THE IMPACT OF LONG-TERM VITAMIN D DEFICIENCY ON THE OFFSPRING METABOLIC HEALTH AND COGNITIVE FUNCTION VIA EPIGENETIC CHANGES

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DECLARATION

I hereby declare that this dissertation is all my own work and includes nothing which is the outcome of work done in collaboration except where specifically stated in the text. The work presented here is not substantially the same as any I have submitted for a degree or diploma or other qualification at any other institution.

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To my amazing mum and dad, for being the wonderful parents they are, for making it all possible

Abstract

Exposure to adequate Vitamin D during early development is crucial for neurodevelopment. However, the prevalence of Vitamin D deficiency (VDD) among women of childbearing age is on the rise. This study aims to investigate the impact of maternal VDD on postnatal neurodevelopment and delve into potential underlying mechanisms.

Previous research has linked chronic VDD to an increased risk of metabolic disorders like obesity and diabetes, collectively termed Metabolic Syndrome (MetSyn). To ascertain the relationship between low vitamin D levels and MetSyn, a systematic review and meta-analysis were conducted. The results revealed a negative correlation between low serum vitamin D concentrations and MetSyn, impacting offspring development from pregnancy through adulthood. We then employed an animal model of VDD to measure the molecular impacts of low serum VD concentrations. Female C57BL/6J mice received either a Vitamin D-sufficient (2.2 IU D/g; CD) diet or others a deficient (0.0 IU D/g; VDD) diet for 6 weeks before mating. Their offspring received their respective maternal diet for an additional 8 weeks after weaning, thus, experiencing VDD during both pre-postnatal stages. We assessed the offspring's metabolic health (changes in whole body physiology and feeding behavior) using the CLAMS method. VDD offspring displayed lower birth weights but experienced increased body weight in adulthood. This weight gain was attributed to reduced oxygen consumption during the dark phase, rather than alterations in food intake or ambulatory activity. In the realm of neurodevelopment, behavioral tests were

administered. VDD mice exhibited a significant increase in anxiety-like behavior, though no significant differences in memory and learning were observed compared to the control group. Notably, the effects of VDD seemed to vary based on gender and age, with female 3-week-old female mice and male 8-week-old male mice responding differently.

The VDD mouse model was also used to explore potential links between altered behaviour and neurodevelopment by assessing the rate of cell proliferation and differentiation in the dentate gyrus (DG) and subventricular zone (SVZ). While VDD had no impact on Ki-67 immunoreactivity at either 3-week-old female mice or 8-week-old male mice, it significantly reduced DCX immunoreactivity at 3 weeks. Ki67 immunoreactivity was notably altered in the SVZ at both time points.

Additionally, VDD led to a substantial increase in the expression of KDM6B and UTX, enzymes involved in neurogenesis, in the hippocampus of both 3-week-old female and 8-week-old male VDD offspring. While KDM5A exhibited no significant changes, BDNF expression significantly increased in 8-week-old male VDD mice but not in the 3-week-old female mice. Furthermore, the hypothalamic expression of non-acronymic VGF decreased significantly in both VDD 3-week-old female and 8-week-old male mice, compared to controls.

Experiments were also conducted ex vivo to corroborate the findings. The MTT assay demonstrated that vitamin D significantly influenced cell proliferation, and VD-treated N2a cells exhibited increased axonal projection. NeuroD1 gene expression

ASDCEX4RF remained constant across groups, while Syp expression increased on day 1 in VD-treated cells and gradually declined over 3 days.

Collectively, these results indicate that VDD during early development influences adult neurogenesis in the SVZ and DG of the hippocampus. These effects might be modulated by changes in the gene expression of histone (de)methylases, as detailed. Consequently, this study highlights alterations in gene expression pertinent to epigenetic programming within the hippocampus and hypothalamus.

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Abbreviations

AD	Alzheimer's disease
ARC	Arcuate nucleus
BDNF	Brain-derived neurotrophic factor
BrdU	Bromodeoxyuridine
CCK	Cholecystokinin
DAB	3,3'-diaminobenzidine
DBP	Vitamin D binding protein
DCX	Doublecortin
DG	Dentate Gyrus
DMN	Dorsomedial nucleus
DMNT	DNA methyltransferase
dNTP	Deoxyribose nucleoside triphosphates
DMT2	Diabetes Mellitus Type 2
EDTA	Ethylenediaminetetraacetic acid
GLP-1	Glucagon-like peptide-1
HAT	Histone acetyltransferase enzyme
IPC	Intermediate progenitor cells
miRNA	Micro-RNA
mRNA	Messenger RNA
MetSyn	Metabolic Syndrome
NPC	Neural progenitor/stem cell
PBS	Phosphate-buffered saline
PD	Parkinson’s disease
PFA	Paraformaldehyde
PPS	Pre- and postnatal stages combined
PYY	Peptide YY
qPCR	Quantitative polymerase chain reaction
SVZ	Sub-ventricular zone
VD	Vitamin D
VMH	Ventromedial nucleus

CHAPTER 1. GENERAL INTRODUCTION

VD is a hormone precursor. Humans can fulfil their VD requirements by either ingesting VD through diet (plants and some fish) or being exposed to the sunlight (UVB). VD controls calcium absorption in the small intestine and works with parathyroid hormone to mediate skeletal mineralization and maintain calcium homeostasis in the blood stream.

Exposure to sufficient VD during early development is thought to be critical for neurodevelopment. In 2016, Saraf and colleagues reported that 54% of the pregnant women, 75% of the new-borns have insufficient serum VD levels (Saraf R, 2016). Multiple evidence supports the link between VDD and pre/post-natal neurodevelopment. VDD, from pregnancy to adulthood, has been implicated in number of complications such as pre-eclampsia, gestational diabetes, vaginosis, and premature birth during pregnancy, as well as a range of neuropsychiatric and neurological disorders during offspring's life span (Mullighan *et al,* 2010: Belenchia, 2017).

The effects of maternal VDD seems to be different at different stages of life: in the embryo, at birth and throughout adulthood. Offspring that are exposed to insufficient maternal VD levels have; low birth weights, are small for gestational age, have reduced bone growth, poor skeletal mineralization, and lower bone mineral content (Wang H, 2018). Additionally, maternal VDD has been associated with higher adiposity, asthma, schizophrenia, and type I diabetes in offspring by the age of 9 (Kaushal and Magon, 2013). Rodent embryos from VDD pregnancies have enlarged brains (Eyles

D, 2015). Also, in early pregnancy, the active metabolite of VD, 1.25OH2D3, induces decidualization, which is key to implantation (Shin *et al.,* 2011). Yet, various authoritative bodies have established different cut-off points for defining VDD. For instance, the Institute of Medicine (IOM) in the United States defines deficiency as a serum $25OHD_3$ concentration below 20 nanograms per milliliter (ng/mL). However, a more conservative threshold is suggested by the Endocrine Society, which sets the cutoff at 30 ng/mL to ensure optimal bone health and overall well-being. The Vitamin D Council considers levels below 30 ng/mL as deficient and levels between 40 to 80 ng/mL as more desirable. These variations in cutoff points highlight the ongoing debate surrounding optimal vitamin D levels and underscore the importance of considering a range of factors including age, health conditions, and geographical location when assessing an individual's VD status.

