X Sex interaction (*p<0.07*), and significant Time x Diet (*p<0.005*) and Sex x Diet (*p<0.05*) interactions. For males in both diet groups, *n=7* and *n=4* for both female groups. **B)** For control males, *n=4; control* females, *n=6*; VDD males, n=5; and VDD females, n=8. Plasma glucose concentrations in 12-week-old mice post-insulin injection showed a significant effect of Time (*p<0.001*) and Diet interaction (*p<0.05*), with no significant influence of sex. **C)** Fasted (6-hours) glucose concentrations in 6-week mice. There was a significant Sex effect (*p<0.05*), significantly different in the control group between the sexes. **D)** Fasted (6-hours) glucose concentrations in 12-week mice. No significant differences were observed.

5.4.7 VDD Influences Brain Weight in a Sex-Specific Manner but not Ventricle Size

There was a notable Time x Diet interaction (*P<0.05, Figure 5.4.6.A*) in the sizes of brain weights, with brain weights increasing with age from 3 to 12 weeks across all mice (*p<0.05*). However, overall, mice on the VDD diet had significantly lighter brains than those on the control diet (*p<0.05*); specifically at 3 weeks, VDD diet mice had lighter brains than controls. However, by 6 weeks, brain weights were comparable between groups, but by 12 weeks, VDD diet males showed a significant reduction in brain weight relative to controls (*p<0.05*). When controlling for body weight, the brain-to-body weight ratio declined over time across all groups (*p<0.01*). VDD diet offspring exhibited marginally higher brain-to-body weight ratios, attributed to their lower body weights, confirming a Time x Diet interaction (*p<0.05*, *Figure 5.4.6.B*). At 3 weeks, control diet males had the lowest brain-to-body weight ratios, significantly differing from VDD diet males (*p<0.05*), whose brain weights were closer to those of females. Generally, females presented higher brain-to-body weight ratios than males, indicating a Sex x Diet interaction (P<0.05, *Figure 5.4.6.B*).

Neither VDD diet nor sex influenced the proliferation/differentiation cell ratio or the Ki-67/B3-Tubulin ratio in brain sections, including the hippocampus and dentate gyrus, of 6-week-old mice *Figure 5.4.6.C*). Similarly, ventricle sizes in VDD offspring did not significantly differ (*Figure 5.4.6.D*). The tissue quality was suboptimal, necessitating caution in interpreting these results.

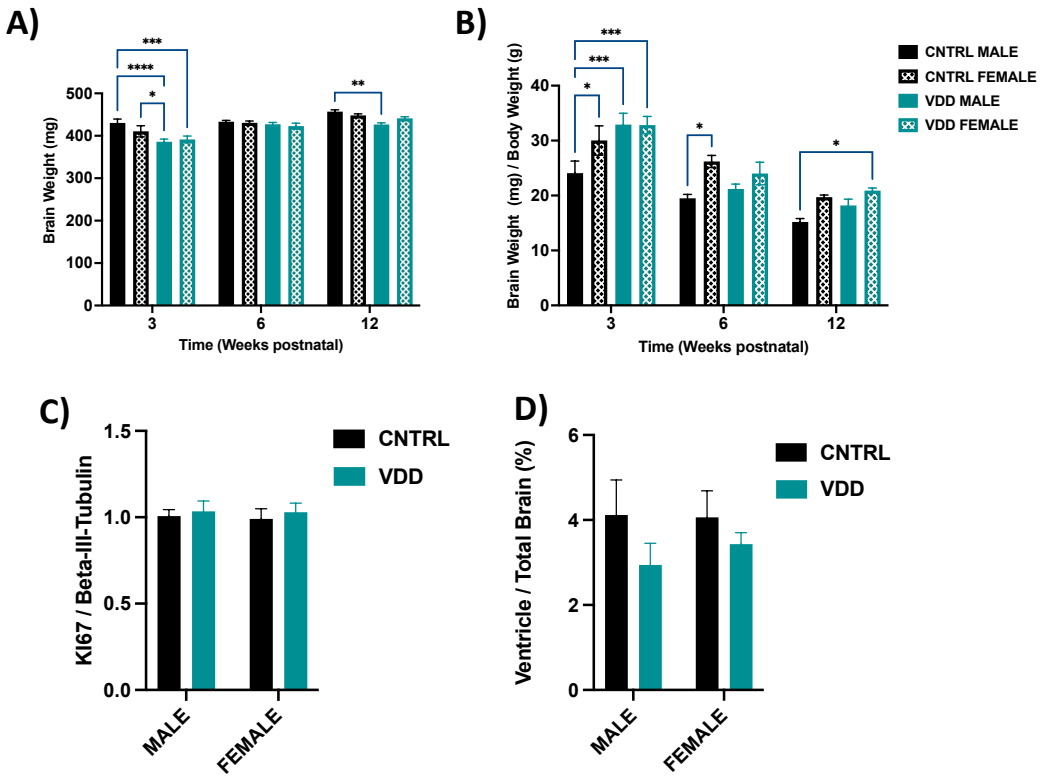

Figure 5.4.6 Offspring Brain Outcomes following VDD Diets

Values are the mean ± SEM. **A)** Brain weights at cull (*N = 4-17*). There was a significant Time x Diet interaction (*p<0.05*; 3-way ANOVA). At 3 weeks, VDD diet mice had significantly lighter brains than controls *(p<0.05)*. At 6 weeks, brain weights were comparable between groups. But at 12 weeks, VDD diet males showed a significant reduction in brain weight relative to controls (p<0.05). **B)** Offspring brain weight divided by total body weight (*N = 4-17*). There were significant Time x Diet and Sex x Diet interactions (*p<0.05*; 3-way ANOVA). The brain to body ratio declined over time in all groups (*p<0.001*). At 3 weeks, the VDD diet offspring had significantly higher brain-to-body ratios compared to the control diet males (p<0.01), and females presented with the higher brain-to-body ratios than males within the diet groups (*p<0.05*) **C)** Ratio of Ki-67 and B3-Tubulin expression in 6-week-old offspring. 2-way ANOVA revealed no significant effects (*n=3-4*). **D)** Proportional Volume of Lateral Ventricles in the Brain. No significant effects were observed (*n = 3-4*) in 2-way ANOVA.