Epigenetic mechanisms play a crucial role in long term programming (which occurs*in utero*) of gene expression. For example, recent studies have shown that prenatal exposure to famine in humans has persistent epigenetic effects in the offspring, impacting metabolism and cardiovascular health (Heijmans *et al.,* 2018). VD interacts with the epigenome at multiple levels: a) Vitamin D receptor (VDR) and its ligands target histone demethylases and b) some VDR ligands demethylate DNA (Fetahu *et al.,* 2014). Thus, it is highly possible that VDD *in utero* will produce epigenetic changes, having life-long effects on the health of the offspring. This study will focus on the impact of VDD at PPS on the offspring's metabolic and cognitive health and explore the potential mechanisms of how these might occur.

1.1 Vitamin D – An Overview

1.1.1 Production and Metabolism

VD is a fat-soluble steroid obtained via the diet or synthesized endogenously by sun exposure. The two major isoforms of VD are D_2 (ergocalciferol) and D_3 (cholecalciferol). D_2 is the first analogue of VD and is produced by exposing the ergocalciferol in plants and fungi to ultraviolet B (UVB) radiation (Bikle, 2014). Viadiet, fatty fish is the richest and one of few sources of D_2. Foods can also be fortifiedto contain D_2, and it can also be supplemented. D_2 differs from D_3 in chemical structure.

The production of D_3 in the skin is not an enzymatic process but a thermo-sensitive,non-catalytic photochemical reaction occurs by 7-dehydrocholesterol converted into D_3 by skin exposure to solar UVB radiation. Metabolism of VD is catalysed by P450 cytochrome enzymes. These are found in the endoplasmic reticulum (CYP2R1) and in the mitochondria (CYP27B1 and CYP24A1) (Bikle, 2014).

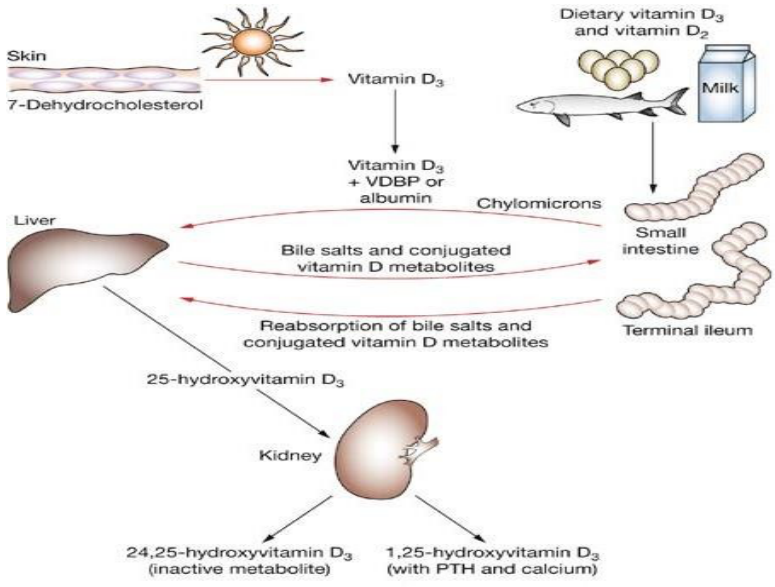


Figure 1.1 Figure showing the overview of VD synthesis.

Modified from Crawford, B., Labio, E., Strasser, S. *et al.* Vitamin D replacement for cirrhosis-related bone disease. *Nat Rev Gastroenterol Hepatol* **3,** 689–699 (2006). https://doi.org/10.1038/ncpgasthep063

The cholecalciferol produced in the skin undergoes first hydrolysis at its' 25th carbon in the liver. During this reaction, 25-hydroxylase is catabolized by number of CYP enzymes. In the mouse microsomal fractions, this reaction was found to be catabolised by the enzyme CYP2R1 to produce its' inactive metabolite form 25-hydroxyvitamin D ($25D_3$) (Chen *et al.,* 2014). In the mitochondria, it is CYP27A1 that induces this reaction. The $25D_3$ circulates in the blood and is distributed to the organs by forming a complex with Vitamin D binding protein (DBP) and albumin. There are very low levels of VD circulating in the free form. The liver produces theDBP and albumin (Bikle, 2000) and $25D_3$ is found bound to them. The $25D_3$-DBP complex is transported to the kidney and is internalised by endocytic receptors named megalin\cubilin in the proximal tubule cells. $25D_3$ undergoes a further hydrolysis at the 1st carbon by the enzyme 1alpha hydroxylase (CYP27B1) to formthe active form calcitriol (1.25 dihydroxyvitamin D_3). The kidney is the major sourceof the active form $1.25OH_2D_3$ (Anderson *et al.,* 2003). Once synthesized, $1.25OH_2D_3$ acts as a ligand by binding to the VDR that is foundacross all cells in the body. VD has genomic and rapid non genomic actions (Haussler, 2011). The genomic actions of VD are directly mediated by the nuclearVDR: upon binding to $1.25OH_2D_3$, VDR heterodimerizes with other nuclear hormone receptors, mainly the retinoid X receptor (RXR). This VDR-RXR complexthen binds to Vitamin D response elements (VDREs), special DNA sequences within the gene it regulates, to exert its transcriptional activity (Carlberg,

1993). The rapid non-genomic actions of VD can happen downstream of VDR, or by membrane receptors; these non-genomic actions are rapid and do not necessarily require nuclear transcriptional activity/ gene regulation. These include interaction with the signalling molecules such as phospholipids A and C, rapid generation of the secondary messenger pathway signalling such as PKC (Hii and Ferrante, 2016). Another non-genomic action of VD includes opening of Ca+ and Cl- channels (Norman *et al*., 1994).

1.1.2 Molecular actions of VD

$1.25OH_2D_3$ exerts most of its physiological actions through its high affinity bindingto VDR. VDR is a member of the steroid hormone nuclear receptor subgroup (NR11). VDR is expressed in many tissues throughout the body suggesting a widerange of biological functions. It is currently estimated that VD modulates the expression of many genes, about 10% of the whole human genome (Reichetzeder*et al.,* 2014). Due to VDR's presence in over 30 tissues, it can affect cardiovascularmortality, cancer prevention, autoimmune diseases, asthma, diabetes mellitus type 2 (DMT2), multiple sclerosis, and have anti-inflammatory properties (Olliver, 2013; Jeon, 2018; Gonzalez *et al.,* 2015). After binding of VDR to $1.25OH_2D_3$, it forms the VDR-RXR heterodimer, migrates into the nucleus where it binds to VDREs. VDR binding to VDREs trigger the expression of many target genes thus, generating downstream biological responses. VDR acts as a transcription factor which binds to other transcription factors and co-regulatory complexes that mediate changes in gene expression. These collectively mediate the transcriptional processes, enhancing or suppressing the expression of the

target genes. Many target genes of $1.25OH_2D_3$ has been identified that are important in regulating mineral metabolism, calcium, and phosphorus homeostasis. These coregulatory complexes include components which can have enzymatic activities such as an ATPase containing nucleosomal remodeling ability or enzymes that have selective epigenetic capabilities (Pike and Meyer, 2010). Upon $1.25OH_2D_3$ administration 8,000 VDR binding sites have beenidentified in osteoblasts across the mouse genome (Meyer, 2010). VD modulates the expression of the genes in many cells/tissues, thus, affecting their function. Inlymphocytes and monocytes,$1.25OH_2D_3$ administration stimulated 2776 and 1820VDR binding sites, indicating VD's role in the adaptive and innate immune system(Heikkinen *et al.,* 2011). In addition to above, CHIP-seq microarray analysis reported VDR target genes in colon (Palmer *et al.*, 2003), prostate (Krishnan, 2004) and breast (Swami *et al.,* 2003). VD modulates the expression of the genesin these cells, thus, affecting their function. Moreover, VD and VDR can directly regulate the expression of the enzymes that are involved VD's production and degradation. CYP27B1 is important for 1-hydroxylase, a key step in VD production.Also, both 25D3 and 1.25D3 is catabolised by CYP24A1 (Bikle, 2014).

VD maintains the level of its biologically active form because it has been found to suppress the renal production of CYP27B1 and induce CYP24A1 (Prosser and Jones, 2004), the enzymes needed for VD metabolism. In addition, an *in vitro* studyshowed that VD can autoregulate the expression of VDR (Costa *et al.,* 1985).

1.1.3 Physiological roles of VD

1.1.3.1 Calcium homeostasis and bone metabolism

One of the first identified, well-known role of the VD is that it is the major controlling hormone for calcium homeostasis. VD regulates all the processes of intestinal calcium absorption and transport via both genomic and non-genomic ways. VD has been reported to induce the expression of apical membrane calcium channel TRPV6, the calcium binding protein calbindin-D_{9k} through genomic actions (Bolt, 2005). And it regulates the opening and transport of the Ca channels via non- genomic, rapid actions. VDR is expressed in osteoblasts, chondrocytes and in largesegments of small and large intestines. Therefore, it contributes to bone development and health (Kitanaka, 1998; Suda ,2015). Despite being normal at birth, VDR null mice developed abnormalities of mineral ion homeostasis due to decreased intestinal calcium reabsorption, therefore prone to manifesting skeletaldisorders (Li, 1998). In addition, mutation in CYP27B1, an enzyme needed for VDmetabolism, resulted in VD dependent rickets in humans (Kitanaka, 1998). When it is not possible to maintain normal serum calcium levels by intestinal calcium absorption, VD acts in concert with parathyroid hormone (PTH) to maintaincalcium and phosphate reabsorption in the intestines, kidney, and bone (Christakos, 2014). As a result, they increase calcium absorption from the renal distal tubule and remove calcium from the bone. However, when the PTH levels increase, removal of the calcium from the bone leads to increased risk of bone fracture and significant amount of bone loss (Sahota, 1999). This shows that VDis needed not just for maintaining calcium homeostasis but also for optimum bone health.

1.1.3.2 Non skeletal roles of VD

Due to VDR expression in over 30 tissues, VD is linked to cardiovascular function, cancer, neurological and neuropsychiatric conditions, and anti-inflammatory properties (Olliver, 2013; Jeon, 2018; Gonzalez *et al.,* 2015).

VD and cancer: The VD biosynthesis occurs in cancer tissue due to presence of VDR and the metabolizing enzyme, CYP27B1. The molecular mechanisms of anti-cancer effect of VD are anti-proliferative, anti-inflammatory effects (Feldman,2014). Epidemiological studies suggest that VD has a role in prevention of variouscancer types (Garland, 1989; Ahonen, 2000; Bertone-Johnson, 2005).

VD and the Immune System: VDR and the CYP enzymes are present in T cells, B cells, antigen-presenting cells (APCs), macrophages and monocytes. VDR overexpression in T and B cells activates many VD responsive genes that are responsible for differentiation and proliferation. VDR-RXR complex in the nucleusof the immune system cells also directly influence transcription of antimicrobial proteins such as defensin and cathelicidin (Wang *et al.*, 2004). $1.25OH_2D_3$ has a direct effect on T cells to inhibit the production of pro-inflammatory cytokines, IL-2and IFN- alpha, and promotes the secretion of anti-inflammatory cytokines (IL-4, IL-5, IL-13) (Harinarayan, 2014).

VD and Neurological Diseases: The presence of the VDR and CYP enzymes across the brain suggests its role in brain function. (Schlögl, 2014). The VDR has been shown to be highly expressed in substantia nigra, caudate putamen and nucleus accumbens. VD crosses the blood-brain barrier, enters the glial and neuronal cells to be converted into the active form of VD, $1,25OH_2D_3$, to exert numerous

functions (Cui, 2015). The understanding of the potential mechanisms behind VD's role in the neurological diseases, however, is still limited. It is involvedin crucial actions throughout brain development including cellular differentiation, proliferation and apoptosis, neuroprotection, anti-inflammation and modulating DAand 5HT signalling pathways (Umar, 2018: Kesby, 2017). Gezen-Ak and colleagues observed that VD plays an important role in the clearance of the amyloid, Aβ, processing pathways, which are considered a causeof Alzheimer's disease (AD), when they prevented the genomic and non-genomic actions of VD by disrupting its associated receptors (Gezen-Ak, 2017). The neuroprotective and anti-inflammatory actions of VD is also linked to AD pathology.VD increases the production of macrophages which act in the clearance of Aβ plaques (Masoumi, 2009). VD reduces amyloid-induced cytotoxicity (Dursun, 2011) and inhibits Aβ stimulation of iNOS (Dursun E, 2013) *in vitro*. The change inturn contributes to the inflammatory process related to AD. VD induces neuroprotection by modulating release of nerve growth factors (Eyles, 2003). VD is suggested to be considered in therapeutic prevention against AD. Parkinson's disease (PD) is a progressive neurodegenerative disorder characterised by selective dopaminergic neuronal loss in substantia nigra. Symptoms include dysfunctional motor skills such as tremor, rigidity, and dyskinesia. People with PD showed lower serum VD levels compared to controls.Zhang and colleagues the CSF of PD patients were significantly increased (Zhang, 2008). Moreover, people with PD were observed to have increased rates of hip fractures compared to non-PD subjects (Chiu, 1992) which may be due to decreased calcium metabolism linked to low levels of VD in the body. Furthermore, population studiesin Chinese, Collectively, these studies link VD to PD. However, it is important to consider that the impaired motor skills in patients

with PD may prevent them to spend time outdoors, thus, this may also be the reason they have decreased serum VD (Zhu *et al.,* 2014). Many in vitro and human studies confirmed that VDD increases the susceptibility to develop autoimmune diseases such as Multiple Sclerosis (MS). There is a causal relationship between low VD levels and MS. It is shown that the anti- inflammatory status is enhanced in murine MS models of VD (Chang, 2010). Epidemiological studies have also shown that the prevalence of MS peaks in areaswhere sunlight is minimal, at higher latitudes (Swank *et al.,* 1952; IA van der Mei *et al.,* 2001) The risk decreases when people migrate to lower latitudes (Kurtzke *et al.,* 1985). However, results of a study investigating CNS demyelination concluded that the effects might be because of UV light itself, independent of VD (Lucas *et al.,* 2001). VD supplementation can reduce the risk of developing MS and the clinical activity established in patients such as decreased risk of relapse and reduction in disease activity on brain MRI (Munger *et al*., 2004). However, ina meta-analysis conducted by James and colleagues on the effect of VD in MS relapses has reported mixed reported results with no significant results (James *etal.,* 2003). Some limitations included small sample size, cohort loss at follow-up duration, treatment with IFNB. Therefore, there is a need for prolonged and larger controlled clinical trials to authorize VD supplementation as a care for MS patients.