5.4.8 Alternation Behaviour (Y-Maze) is changed with maternal VDD.

The Alternation behaviour was measured using a Y-maze, and a summary of the results can be seen in *Figure 5.4.7*. The significant differences were analysed using a 3-way ANOVA, which revealed a significant influence of diet (*p<0.01, Figure 5.4.7.A*). Mice on the VDD diet exhibited fewer alternations. In contrast, sex and time did not have significant effects, suggesting that the mice fed a control diet had better short-term memory, regardless of age or sex. Additionally, the mean velocity, which reflects the average speed of each mouse in the maze, did not show significant differences attributable to diet, sex, or time. However, the diet significantly affected the total distance moved within the maze (*p<0.05, Figure 5.4.7.C*), with mice on the VDD diet moving less than those on the control diet. Similar to velocity, the total distance moved did not show significant effects related to sex and time. There were no interactions observed in these data sets.

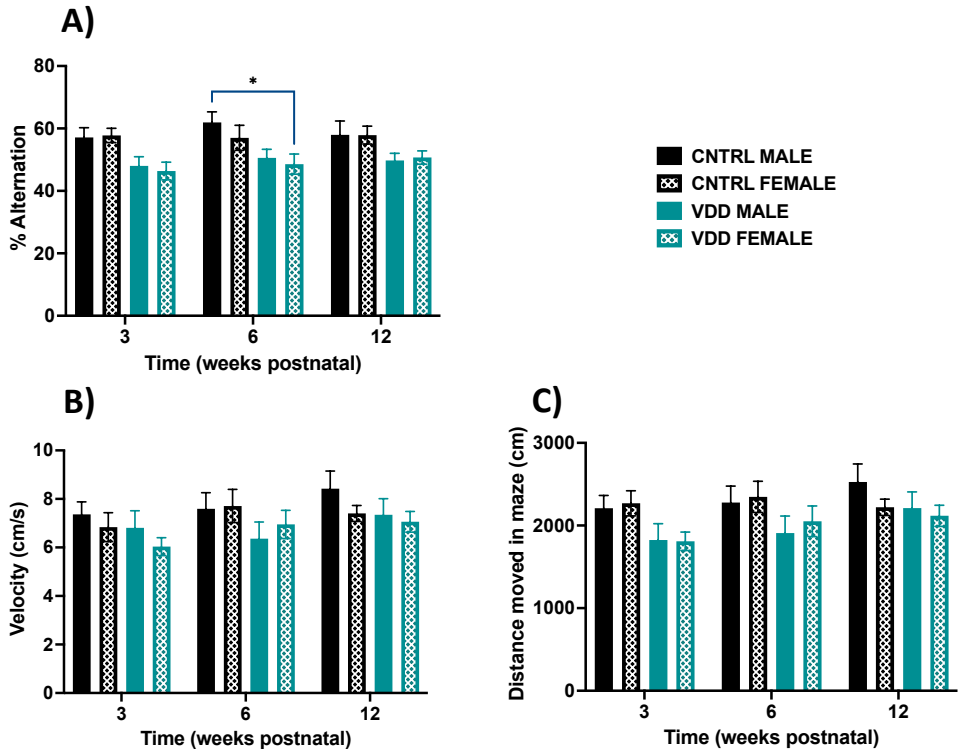

Figure 5.4.7. Offspring Alternation Behaviour and VDD

Values represent the mean ± SEM (*n=8*), analysed using a 3-way ANOVA. **A)** "% Alternations" was the ratio of correct consecutive alternations to the total possible alternations multiplied by 100. Diet significantly influenced % alternation ($p<0.01$), with VDD mice showing a lower percentage, while sex and time showed no significant effects. **B)** The mean velocity, calculated as the average speed of each mouse in the maze, showed no significant effects of diet, sex, or time. However, marginally significant differences were observed with time (*p<0.09*). **C)** Total distance moved within the maze was significantly affected by diet (*p<0.05*), with VDD mice moving less than control mice, with no significant effects of sex or time and no significant interactions.

5.4.9 VDD Influences Anxiety-Like Behaviour in Mice.

VDD influences all the parameters of the elevated plus maze, as seen in *Figure 5.4.8,* with every parameter being tested for significance using a 3-way ANOVA. Analysis showed that diet significantly affected the percentage of total time mice spent in open arms. VDD animals displayed notably less time in these exposed areas (*p<0.005, Figure 5.4.8.A*), suggesting increased anxiety-like behaviour. Additionally, the cumulative time in open arms, reflecting the aggregate duration during the test, diminished consistently over time and was further reduced by dietary treatment (*p<0.0001* for time, *p<0.05* for diet, *Figure 5.4.8.B*), with VDD mice exhibiting this trend at weeks 3, 6 and 12. The total distance travelled in the maze was also significantly shorter for VDD animals (*p<0.05, Figure 5.4.8.C*), indicative of a lower level of overall exploratory activity, with a marginally significant interaction between time and diet (*p=0.053*).

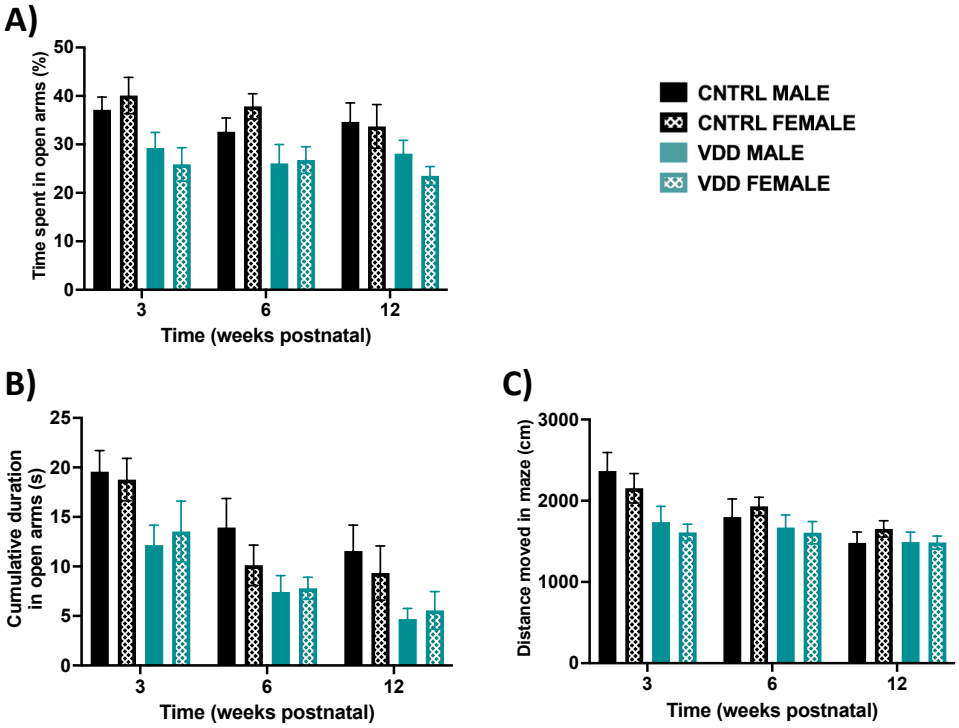

Figure 5.4.8. VDD Influences Anxiety-Like Behaviour

Values are presented as mean ± SEM (n=8), analysed using a 3-way ANOVA. **A)** The percentage of total time spent in open arms was significantly influenced by diet (*p<0.005*), with VDD animals spending a smaller percentage of time in the open arms. **B)** Cumulative time spent in open arms, indicating the total duration within each test, was influenced by both time (*p<0.0001*) and diet (*p<0.05*). However, there was no interaction. This resulted in a consistent decrease in time spent in open arms at each successive point, with VDD spending less time in the open arms at all time points. **C)** Total distance moved in the maze was significantly influenced by diet (*p<0.05*), with VDD mice spending less time in the open arms than the controls. Also, there was a marginally significant Diet x Time interaction (*p=0.053*), which suggested that diet influenced the time spend in arms over time.

5.5 Discussion

As expected, mothers and offspring on VDD diets exhibited significantly lower VD levels, with offspring showing time and diet-dependent variations. Notably, VDD dams were more likely to get pregnant. However, maternal VDD profoundly impacted offspring development, with the offspring having lower survival rates and exhibiting significant changes in body composition, including variations in body weight and adiposity, as well as reduced insulin sensitivity. Furthermore, VDD affected brain weight, in a sex-specific fashion while not significantly altering ventricle size. Behavioural assessments indicated that offspring from VDD diets experienced a reduction in memory and a increased anxiety behaviour, evidenced by their performance in maze tests.

5.5.1 Impact of Maternal VDD on Offspring Vitamin D Status and Body Composition

As anticipated, maternal serum VD levels were markedly lower in the VDD group compared to the control group, aligning with findings by Hawes et al., (305). Given that the foetus's only source of VD is maternal, it can be inferred that the offspring of VDD mothers had reduced VD transference via the placenta (66). The offspring from VDD mothers were also maintained on a postnatal VDD diet and exhibited predictably lower VD levels.

In humans, VDD and obesity often come hand in hand due to high adiposity reducing circulating VD, and have been well-documented, though the impact of VD supplementation on obesity remains unclear (519). The link between VDD and adiposity is also inconsistent in mice, with varying results reported in the literature (519). The review by Bennour et al., (519) found that feeding rodents

a high-fat diet (HFD) to induce obesity leads to a decrease in plasma VD in studies which used ELISA quantification, but studies which used mass spectroscopy did not find a significant reduction in VD. It is also interesting that VDR null mice do not show increased adiposity when provided a HFD, which may be attributed to higher energy expenditure (suspected to be due to increased fatty acid oxidation, uncoupling proteins and alopecia) (484, 520, 521). Our findings of reduced adiposity in VDD male mice resonate with this explanation, as our VDD males had significantly less abdominal WAT, and had smaller overall body weights.

Our results and other similar studies raise questions about the suitability of mice as models in this context, particularly considering their brown fat content, which could affect total white adipose tissue weight (484, 520). The frequent co-occurrence of obesity and VDD in humans underscores the need for more focused human studies to determine if VDD contributes to increased fat mass, as current research in this area points to vice versa: that increased adiposity leads to reduced circulating VD.

5.5.2 Survival and Developmental Outcomes in Offspring

In our trial, females on the VDD diet exhibited a higher pregnancy rate, which led to a higher number of offspring compared to the control group. This observation suggests that the VDD diet may influence reproductive behaviours or fertility rates. Despite the increased pregnancy rate in the VDD group, the average litter size remained consistent across both groups, similar to the results in the study by Hawes et al., (305). The opposite was found by Liu et al., (522),

who found that a lower VD dose leads to a smaller litter size compared with a high dose. In their study, Liu et al., (522) also reported a significant increase in mortality rates within the VDD, which is consistent with our findings. This finding is in line with broader human research that has linked VDD during pregnancy to various adverse outcomes for offspring (66). These outcomes encompass low birth weight, small size for gestational age, and preterm birth, all of which are associated with higher postnatal mortality (495, 523, 524). Moreover, a recent observational study conducted by Eves et al., (525) uncovered that infants born small for gestational age (SGA) exhibited lower IQ scores compared to their non-SGA counterparts, with these differences persisting up to the age of 26. It is plausible that VD's influence on brain development may be partly connected to its association with SGA (491, 526). One potential explanation for this link is the impact of VD on placental inflammation, which is known to be associated with intrauterine growth restriction (IUGR) and SGA. Indeed, several studies have demonstrated that women with heightened levels of placental inflammation tend to have a higher incidence of SGA infants than women with lower inflammation levels (527-530). Given that VDD is associated with placental inflammation, this connection could be a mechanistic explanation for the observed effects of VDD on behaviour and brain size (531).

5.5.3 VDD's Influence on Insulin Tolerance

At both 6 and 12 weeks, VDD mice exhibited a weaker insulin response compared to control mice, demonstrated by their lesser decrease in glucose

levels following insulin injection. However, the exclusion of animals that experienced hypoglycaemia at the 90-minute mark could introduce attrition bias, particularly affecting the data for 6-week-old VDD females, who exhibited the highest incidence of hypoglycaemia. Therefore, the evident lack of insulin response in the VDD female group should be carefully considered. In the study by Maia-Ceciliano et al., (532), adult mice were fed either a VDD or a VD-sufficient diet for 10 weeks. The findings showed that VDD mice had significantly higher glucose levels following an insulin injection, mirroring our observed results. Importantly, our research represents a novel investigation into how developmental VDD influences ITT outcomes, as no prior studies have explored this specific aspect in rodents. Generally, rodent studies concentrate on glucose tolerance testing rather than ITT, with most research indicating that VDD mice typically exhibit elevated glucose levels following glucose administration, which indicates reduced insulin sensitivity (520, 533). The potential mechanism by which VD affects glucose control is likely through its ability to enhance insulin secretion, as highlighted in the comprehensive review conducted by Tai et al., (478). This aligns with the findings of various studies on glucose tolerance testing. However, it doesn't fully elucidate why our VDD mice exhibited diminished responses in the ITT. To explain why VD influences insulin tolerance, authors propose another contributing factor: PTH, as VDD is linked to elevated PTH levels, and increased PTH is associated with decreased insulin sensitivity (478). However, we did not measure PTH, and it is evident that further investigation is warranted to comprehensively understand the complex interplay between VD and insulin in glucose regulation.