VD has been reported as an important factor in prevention and treatment of several chronic, cognitive disorders. Autism spectrum disorder (ASD) is a common neurodevelopmental disorder characterised by impaired communication and repetitive behaviour. Preliminary research shows a relationship between VD and ASD. Treatment of ASD patients with VD improved core symptoms of ASD in few clinical studies. (Jia *et al.,* 2015; Feng *et al*., 2017).

Mood disorders such as depression and anxiety are common causes of disability. According to World Health Organization, 2008, 121 million people are affected worldwide. Depression is a socio-economic burden costing $36.6 billion and 225 million lost workdays annually in United States (NIMH, 2006). VDD has been associated with depression. A randomised study observed adecrease in depression in patients with seasonal affective disorder who were treated with VD with a one-time dose of 100,000IU (Gloth *et al.,* 1999). VD is related with anxiety in patients with anxiety disorders (C M *et al.,* 2015).

1.2 Vitamin D deficiency (VDD)

VD status is determined by the measurement of serum level of $25D_3$, and VD status can be described as deficient, insufficient, or normal. However, assessments of VD status can vary as the cut-offs are not agreed upon by all authoritative bodies, thus reporting different effects of VD as different levels are considered as deficiency (Hollis and Wagner, 2013). Although no consensus has been reached yet by the authorities, most experts describe the values of the VDD below 50nmol/l as deficiency, the range between 30nmol/l and 50nmol/l as VD insufficiency and the range between (50nmol/l) and 125nmol/l as optimal VD levels (Amrein *et al.,* 2020). Vitamin D deficiency has been defined by Institute of Medicine (IOM) as a 25(OH)D of less than 50nmol/L as this results in unfavourable skeletal outcomes (Holick, 2007; Malabanan *et al.,*1998). VD toxicity is thought to occur after 375nmol/L, although the views are inconsistent with this too (Marcinowska-Suchowierska *et al.,* 2018). VD toxicity is associated with hypercalcemia. Furthermore, there are obvious differences between the ‘sufficiency’ cut-off between authoritative bodies as IOM defines it as50nmol/L and ‘Endocrine

Society Clinical guidelines' suggests 75nmol/L. IOM established these levels since 1997 solely based on effects on bone health. In 2011, they concluded that the evidence regarding non-skeletal roles of VD is inconclusive. For vitamin D, RDAs of 600 IU/d for ages 1–70 year and 800 IU/d for ages 71 year and older, corresponding to a serum 25-hydroxyvitamin D level of at least 20 ng/ml (50 nmol/L), meet the requirements of at least 97.5% of the population (IOM,2011). The other authoritative bodies suggests that higher amounts are necessary to avoid VDD (Hanley *et al.,* 2010).

VD status can vary due to different ethnic backgrounds; darker skin pigmentation, season, diet, latitudes, and usage of supplementation (Holick *et al.,* 2006). At high latitudes serum VD levels vary throughout the year, peak in mid-summer and lower in late winter due to the ultraviolet radiation exposure. The level of VD synthesis through the skin decreases due to varying angle of sun rays and decreased exposure to sunlight in the winter months. In addition, skin pigmentation has an important role in the deficiency: high levels of melanin pigment prevent the efficientabsorption of the UVB rays in dark-skinned people. The countries in Middle East, VDD is common due to the cultural clothing style covering most of the body (Correia *et al.,* 2014). In Norway, however, there is a lower frequency of VDD (Holvik *et al.,* 2005), probably due to the diet or use of supplementation, compared to the countries such as Mediterranean. Epidemiological studies often observed insufficient circulating levels of VD with increasing age and the prevalence is as high as 80% in most European countries (Palacios *et al.,* 2014). It has also been reported that with ageing, the $1,25OH_2D_3$ absorption in the intestines decreases. Since VD is absorbed from the ileum, it can lead to deficiency. Crohn's disease and celiac disease which are

malabsorption syndromes, are correlated with VDD. Epidemiological studies show that higher levels of VD are associated with lower incidence of IBD, especially Crohn's disease (Joseph *et al.,* 2008). In addition, individuals who had <50nmol/L VD levels had increased risk of CD surgery and hospitalization (Ananthakrishnan *et al.,* 2013). In a cross-sectional study, higher levels of faecalcalprotectin, which is a marker for intestinal inflammation, is associated with low VD levels (Garg *et al.,* 2013). In colitis, defective autophagy has occurred upon deletion of the VDR. Therefore, one can say that there is an essential correlation between intestinal autophagy, VDR and gut microbiome. VDR activity is also affected by certain strains of bacteria (*Chlamydia trachomatis)* (Wu *et al.,* 2015).Since the expression and activity of VDR is established, the use of VD as a therapeutic in immune regulation and associated diseases can be considered.

Collectively, the risk factors of VDD include ageing, clothing style, excessive sunscreen use, sedentary lifestyle, smoking, air pollution, renal and liver diseases,and medication such as glucocorticoids which effects the VD metabolism (Lavie *et al.,* 2011).

1.2.1 Maternal and longer-term impacts of VDD

VDR and the enzymes CYP27B1 and CYP24A1 are present in placenta during pregnancy: human placental decidua and foetal trophoblasts synthesize $1.25OH_2D_3$ which in turn contribute to foetal skeletal ossification (Weisman *et al.,* 1979). The need for VD production doubles during early stages of pregnancy for foetal development and return to normal during lactation. Therefore, the optimal intake of VD during pregnancy is crucial.

The VD circulation in the maternal-foetal blood is highly important regarding both the complications caused during pregnancy and the adverse outcomes on offspring health. VDD is more common in expectant women then non-pregnant women (De-regil *et al.,* 2012). Due to the increase in demand for VD during the 3rd trimester of pregnancy, without optimal intake of VD in pregnant women, many complications may occur such as pre-eclampsia, gestational diabetes, vaginosis, and premature birth, depression after pregnancy, infant height, and weight (Lewis *et al.,* 2010). Rickets, wheezing, allergy, respiratory tract problems i.e., asthma, bone fragility andincreased susceptibility to autoimmune diseases may occur later in life (Shin *et al.,* 2010).

VDD during early life leads to rickets. And in adulthood, VDD may lead to osteoporosis and/or osteomalacia due to physiological actions of VD mentioned in section 1.1.3.1. Besides bone health, VD is also known to effect skeletal muscle mass and strength with increasing age (Wintermeyer *et al.,* 2016). Indeed, in a studyinvolving people aged 10-65 with diverse ethnic backgrounds, 90% of the patients with musculoskeletal pain had VDD (Plotnikoff and Quigley, 2003).

Studies have also shown that low maternal circulating $25D_3$ in late pregnancy to be associated with lower fat mass at birth but with greater fat mass at ages 4 and 6 years (Crozier *et al.,* 2012). The greater body fat mass during adolescence can further lead to high susceptibility for diabetes, hypertension, and cardiovascular diseases. In addition, a recent study in children and adolescents suggests a positivecorrelation between VD levels and serum levels of total cholesterol, apolipoprotein A1, apolipoprotein B, and triglycerides (Delvin *et al.,* 2010). In adults, many studies suggest an inverse association between Metabolic Syndrome (MetSyn) components

such as BMI, waist circumference, insulin sensitivity and glucose levels with serum VD levels (Diaz *et al.,* 2016).

In the rat, VDR expression starts in the developing brain just after neural tube closes,at embryonic day 12. Cui and colleagues showed that VDR is also localised in neuro proliferative regions of the neonatal rat brain and maternal VDD altered adult neurogenesis (Cui *et al.,* 2007). VD is shown to affect cell cycle. The offspring born from VDD mothers had an abnormal anatomical brain development compared to controls. The deficient offspring had enlarged brains; enlarged lateral ventricles, decreased width of the neocortex (Eyles *et al.,* 2003). Due to the structural changes occurring in hippocampus of the developing brain, alterations in motor function, memory and sensory motor abilities and behaviours of the VDD animals has been observed (Byrne *et al.,* 2013). Data from human studies showed that the expectant mother with insufficient levels of VD has a higher risk of having children with reduced social development, cognitive and motor skills in childhood (Darling *et al.,* 2017). Following the effects of VD mentioned above, there have been many studies linking VDD to the nervous system disorders. Low levels of VD are associated with AD and dementia (Anastasiou *et al.,* 2014; Landel *et al.,* 2016), in addition to PD (Shinpo *et al.,* 2000) and depression (May *et al.,* 2010). A lower risk of developing AD is found to be associated with higher dietary intake ofVD in older women in a seven-year follow up study (Annweiler *et al.,* 2012). Agingis a strong risk factor for developing VDD due to the increased skin thickness. Llewellyn and colleagues observed an increased risk of cognitive decline in elderly with VDD compared to ones with sufficient levels of (Llewellyn *et al.,* 2010).

Higher percentage of people with schizophrenia were born during winter and spring with maybe associated with VDD due to lack of necessary sunshine (McGrath *et al,* 2010). The winter\spring births correlates with the last trimester of the pregnancy, where there is less sunshine therefore increased risk of mothers being VDD. An epidemiological study showed a 5-8% excess in the number of patients with schizophrenia and/or bipolar disorder in winter\spring births. In addition, an increased prevalence of depression and autism (Torrey *et al.,* 1997). Furthermore, low levels of serum VD were associated with depression in overweight and obese rats (Jorde *et al.,* 2008). And, in people with cardiovascular diseases (May *et al.,* 2010).

In conclusion, many studies show that VDD might increase the susceptibility to develop chronic, autoimmune, and neurological diseases due to diverse actions of VDR throughout the body (Holick, 2005). Therefore, optimal intake during pregnancy is crucial to long-term health of the offspring.

1.3 Brain Function

The adult human brain is arguably the most complicated organ of all biological systems. Due to VD's many functions regarding metabolic health and neurogenesis described above, we will focus our study on two brain regions: hypothalamus and hippocampus.

1.3.1 Hypothalamic regulation of energy homeostasis

The brain, particularly the hypothalamus has a key role in the homeostatic regulation of energy and glucose metabolism. The crosstalk between the peripheral metabolic organsand the hypothalamus is an integral part for the control of food intake, insulin

secretion, energy metabolism, fatty acid/glucose metabolism in adipose and skeletal tissues (**Figure1.2**).

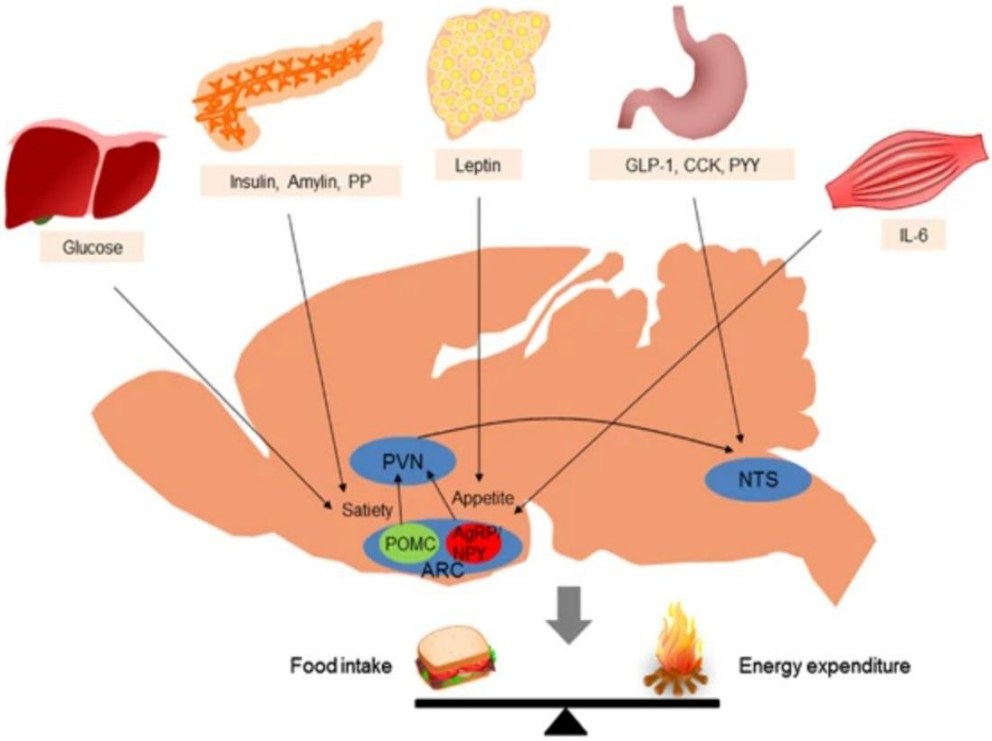


Figure1.2 The central nervous system and the peripheral metabolic signals involved in energy homeostasis. The brain integrates metabolic signals from peripheral tissues such as liver, pancreas, gut, muscle, and adipose tissue. Neuronal networks in the brain coordinates food intake and energy homeostasis upon altered metabolic conditions. arcuate nucleus (ARC); cholecystokinin (CCK); glucagon-like peptide-1 (GLP-1); interleukin-6 (IL-6); pancreatic polypeptide (PP); paraventricular nucleus (PVN); PYY, peptide YY (PYY) *(Roh Eet al, 2016).*

Substantial evidence suggests that homeostatic mechanisms which controls food intake and energy expenditure in humans are modulated primarily in hypothalamus. In 1849, Claude Bernard implicated that the brain is central to the regulation of energy homeostasis with his first ever clinical description that the injury to hypothalamic-

pituitary axis caused obesity. Since then, the field of obesity and diabetes has contributed to the understanding of the neural networks controlling energy and glucose metabolism (Williams *et al.,* 2010), in addition, withthe recent advancements in technology such as the field of optogenetics, understanding detailed hypothalamus-based networks became easier (Aponte *et al.,* 2011).

Hypothalamus is made from multiple nuclei. Arcuate nucleus (ARC), located at the base of the hypothalamus, is a major hub for integrating nutritionally relevant information from all peripheral organs via circulating hormones, metabolites, and/or neural pathways. It is located neighbouring to the median eminence and surrounds the third cerebro-ventricle. Therefore, it is accessible to the signals in the systemic circulation. ARC contains of two populations of neurons: activation ofone expressing orexigenic peptides neuropeptide Y (NPY) and agouti related protein (AGRP). AGRP leads to increased food intake and the activation of the other expressing anorexigenic peptides proopiomelanocortin (POMC) andcocaine-amphetamine regulated transcript (CART) leads to decreased food intake. These neuronal populations are interconnected with multiple brain structures so they can be activated by the signals received from other neuron groups. POMC neurons project axonal processes to other neuron groups in other areas of hypothalamus such as paraventricular nucleus (PVN), lateral hypothalamus (LH),ventromedial hypothalamus (VMH), brain stem and spinal cord (Hill, 2010; Lanfray*et al.,* 2017). These neurons that are involved in nutritional and hormonal signals are also influenced by the expression of the pro-inflammatory cytokine, IL-6 (Señaris *et al.,* 2011). The activation of the PVN neurons has an inhibitory role in food intake and weight gain. Activation of these neurons results in increased fatty acid oxidation and lipolysis (Foster *et al.,* 2010), malfunction of PVN

therefore leads to hyperphagia and obesity (Leibowitz *et al.,* 1981). VMH receives neuronal projections from ARCand sends projections to DMN, LH and brain stem. VMN neurons senses glucose (satiety) and leptin (appetite). Therefore, these functions make VMN to be an important area for maintaining food intake. Destruction of VMN and/or DMN lead to hyperphagia, obesity and hyperglycemia (Shimizu *et al,* 1987; Bernardis and Bellinger, 1986). Studies have also shown that VMN produces the neuropeptide brain-derived neurotrophic factor (BDNF) which has a role in regulation of energy metabolism (Xu *et al.,* 2003). VMN and DMN are the feeding centres and destruction leads to changes in food intake.