Altered glucose control can potentially influence brain development, particularly in children. In a recent review conducted by Nevo-Shenker et al., (534), the authors concluded that hyperglycaemia and blood glucose level fluctuations affect brain structure and cognitive function, however, the focus was on type 1 diabetes. In another review by Liu et al., (535), the investigation delved into the role of insulin and insulin-like growth factor (IGF) in NSC proliferation and differentiation. This analysis revealed a positive influence of insulin signalling on NSC proliferation (535). It raises the possibility that VDD could also affect maternal insulin tolerance during pregnancy, potentially leading to elevated maternal insulin levels and subsequently increased NSC proliferation. While insulin doesn't cross the placenta, elevated maternal glucose levels resulting from insulin intolerance can pass through it. This exposure to higher glucose levels can stimulate the offspring to produce increased insulin and downregulate glucose transporters GLUT1 and GLUT4, potentially contributing to their insulin resistance (536). This phenomenon could explain the increased brain-to-body ratio in VDD offspring at three weeks of age. It is important to note that in mice, offspring of insulin-resistant mothers do not exhibit increased size compared to normal, unlike human offspring, who are typically heavier at birth (537). This observation suggests a potential link between VD and its influence on neurodevelopment, although we did not conduct ITT on the mothers. Consequently, the observed reduction in insulin sensitivity among VDD offspring should be considered when assessing overall offspring health, including potential implications for brain health and development.

5.5.4 Effects on Brain Development and Behaviour

5.5.4.1 Brain Morphology and Locomotion

Brain size, relative to body weight, was significantly larger in VDD male offspring than in control male offspring at 3 weeks; however, at 6 and 12 weeks, brain sizes were the same in both groups. This observation is supported by Eyles et al., (205), who noted increased brain sizes in 12-hour-old VDD offspring. However, Hawes et al., (305) reported no significant differences in total brain volume at the embryonic stages E14.5 and E17.5. Our data also indicated that the diet did not impact the size of the lateral ventricles in 6-week-old mice. This aligns with findings from Féron et al., (204), although they did document changes in lateral ventricle volume due to maternal VDD in 3-week-old offspring. The relevance of this enlargement to mental health conditions is notable, as excessive brain size has been linked to disorders such as autism and schizophrenia (538, 539). Macrencephaly (defined as a head circumference in the 97th percentile) is present in approximately 15.7% of individuals with ASD (540). The most consistent and widely accepted explanation is increased neuronal proliferation coupled with decreased synapse pruning, leading to an excessive neuronal population (540).

VD influences brain cell growth and differentiation (205). Although our study did not detect ventricular enlargement or behaviours typically associated with schizophrenia, the theoretical link between VD and these schizophrenia-related brain changes warrants further examination (205). Regarding locomotor activity, our VDD mice displayed no significant changes in mean velocity within the Y-maze, suggesting that gross motor functions remained

intact; this finding is notable given that altered locomotor activity can be a feature of schizophrenia (541). To enhance the specificity of our findings, future research might incorporate the Open Field Test (OFT), which could reveal subtle locomotor anomalies often observed in schizophrenia models and potentially associated with changes in ventricle volume (542, 543). It would be insightful for future research to focus on measuring gene expression, morphology, and cell types in the third week of mouse brain development. Brain volume increased significantly in VDD offspring, which might be linked to schizophrenia-like traits. Also, the hippocampus, which is important for memory and navigation, starts developing rapidly in rodents after 3 weeks (192). Understanding that early stage can help reveal how VDD influences gene expression, brain shape, and behaviour in that time and the future.

5.5.4.2 Memory

In the hippocampal dentate gyrus, we found no difference in the ki-67 to ß3-tubulin ratio, mirroring our findings with lateral ventricle volume. However, the quality of the brain tissue analysed was compromised due to enlarged vacuoles in the tissues, suggesting inadequate fixation, casting doubt on these results. Nonetheless, other studies have reported similar effects, such as Groves et al., (544), who found no difference in ki-67 expression in VDD adult mice. Using a different measure of proliferation, Zhu et al., (545) used 1α-hydroxylase knockout mice and measured BrdU positive cells (indicative of proliferation) and NGF mRNA (indicative of differentiation) in the dentate gyrus. They found that these mice had significantly more BrdU-positive cells without VD supplementation and significantly less NGF mRNA. Conversely,

when VD was applied (in its active form), both levels returned to the same levels as control mice (545). This paper indicates that VD supplementation post-gestation can mitigate certain deficiency effects, but without assessing behaviour or brain physiology, it cannot conclude on long-term brain function impacts.

In the Y-maze, specifically designed to assess spatial memory and decision-making, mice with VDD showed reduced alternation behaviour. This finding aligns with previous research on rats. However, these studies differed by administering VD during adulthood for 4 and 8 weeks rather than during pregnancy, as in our study (546, 547). In humans, a handful of studies have investigated maternal VDD and offspring cognitive outcomes, such as the study by Specht et al., (266), who found that newborn's associated with the lowest maternal VD levels also had lower IQ, and the study by Zhang et al., (548) who found a positive correlation between maternal VD status and cognition, language and motor development in children, with a comparable study by Voltas et al., (549) finding a similar result in infants. In contrast, the systematic review by Mutua et al., (550) highlighted inconsistencies in findings related to human offspring and underscored the need for higher-quality studies in this domain. Notably, the most substantial evidence in their review pertained to the correlation between VD levels and language development (550). Given the established links between delayed or altered language development and autism, as well as between VDD and autism, it is plausible to suggest a potential association between VD levels and autism-related language development (551, 552).

5.5.4.3 Anxiety

In our study, VDD mice travelled shorter distances in the plus and Y mazes, indicating reduced exploratory behaviour or physical limitations. Conversely, control mice covered greater distances, possibly indicating that the increased movement in control mice might be related to their lower anxiety, not necessarily improved locomotor activity, as movement speed was similar in both VDD and control groups.

A notable observation was the consistent decrease in the time spent in the open arms of the maze at successive time points, regardless of diet. This pattern aligns with a study on rats, which linked higher baseline plasma corticosterone levels in older rats to their reduced willingness to remain in open arms (553). In a study similar to ours, researchers investigated the effects of initiating VDD in dams before pregnancy, with offspring later placed on a control diet post-weaning (554). Their findings revealed that such prenatal VDD exposure led to increased glucocorticoid levels and unusual social behaviours in the offspring, highlighting the profound influence of foetal programming (554). This suggests that early VDD can have lasting impacts on stress hormone regulation and social interactions, reinforcing the importance of prenatal nutrition on long-term health and behaviour (555). Since corticosterone is released in response to stress and chronically elevated corticosterone results in depression and anxiety, there may be an unexplored link here (554). This potential mechanism might also explain the influence of a VDD diet on cumulative time in the open arms, as VD supplementation has been shown to mitigate corticosterone-induced depression (556). However, as highlighted in a review by Casseb et al.,

(557), the role of VD in reducing anxiety in rodent models remains unclear, necessitating further research to understand its effects and underlying mechanisms.