1.3.1.1 Nutrient signals

Nutrient signals play a crucial role in the intricate web of endocrinology, where hormones regulate various physiological processes. Nutrients such as glucose, amino acids, and fatty acids act as signaling molecules that trigger hormonal responses to maintain metabolic homeostasis. For example, after a meal, increased blood glucose levels stimulate the pancreas to release insulin, which promotes glucose uptake by cells and its storage as glycogen. Amino acids also contribute to hormone secretion; their presence signals the release of insulin and other anabolic hormones, promoting protein synthesis and tissue growth. Fatty acids, on the other hand, can influence the secretion of hormones like insulin and glucagon, affecting energy storage and release. In 2002, Obici and colleagues demonstrated that centraladministration of Long-chain fatty acids triggered a hypothalamic response to regulate energy and glucose homeostasis. An increased amount of long chain fatty acids activates

hypothalamic long-chain fatty acid-coenzyme A (LCFA-CoA), and this results in decreased food intake (Obici S et al., 2002).

This intricate interplay between nutrient signals and hormonal responses underscores the dynamic nature of endocrinology, highlighting how our body's hormonal balance is intricately intertwined with the nutrients we consume.

1.3.1.2 Adiposity signals:

The adiposity signals are in proportional with the body adiposity. They enter the brain via the blood-brain barrier and interact with the gastrointestinal signals. Insulin and leptin are well-known adiposity signals. Insulin is secreted by the pancreatic beta cells, and it is the primary controller of blood glucose levels. In the hypothalamus, ARC has high densities of insulin receptor (Bruning *et al.,* 2000). Reduced food intake and body weight was observed after exogenous administration of insulin into the ARC of the animals (van Dijk *et al.,* 1997; Woods *et al.,* 1979). Leptin, which is a circulating hormone produced primarily by adipose tissue. It regulates food intake, and its' production is stimulated by insulin (Majewska *et al.,* 2016). Leptin injection in ARC resulted in reduced food intake and body weight: ARC mediates leptin's actions (Oswal and Yeo, 2009). Although both insulin and leptin are adiposity signals and regulate adiposity, they individually have crucial actions: Insulin, is the key controller of the blood glucose usage throughout the body. Leptin is thought to maintain important physiological systems in case of starvation (Ahima *et al.,* 1996). Upon receiving information about the nutrient state and the balance of the peripheral signals, the brain monitors altered metabolic conditions and coordinates adaptive changes. Any

malfunction in the hypothalamic neuronal circuitry can lead to metabolic diseases including DMT2 and obesity.

1.3.2 VD in hypothalamic regulation of energy homeostasis

VDR is present in tissues that are highly active in energy metabolism of the whole body, such as pancreas (Lee *et al.,* 1994), liver (Duncan *et al.,* 1988), muscle (PikeJW, 2014) and adipose tissue (Ding *et al.,* 2012). There is a relationship between the actions of VD and the nutrient and energy balance. VD regulates glucose homeostasis through activation of the PVN neurons and energy metabolism through activation of the ARC neurons (Sisley *et al.,* 2016). Sisley and colleagues observed that VDR is colocalised with POMC and NPY neurons in ARC. They observed a rapid depolarisation of POMC cells by $1,25OH_2D_3$ and proposed that the mechanism might be through transient receptor potential channels (TRPCs). Administration of VD improved glucose tolerance and insulin sensitivity, an effect modulated by the VDR in PVN (Sisley *et al.,* 2016). It also promoted weight loss by the reduced food intake in obese rodents, again through the actions of VDR in ARC. Animal studies and clinical data linking VDD and VDR gene polymorphisms to metabolic (Holick *et al.,* 2011) and autoimmune diseases (D'Aurizio *et al.,* 2015) suggest an interaction between VD and thyroid hormone. Within the hypothalamic circuitry, neuronal projections in PVN receive direct input from ARC neurons and associated mainly for autonomic and neuroendocrine functions such as thyrotropin-releasing hormone regulating HPA axis and CRH regulating stress-response (Lechan and Fekete, 2006). Thyroid hormone regulates insulin sensitivity, which is important for gluconeogenesis therefore correlates with energy metabolism and glucose homeostasis. Thyroid hormone,

together with VD, regulated the expression of VGF (Lewis *et al.,* 2015). VGF is a nerve growth factor which is abundantly expressed in hypothalamus (Levi *et al.,* 2004). VGF is previously shown to be associated with increase in energy metabolism and food intake. *In silico* analysis of the VGF promoter sequence revealed to have both thyroid response element (TRE) and Vitamin D response element (VDRE) (Lewis *et al.,* 2016). *In vitro,* VD inhibits expression of leptin in human adipose tissue (Menendez *et al.,*2001). Adipose tissue is an active endocrine organ, a major storage site for VD metabolism and VDR has many actions in the adipose tissue. VDR participates in adipogenesis, lipolysis, fatty acid synthesis and cytokine secretion (Kong *et al.,* 2013; Shiba *et al.,* 2001) by interacting with membrane receptors, nuclear receptors, and uncoupling proteins. VDR KO leads to upregulation of uncoupling proteins, leaner body type, increased energy expenditure and secretion of fatty acids (Wong *et al.,* 2009).

1.3.3 Metabolic Diseases

1.3.3.1 Metabolic Syndrome

MetSyn is a collective term used to describe having cardiovascular diseases (CVD), obesity and DMT2 (International Diabetes Foundation, 2006; Alberti *et al.,*2006). The characteristics of MetSyn include insulin resistance, high blood glucose/lipid levels, hypertension, atherosclerosis, and obesity.

The current treatments are mainly lifestyle changes through physical activity and diet and pharmacological treatment for dyslipidaemia, hypertension, and hyperglycaemia. Patients with MetSyn show abdominal obesity, diabetes, atherogenic dyslipidaemia, and hypertension and insulin resistance (Alshehri *et al.,* 2010). The shared pathophysiology and the clustering of the risk factors mightbe indicative of their inter-dependence therefore, they might share the same underlying cause and mechanism (Grundy *et al.,* 2005).