5.5.5 Limitations and Future Directions

The standout finding from our research is the significantly reduced insulin response in VDD mice compared to their control counterparts. This prompts an intriguing question for future research: will the insulin irregularity remain if we transition the offspring to a controlled diet after birth? Answering this could confirm the lasting influence of foetal programming. Additionally, our initial behavioural tests on VDD offspring revealed a notable decline in memory and an increase in anxious behaviour. To delve deeper, we could expand our behavioural analyses to include tests for social interaction and open-field assessments to better quantify locomotion. These further studies would allow us to tease apart the effects of foetal VDD exposure from those that occur after birth, thus contributing data to the DOHaD hypothesis narrative.

However, our study did have limitations. Certain animals developed hypoglycaemia when investigating glucose response to insulin and were excluded from the final analysis. This exclusion of data from animals experiencing adverse effects could lead to attrition bias. Such bias may skew the results and interpretations of the ITT experiments, impacting the overall reliability and applicability of these findings.

Time constraints prevented the exploration of genomic and metabolic impacts of VDD, which would have been particularly relevant in examining

brain proliferation and differentiation rates. In immunohistochemistry, the poor tissue quality significantly hindered the accurate quantification of ki-67 and β3-Tubulin-positive cells. Combining immunohistochemistry with gene expression analysis might provide a more complete understanding of the effects under investigation.

Additionally, the quality of brain tissue samples was suboptimal, and the sample size for each gender was limited. Future studies could benefit from extended paraformaldehyde treatment of brain slices and ensure a sufficient quantity for analysis. A more comprehensive assessment of lateral ventricles across the entire brain, rather than selected slices, would also be ideal to reduce potential human error and improve the reliability of ventricle size measurements.

Our trial design did not allow for the isolation of foetal programming effects. We maintained consistent diets post-birth, making it challenging to distinguish if the observed effects were solely due to VDD during pregnancy or extended throughout the animals' lives.

Our study identified significant and non-significant but notable sex differences in some results. Although research progressively includes both sexes, the knowledge base remains more extensive for males than females. Considering both male and female subjects is crucial, especially since VD is integral to developmental processes and might affect the sexes differently due to hormonal and metabolic variations. For example, VDD has been linked to reduced fertility in both males and females, with obviously very different

mechanisms (558). In addition, many large-scale cohort studies cite different outcomes between sexes when investigating VDD and cardiovascular disease, bone density and autoimmune disease, to name a few (559-562). Future research should strive for gender balance to provide insights representative of the population.

5.5.6 Conclusion

In conclusion, our study sheds light on the extensive impact of maternal VDD on offspring. The effects of VDD are wide-ranging, influencing not only pregnancy success and offspring viability but also leading to marked changes in body composition, reduced insulin sensitivity, and altered behaviour. These changes are notably reflected in the observed deficits in memory and increase in anxiety behaviours in the offspring. Our findings point to the complex and comprehensive nature of VDD's consequences, signalling a clear need for further investigation. Unravelling the intricate mechanisms that connect maternal VDD to the developmental and behavioural alternations seen in offspring is essential, as it could inform strategies to mitigate these effects.

6 General Discussion

6.1 Summary

This thesis explored the role of VD in neurogenesis, behaviour, and inflammation *in-vitro* and *in-vivo*. Given VD's capacity to affect the expression of thousands of genes, its significant impact on neurodevelopmental processes is well-founded and has garnered substantial research interest. We proposed three central hypotheses: first, VD could promote neuronal differentiation while inhibiting proliferation; second, maternal VDD may foster an inflammatory environment detrimental to brain development and neurogenesis; and third, VDD has adverse effects on brain function. However, during the investigation, specifically when studying the impacts of developmental VDD in mice, we found that VDD influenced factors such as body weight, fat mass and an altered response to insulin, which may indirectly impact brain development. This concluding discussion seeks to summarise the findings and address these hypotheses.

6.2 Does Vitamin D Influence Neuronal Proliferation and Differentiation?

Brain development is critically dependent on the tightly regulated stages of cell proliferation followed by differentiation, which are essential for forming a healthy brain (563). Samuel et al.'s (291) review highlights that VD generally reduces proliferation; however, its impact varies by cell type and dose, being more pronounced at higher concentrations in cells like keratinocytes and

squamous cell carcinoma lines (291). This review did not include neuronal cells, which underscores a research gap. Intriguingly, in NSC's from adult mice, VD increased proliferation and differentiation, indicating a distinct response from these cells compared to those in a developing brain, suggesting a potential protective role in mature neural tissue (281). This suggests that VD's role is age-specific and possibly protective in adults, while it may differ during development. Supporting this, VD has been shown to inhibit cell division in embryonic hippocampal cells and neuroblastomas *in vitro* (287, 545, 564), although *in vivo* studies further complicate the narrative. VD supplementation did not alter hippocampal cell proliferation extracted from adult mice, whereas its absence during development was associated with abnormal proliferation and increased brain size (205, 544). These inconsistent findings warrant the studies conducted in this thesis, thus clarifying VD's effects through both *in vitro* and *in vivo* techniques. In addition, the link between VDD and an increased prevalence of neuronal disorders, such as schizophrenia and ASD, prompted us to investigate the potential regulatory role of VD in this context. Indeed, both schizophrenia and ASD are thought to partly arise from an overpopulation of neurones (565-567).