There has been a consensus between different government bodies regarding the accepted diagnostic criteria for the presence of MetSyn. National Cholesterol Education Program 2005, World Health Organization (WHO) 1998, European group for the Insulin Resistance (EGIR) 1998, American Association of Clinical Endocrinologist (AACE) 2003, IDF 2005 and American Heart Association (AHA) 2005 all have different cut-offs for defining MetSyn. NCEP Adult treatment panel III.defined as having MetSyn upon presentation of three or more of the following components: i) waist circumference ≥90 cm for men or ≥80 cm for women; ii) triglyceride ≥1.7 mmol/L; iii) HDL cholesterol <1.03 mmol/L for men or <1.30 mmol/Lfor women; iv)blood pressure ≥130/85 mmHg;

and v) fasting glucose ≥5.6 mmol/L.IDF defined MetSyn as according; if subjects had central obesity (BMI>30 kg/m_2) and any two of the following: a triglyceride level >150 mg/dl, an HDL cholesterol level <40 mg/dl in men or <50 mg/dl in women, systolic blood pressure >130 mmHgand/or diastolic blood pressure >85 mmHg, and fasting glucose >100 mg/dl. Individuality of certain lifestyle factors such as outdoor physical activity and smokingis a limitation that makes it difficult to quantify VD in the body, therefore the treatments are still not efficient.

VD and MetSyn: To examine the role of VD on MetSyn prevalence, several studies have investigated potential associations of VD with the traditional components of the MetSyn. These studies reported significant inverse associations of baseline serum VD levels with several metabolic syndrome components including waist circumference, triglycerides, fasting glucose and homeostatic model assessment of insulin resistance (HOMA-IR) (Ganji *et al.,* 2011; Mellati *et al.,* 2015; Diaz *et al.,*2016). (More detail in chapter 2.1). Nevertheless, the most common diagnosed component of MetSyn is insulin resistance (Grundy *et al.,* 2005: National Cholesterol Education Program, Adult treatment panel III, 2002), even though not every person at risk who fulfils the criteria for MetSyn has insulin resistance and, on the contrary, not all persons with insulin resistance have MetSyn (Kahn, 2007).

1.3.3.2 Obesity

WHO describes obesity as having BMI of 30 kg/m^2 or more Noncommunicable Diseases Country Profiles, World Health Organisation; Geneva, Switzerland: 2011 p. 209) It is described as a chronic progressive disease, independent of being justa risk factor for other disorders by the World Obesity Federation and the American and Canadian Medical Associations. Indeed, most adults in the UK are overweight or obese

with 26% of men and 29% of women being considered obese. According to the latest data from NHS this costs an increasing estimation of £27 billion to society. Obesity affects high percentages of the population both in developed and developing nations (James, 2008). It is a world-wide epidemic which is significantly associated with increased mortality, morbidity and is also the risk of developing number of other metabolic diseases such as DMT2, cardiovascular diseases, several cancer types (breast, ovarian) as well as Alzheimer's disease and depression. Obesity makes a significant impact on one's quality of life, it might impact unemployment, lower productivity and efficiency and lead to many other social drawbacks. Obesity among women of reproductive age is increasing across the globe (Chen *et al.,* 2018). Maternal obesity is associated with several complications during pregnancy including pre-eclampsia, gestational diabetes mellitus, pre-term delivery (Lynch *et al.,* 2008) as well as foetal/neonatal complications such as congenital heart defects i.e., heart septal defects, aortic arch and conotruncal defects (Persson *et al.,* 2019). In addition, excessive weight gain during pregnancy and retaining the pregnancy weight can lead to future risk of obesity in the mother(Rooney and Schauberger, 2002). Furthermore, it has a significant impact on *in utero* environment, thus increasing the risk of obesity in offspring later in life. Indeed, Whitaker and colleagues demonstrated a relationship between maternal and childhood obesity. Among the 8494 children, prevalence of childhood obesity was 9.5%, 12.5%, and 14.8% at 2, 3, and 4 years of age respectively regardless of maternal obesity, of these 30.3% had obese mothers. By the age of 4, maternal obesity more than doubled the risk of obesity (24.1%) compared to others with non-obese mothers (9.0%) (Whitaker, 2004). A potential underlying mechanism of the consequences described above can be the foetal programming in

response to nutritional status (details in 1.4). Foetal development continuously adapts to the nutrients provided and their metabolism is adjusted accordingly (Boo and Harding, 2006). Maternal obesity is characterized by abnormal placental vascular supply and increased placental nutrient transfer (Howell, 2017).

Obesity and VD: Liquid chromatography and mass spectrometry confirmed apositive relationship between VD in adipose tissue and serum VD (Lipkie *et al.,* 2013). Adipose tissue is VD-sensitive. 25-hydroxylation of VD is impaired in obese individuals (Roizen *et al.,* Holick *et al.,* 2008; Vanlint, 2013). The possible mechanisms on how insufficient VD can cause obesity may be the following: VDR is present in adipocytes and activation of VDR by VD leads to adipogenesis and induces adipocyte apoptosis (Ding *et al.,* 2012). Hydroxylation enzymes that are involved in VD metabolism are also impaired in obesity (Bikle *etal.,* 2014). A recent study showed that CYP2R1 was supressed by obesity in mice and humans in addition to CYP27B1 which was repressed by weight loss (Elkhwanky *et al.,* 2020). So, the expression of the CYP genes and consequent VD-hydroxylation is tightly regulated by the metabolic state (Aatsinki *et al.,* 2019).Although, these data do not prove a causative effect, these clearly prove an association between low serum VD levels and obesity.

1.3.3.3 Diabetes Mellitus Type 2

The increase in the prevalence of obesity in women at a reproductive age is the likely cause for the gestational diabetes in the mother during pregnancy and the increased risk of DMT2 in the offspring years later. In fact, according to a systematic review 16% of women aged 20-49 years old worldwide are affected by gestational diabetes during pregnancy (Guariguata *et al.,* 2014): at age 22, the prevalence of DMT2/pre-diabetes

was 21% in the offspring of GDM mothers while 4% in the offspring of mothers with no diabetes (Garcia-Vargas *et al.,* 2012).

DMT2 is a common diagnosed component of MetSyn (Grundy *et al.,* 2005;National Cholesterol Education Program, Adult treatment panel III, 2002). It is characterised by glucose dysregulation, impaired insulin secretion and insulin resistance. Insulin resistance is a phenomenon which is implicated in several adult-onset diseases (Reaven, 2011). It is defined as the condition where the cells in themuscles, adipose and liver does not respond well to the hormone insulin and thus cannot effectively take up the glucose from the bloodstream. Therefore, the pancreas starts producing more insulin to help absorb the glucose into the cells (Lebovitz, 2001). Maternal diabetes during pregnancy can cause neonatal hypocalcemia, seizures, and tetany within the first 24–72 h after birth (Kovacs, 2015). Furthermore, several findings confirm that offspring of the mothers with diabetes, existing or developed during pregnancy, have higher risk of developing cardiovascular disease. (Leirgul *et al.,* 2016).

DMT2 and VD: The effects of VDD on insulin resistance, pancreatic β-cell dysfunction and systemic inflammation have been proposed as causative factors for developing DMT2 in several studies (Grimnes *et al.,* 2010). VD directly enhances the transcriptional activation of insulin in pancreatic β-cells (Bornstedt *etal.,* 2019). One of the mechanisms by which VD regulates insulin is via regulating calcium signalling. Calbindin is a VD-dependent cytosolic calcium binding protein in pancreatic beta-cells. So, VD can mediate intracellular calcium influx in beta cells through production of calbindin, thus insulin release (Johnson *et al.,* 1994). In patients with DMT2, VD inhibits macrophage cholesterol uptake. The link between VD and CVD in diabetics is largely unknown. Therefore, understanding the mechanism by which atherosclerosis

induced by VDD may help treating CVD in diabetics (Oh *et al.,* 2009). Another study reported an increase in calcified plaque formation of the arteries in rodents that were fed high cholesterol diet along with high VD (300,000 IU/kg bodyweight/day) for 4 weeks. This might potentially be through suppressing the vascular smooth muscle cell proliferation. Endothelial dysfunction is the most common cause for structural and vascular alterations. Therefore, the suppression of smooth muscle proliferation might protect against developing MetSyn (El Akoum *et al.,* 2012). Therefore, we could hypothesize that decreased serum VD is a potential risk factor for developing cardiovascular and metabolic diseases. The current research is restricted to identify the causation.