The studies in Chapters 3 and 5 addressed this question. We began our investigation using SH-SY5Y cells as a model to study the effects of VD on neuronal proliferation and differentiation, as seen in Chapter 3. Despite initial challenges in using SH-SY5Y cells to model differentiation effectively, our experiments revealed that a high concentration of VD (10^{-7} M) significantly reduced neuronal proliferation after 7 days of treatment. This finding indicates

the potential of VD to regulate neuronal population size, independent of its effects on differentiation. This observation led us to consider the broader implications of these findings for brain development, specifically the early proliferation NPCs undergo as part of brain development. Given the substantial differences between SH-SY5Y cells and NPCs, the fact that VD impacts SH-SY5Y proliferation does not necessarily imply a similar effect on NPCs. Although focussing on differentiation, in the work by Pertile et al., (288) the researchers demonstrated that while VDR-expressing SH-SY5Y cells exhibited changes in neurone length upon exposure to calcitriol, mesencephalic neurones at E14.5 showed no such response. It is important to note that neurogenesis has only just commenced at the rat embryonic stage of E14.5 (568). Therefore, these cells are unlikely to have been extracted as differentiated (568). This highlights the fundamental differences between these cell types, underscoring the importance of cell-specific studies when examining the effects of VD. Therefore, we also aimed to measure proliferation in our VDD offspring's brains, specifically in the dentate gyrus, an area of the brain related to memory. Ki-67 is a known marker of proliferation in many cell types, including immature neurones, and ß3-tubulin is a microtubule protein mostly only found in mature neurones (569, 570). We attempted to measure neuronal proliferation to differentiation ratio (Ki-67 to ß3-tubulin protein expression) in our 6-week-old offspring but found no significant differences. This may have been due to technical limitations in quantifying Ki-67 and ß3-tubulin levels due to poor brain tissue fixation (571). In addition, we observed significant differences between VDD and control offspring regarding brain weights at week 3, but not week 6.

Had we measured the Ki-67 and ß3-tubulin ratio in week 3 mice, we may have seen differences, although this is speculative. The third week could serve as a pivotal point, distinguishing effects attributable to maternal VDD from those resulting from the offspring's diet. Given that the lactation period in mice is analogous to the third trimester in humans, the alternations observed up to week 3 are likely a consequence of maternal VDD. The observed variance in brain sizes at this juncture suggests maternal VD status plays a significant role in the brain development of offspring, lending support to the DOHAD hypothesis. Furthermore, the behavioural differences emerging as early as week 3 imply changes in brain function, despite the absence of detectable alternations in proliferation or differentiation. Due to time constraints, we could not perform more precise proliferation measurements in our animal models. Consequently, while we observed a significant impact of VD on the proliferation of SH-SY5Y neuroblastoma cells, our results do not definitively conclude VD's effect on neuronal proliferation in animal models. However, our findings contribute valuable insights into the complex role of VD in neuronal development, highlighting the need for further research to elucidate this relationship.

VD, primarily through its active form calcitriol, induces differentiation in various cell types, including benign and malignant cells (291). *In-vitro* studies in cultured neurones have shown that VD can induce the synthesis of NGF and BDNF, which support neuronal differentiation and GDNF, which promotes the development of dopaminergic neurones (282, 284, 572). Our investigation into the role of VD in neuronal differentiation presented a multifaceted picture.

When investigating SH-SY5Y cells, we noted an upregulation in β3-Tubulin expression with VD treatment at 10^{-10} M and more markedly at 10^{-7} M concentrations. However, this did not translate into an enhanced differentiation effect when combined with RA in SH-SY5Y cells. This suggests that VD might marginally trigger differentiation in our experimental conditions, but it does not enhance the differentiation process stimulated by RA in these cells.

Gene expression analyses provided a further layer of complexity. The effects of VD on critical neuronal genes such as NeuroD1, MAPT, and NRXN1 were inconsistent, exhibiting time-dependent fluctuations that imply a non-linear influence of VD on neurogenesis. The variability in gene expression changes despite clear morphological evidence of differentiation highlights the complexity of neuronal differentiation processes. It suggests that other genes or pathways might be more indicative of VD's effects on neuronal differentiation. In addition, our animal study did not observe the expected shifts in the β3-Tubulin to Ki-67 ratio. This absence of a clear pattern echoes the findings from our cell culture experiments and underscores the need for a more granular analysis to ascertain VD's effects with confidence.

VD has been shown to suppress proliferation in various cellular systems and simultaneously induce differentiation (573). However, the relationship between these two processes is inconsistent; VD can decrease cell growth without prompting differentiation or enhance differentiation independently of growth inhibition (291, 574). VD's selective regulation of cell cycle inhibitors

like p21waf1 and p27kip1 can explain its distinct effects on cell proliferation without altering differentiation (291). These inhibitors block cell cycle progression, a necessary step for proliferation, but differentiation typically involves cells exiting the cell cycle, a process that can proceed unaffected by VD's influence on cell cycle regulation (291). Another way in which VD's supportive role could manifest is by providing an optimum environment for brain development (involving proliferation and differentiation) during pregnancy, rather than directly engaging with cellular differentiation (68). We did not investigate physical synapse formation, plasticity, and pruning, key parts of brain development. However, evidence suggests that VD plays a crucial role in synaptic plasticity and brain development (575). Animal studies have found that insufficient VD during pregnancy can lead to permanent changes in brain structure, which include a thinner cortex and enlarged lateral ventricles (which we did not observe in our animal trial) (575). VD also influences brain communication by regulating neurotransmitter systems, affecting transporters, receptors, and synthesis enzymes for key neurotransmitters like glutamate, GABA, and dopamine (576). This is critical in maintaining balanced synaptic transmission and brain health (576). As we had conflicting results regarding gene expression during differentiation, could it be possible that the influence of VD is more aligned with regulating synaptic functions in the brain rather than affecting neuronal differentiation? A more effective approach to studying the differentiation of these cells might involve quantifying the expression of proteins indicative of mature neurone development and function. Key markers to consider include tyrosine hydroxylase, neuron-specific enzyme, and

neuronal nuclei. Additionally, assessing cellular excitability could provide valuable insights into neuronal functionality. This could be a subject for future work.

6.3 Does Vitamin D Deficiency Foster an Inflammatory Environment during Pregnancy, Detrimental to Brain Development and Neurogenesis?

VD has been established as a promoter of anti-inflammatory states in biological systems (577). Past investigations have shown that maternal inflammation, whether chronic or as an acute response to infection, can have significant repercussions on brain development in offspring (347). A possible mechanism by which inflammation affects brain development is outlined by Jonakait (578), which suggests that maternal inflammation triggers the release of cytokines like IL-6 and BMPs, which have been documented to cross through the placenta (579, 580). These cytokines then activate microglia in the foetal brain, disrupting normal development processes and leading to abnormal brain development due to altered neuroimmune responses and cell differentiation pathways (578). An increase in maternal IL-6 secretion has also been known to cause a non-reversible increase in proliferation in the offspring's brains (581). In addition, a systematic review of studies on pregnant women found strong evidence that lower VD levels are linked to increased inflammation, as indicated by higher levels of hs-CRP, IL-8, and TNF-a (582). Studies using animal models have also shown that VD can reduce placental inflammation by

activating the VDR/NF-κB pathway, indicating a link between VD deficiency and placental function (123, 531). Based on this understanding, the hypothesis was established that a sufficient maternal VD status contributes to optimal brain growth and development by modulating inflammatory processes. The absence of an experimental model applying inflammatory stress necessitated a scoping review, as seen in Chapter 4, which explored the relationship between maternal VD levels during pregnancy and subsequent brain development and neurogenesis in offspring. The review examined studies that manipulated VD status during gestation, measured or induced inflammation and assessed brain development outcomes through behavioural tests or brain metabolism evaluation. The studies within the review collectively indicated that maternal VD supplementation or adequate status reduces inflammatory states during pregnancy. Specifically, the literature demonstrated that adequate levels of VD are associated with a decrease in pro-inflammatory cytokines such as IL-1β, IL-6, and MCP-1. This suggests that VD plays a role in creating an anti-inflammatory environment conducive to healthy foetal brain development. Research suggests that pro-inflammatory cytokines can modify VDR activity and VD metabolism, leading to divergent effects on cell differentiation (572, 573) This modulation by inflammation could account for the observed variability in VD's differentiation outcomes in our *in-vitro* experiments, as the inflammatory state dictates the cellular response to VD (291). Further, the studies reviewed revealed that VD enhanced neurobehavioral outcomes. These positive outcomes manifested as decreased pain behaviours, lower anxiety and depressive-like behaviours, and improved social interaction and memory

performance in mice and rats. In the subsequent animal trial detailed in Chapter 5, findings paralleled those of the scoping review, although direct measurements of inflammation were not conducted. We observed that offspring experiencing VDD presented with impaired memory and heightened anxiety. This raises the question of whether the maternal VDD creates a stressed intrauterine environment, consequently impairing optimal brain development. Our trial's lack of direct inflammation or oxidative stress analysis limits our ability to speculate or confirm our hypothesis. Furthermore, the scoping review (Chapter 4) stresses the potential for VD to exert wide-ranging effects on brain development, not just through one pathway but by influencing many genes. This is supported by numerous VDREs across the genome, which are responsive to the VDR (583). Such widespread genomic effects of VD suggest a comprehensive role in shaping neurodevelopment beyond anti-inflammatory actions. The narrative that emerges from the scoping review in Chapter 4 is that VD has a dual role during pregnancy: it dampens the inflammatory response, and it supports the neurogenesis process, leading to better brain outcomes in the offspring. Therefore, we suspect that VD has two roles in protecting the brain: modulating inflammation and modulating proliferation/differentiation. This link establishes a compelling argument for maintaining VD sufficiency during pregnancy. However, despite these associations, the scoping review identified a gap in the literature regarding the detailed mechanisms through which VD mediates these effects. There is a need for further research to dissect the specific genetic and metabolic pathways by

which VD influences both the inflammatory environment and neurodevelopmental processes.

6.4 Does Maternal VDD Cause Altered Behaviour in the Offspring?

In a recent systematic review of human longitudinal studies which focussed on maternal VD status and offspring psychiatric outcomes, the authors found small amounts of evidence that VDD was associated with ASD, ADHD and schizophrenia, although they stressed the evidence was sparse (208). Clearly, studying this topic is much more challenging in humans, especially since VD status is associated with external factors such as income, skin colour and frequency of holidays abroad (584, 585). Therefore, animal studies are ideal models for controlling VD status and environments without those external factors, despite the obvious differences in brain complexity and behaviour between species. Previously, studies using the offspring of VDD mice and rats have reported that VDD leads to alternations in memory and learning (586). The findings from our own animal trial (Chapter 5) and the insights gathered from the scoping review (Chapter 4) also support these findings. The scoping review found that increased maternal VD levels were associated with positive neurodevelopmental outcomes in offspring, including behaviour and increased BDNF levels, essential for neuronal development and survival, and a reduced incidence of neural tube defects (NTDs). The review also highlighted that maternal VD supplementation was linked to lower cell proliferation rates in offspring's primary microglia, as evidenced by decreased Ki-67 levels (although we did not manage to see this in our own work in Chapter 5) and reduced

myelin loss, underscoring VD's protective role against neuronal damage. Our animal trial presented complementary findings, demonstrating that a VDD diet resulted in behavioural changes indicative of deficits in spatial learning and memory, as observed through altered performances in the Y-maze and increased anxiety-like behaviour, evidenced by reduced exploration of the open arms in the elevated plus maze. In addition to the animal studies reviewed, the scoping review examined two human cohort studies. The first study, involving a Turkish cohort, linked lower maternal VD levels to a higher incidence of HIE in neonates, suggesting VD's potential protective role against brain injury. Based on a Chinese cohort, the second study associated lower maternal VD levels with neurodevelopmental delays, utilising the DDST-II and GDS for assessments. These findings from human studies support the animal trial results and the broader narrative established in the scoping review about the importance of adequate maternal VD levels for healthy brain development.

Since the scoping review had a limited focus on inflammation's role in the neurodevelopment of VDD offspring, numerous studies were excluded to maintain a focus on this alone. However, a recent systematic review by Mutua et al., (550), focussing on human studies, found that there was a significant link between low maternal VD status and language impairment in children, suggesting that insufficient in-utero VD levels may impair the development of language-related brain structures. The analysis proposed maternal VD deficiency could alter critical brain structures and genes involved in neuronal survival and speech and language development, specifically BDNF and forkhead box protein P2 (Foxp2) genes. Given that our animal trial did not examine social

interaction, BDNF, or Foxp2 gene expressions, this represents a limitation and an opportunity for future research. This delineation between our direct experimental work and the broader literature review underscores the need for further investigation to fully understand the mechanisms through which maternal VD status affects offspring neurodevelopment and behaviour, and to assess the potential of maternal VD supplementation as a preventive strategy against neurodevelopmental disorders.

6.5 Maternal VDD Affects Body Composition – What are the Links to the Brain?

In addition to direct effects on the brain, our animal study suggests potential indirect pathways through which VD may influence neurodevelopment and function. We observed that VDD offspring weighed significantly less at three weeks than control mice, a developmental stage in mice akin to the end of the third trimester in humans when organ development is taken into account (587). Since infants with IUGR or born SGA often exhibit poorer neurodevelopmental outcomes, the lower weight of our VDD mice may be linked to their increased anxiety and diminished memory performance (588). This hypothesis is supported by studies of SGA infants showing higher levels of inflammatory markers in cord blood, which could tie back to the anti-inflammatory effects of VD on brain development discussed in the scoping review in Chapter 4 (589).

Further, our VDD mice demonstrated a degree of insulin resistance when compared to their control counterparts, indicating disrupted glucose regulation that could affect brain development. We did not measure insulin resistance in the mothers of the offspring; however, although insulin does not cross the placenta, high maternal glucose levels can lead to potential insulin resistance in offspring (590). Studies also show that poor insulin control in mothers is associated with poor cognitive function in the offspring (534, 535). Therefore, it is possible that this also contributed to the poor behavioural outcomes in our VDD mice. The VDD mice, especially males, had significantly lower fat mass than their control counterparts, which may influence the secretion of hormones like adiponectin from WAT (591). Adiponectin has anti-inflammatory properties and enhances glucose uptake, but its release is significantly decreased in obesity (591, 592). Intriguingly, research has shown that, paradoxically, children born SGA have markedly reduced adiponectin release in adulthood, with low levels associated with neurodegenerative disorders such as MS, Alzheimer's disease, and epilepsy (593, 594). However, as adiponectin levels were not measured, this remains speculative and potentially one of several factors that influence inflammation, insulin resistance, and body weight, which are also affected by VDD.

6.6 Conclusion

In conclusion, this thesis has established that VD exerts a significant regulatory effect on neurodevelopmental processes, impacting neuronal proliferation, differentiation, inflammation, and subsequent behavioural outcomes. We conclude from the evidence presented in this thesis that VD acts through two mechanisms to aid brain development: regulating inflammation and controlling differentiation and proliferation, as summarised in *Figure 6.6.1*. VD reduced proliferation and increased some differentiation markers in vitro, which indicates that it can regulate the cell cycle and directly affect neurone-like cell physiology. The scoping review revealed an association between maternal VDD, increased inflammatory markers, and altered foetal brain development, suggesting that VD's role extends to modulating the prenatal inflammatory environment. Moreover, in addition to the increased anxiety and reduced memory, VDD offspring exhibited altered insulin responses and body composition in the animal trial, indicating that VD's impact on brain development may involve metabolic and endocrine pathways. Currently, in the UK, many people, including pregnant women, are VDD. The research presented here underscores the potential long-term consequences of VDD, including an elevated risk of neurodevelopmental disorders such as ASD, MS, and schizophrenia. This thesis reinforces the importance of sufficient VD levels during pregnancy, providing further evidence that VDD is a modifiable risk factor for neurodevelopmental disorders.

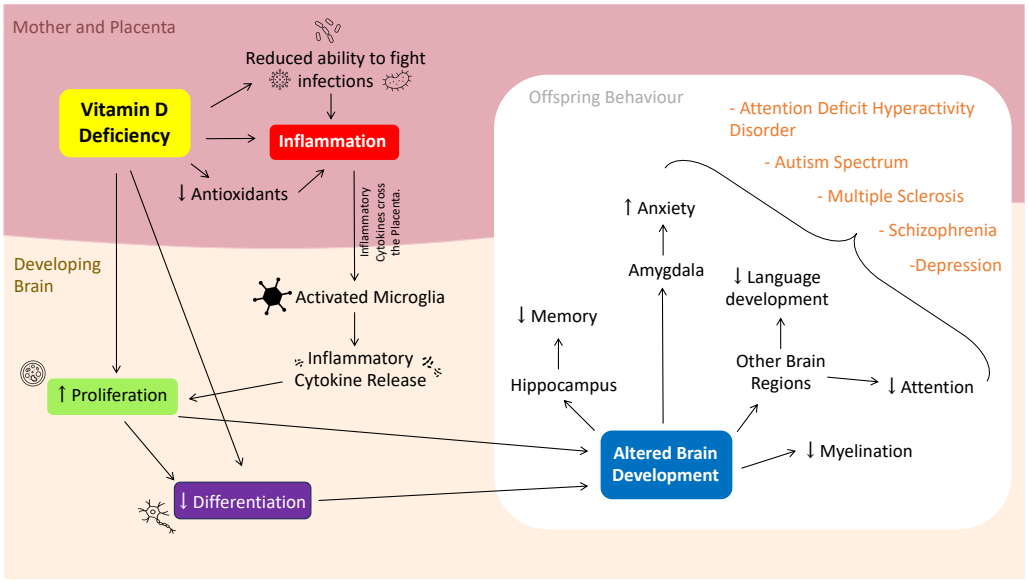

Figure 6.6.1. A Summary of the Findings of this Thesis

6.7 Limitations and Future Work

The in-vitro work conducted during this research has illuminated significant gaps in the current understanding of neurogenesis, particularly with utilising the SH-SY5Y cell line. While this cell line has proven invaluable for preliminary investigations into neuronal behaviour and development, it falls short of capturing the full spectrum of neuronal functionality in the brain. One of the critical findings from this research was the observation that high concentrations of calcitriol (10-7 M) reduced proliferation and facilitated protein expression associated with differentiation in SH-SYSY cells, albeit with concerns regarding the potential toxicity of such concentrations in vivo for both mice and humans. This highlights a pressing gap in translating in vitro findings to physiological contexts, underscoring future research's need to explore calcitriol's effects across a wider range of concentrations in models that more accurately reflect biological systems. In addition, it highlights the gap in knowledge of the actual physiological concentrations of calcitriol in the brain. To date, this has not been measured in humans or mice. The exclusive use of SH-SY5Y cells in the in-vitro work presented here has identified the need for a more comprehensive approach that includes primary neurons, such as the neurones extracted from the VDD and control offspring in our animal trial. Combining those two in-vitro systems would allow better comprehension and accuracy of the data.

Due to our findings in the animal trial, the interaction between VD and insulin

signalling in neurones is also a compelling area for further investigation. As mentioned previously, factors such as adiponectin could, in future, be investigated to form a more complete picture of how VDD resulted in poorer insulin sensitivity. Research into how VD influences neuronal responses to insulin through the proliferation and differentiation of neuronal cells under varying VD levels and in the presence of insulin could shed light on the mechanisms through which VD affects glucose control and neuronal function.

A popular and well researched concept within the nutrition and public health sphere is the DoHAD hypothesis (463). The DOHAD hypothesis is essentially the reason for this research, as we hypothesised that maternal VD status would dictate developmental effects. To further with this line of thought, though, an animal trial similar to ours but which involved switching the diets after weaning would be a lot more illuminating of the hypothesis, as we would be able to tell between the effects of maternal VDD or just lifelong VDD on behaviour and brain health. In addition, an area which we have not touched on is brain ageing. Globally, we are facing an ageing population. Could the effects of maternal VDD influence outcomes which last all the way into our older years? In addition to switching diets, future in-vivo work could also study the effects of VDD in old age, potentially uncovering the permanent effects that maternal VDD has on the brain.

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