1.3.4 Neurogenesis

Neurogenesis is an orchestrated mechanism of the production of new neurons by either neuronal stem cells (NSCs) or neural progenitor cells (NPCs) and their integration into the brain circuitry. It starts through embryonic stage and continues through perinatal stages and throughout life. During embryogenic development, pluripotent stem cells are only present briefly, then differentiate into number of somatic cells, including neurons and glia, astrocytes, and oligodendrocytes, to be integrated into the nervous system. NSCs make up the neural tube that forms early in the brain development and become radial glial cells (RGCs) during the embryonic neurogenesis. The multipotent NSCs differentiate to become fully matured neurons during embryonic neurogenesis while some remain quiescent. In the adult brain, most of the NPCs are in a quiescent state. Active NPCs are highly proliferative and once they differentiate into neuroblasts, they either become fully mature neuronal cells or glial cells (Kriegstein and Alvarez-Buylla, 2009).

There are neurogenic areas of the brain remain active during adulthood: SVZ andSub granular zone (SGZ) of dentate gyrus of the hippocampus and hypothalamus,where adult neurogenesis occurs (Eriksson *et al.,* 1998; Cheng, 2013). The adult neurogenesis process is different to embryonic neurogenesis (Urban and Guillemot, 2014). The NPCs of the adult hypothalamus are ependymal-glial cells, called tanycytes. They share the characteristics of the SGZ and SVZ neurogenic cells such as expression of the astroglial and stem cell markers and they are capable of neurosphere formation. Hypothalamic progenitors begin to express proteins characteristic of migrating immature neurons including Doublecortin (DCX) (Batailer *et al.,* 2014). They migrate to ARC and differentiate into anorexigenic and orexigenic neurons, therefore, have important role in energy balance and instinctive behaviours.

1.3.4.1 Adult Neurogenesis

In the adult brain, the RGCs proliferate to either directly differentiate into neurons or generate intermediate neural progenitor cells (IPCs) to divide one or more times to then differentiate into neurons. In mice, this process occurs in a shorter period compared to humans (Yao B et al., 2016). Proliferation in SVZ occurs in the lateralventricles (LV) in which stem cells divide into transit amplifying cells that in turn differentiate into neuroblasts that migrate along the rostral migratory stream to olfactory bulb. The fate determination of a new-born cell and the proliferation rate is regulated by several intrinsic and extrinsic mechanisms. The migration process along the rostral migratory system (RMS) is also controlled by extrinsic factors that are produced by surrounding tissues to act on progenitor cells neurogenic processes, that involves neurotransmitters, hormones, and growth factors.

The olfactory bulb (OB) undergoes some changes during adulthood and in pregnancy. In pregnancy, proliferation is enhanced thus, the production of new olfactory interneurons contributes to the formation of the new olfactory memories.This process is highly important for maternal behaviour such as offspring recognition and rearing and even for adaptive behaviours in mating and pregnancy. Rochefort and colleagues observed cell proliferation and survival rate in the olfactory bulb by exposing the mice to an odour-enriched environment. Theyfound a region-specific increase in cell survival coupled with improved olfactory memory (Rochefort *et al.,* 2002). In the adult brain, neuroblasts travel through the RMS to the OB continuously following structural and molecular cues. They continue to migrate to mature into interneurons. In the DG, neuroblast also migrate from SGZ to granule cell layer to become mature neurons. In this time, the NPCs become dentate granule cells (DGCs) and go through morphological changes to differentiate and migrate into granule cell later. All these processes signify certain functional changes. SVZ provides GABAergic granule interneurons to the OB. New-born dentate granule neurons in the CNS, however, are glutamatergic neurons which can also express GABA. In this, they differ from the mature DG neurons. They form both afferent and efferent synapses before they fully mature and integrate into the neural circuitry (Toni and Schinder, 2016). DGCs are excitatory but they can also act as inhibitory cells. Due to this feature, these cells have been shown to be involved in hippocampus-dependent functions involving learning and memory (Shors *et al.,* 2001). Beyond this, Gould and colleagues demonstrated that learning enhances the adult hippocampal neurogenesis, aiding the survival of the adult-born neurons (Gould *et al.,* 1999).

1.3.4.2 Markers of Neurogenesis

In the adult brain, neurogenesis occurs in a sequential manner. Quiescent stem cells can be marked by a glial fibrillary acidic protein, (GFAP), an astrocyte marker.These cells are multipotent, they can differentiate into neurons and glia. Amplifying NSCs are unipotent and their fate is pre-determined. They are committed to neuronal differentiation and proliferation. Neuroblasts develop qualities of a neuronand mature into a differentiated neuron (see Figure 1.3), a process that takes 28-days (Dayer *et al.,* 2003).

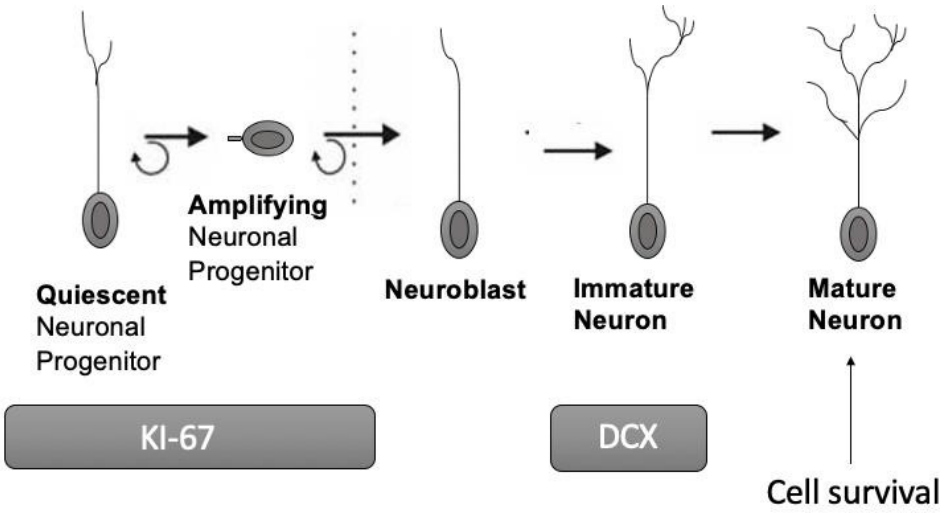


Figure 1.3 The stages of differentiation, from quiescent stem cell to mature neurone, and the various markers for its phases. Image modified from Von Bohlen Und Halbach (2007).

As neurons continue to differentiate, they express different proteins at each stage of differentiation. Different stages of development can be identified by the markers expressed. Ki-67 is a nuclear-based protein that is expressed by the amplifying progenitors and neuroblasts in all active cell cycle phases (G1, S, G2, M) except for the resting phase. Antibodies for Ki-67 is therefore a reliable endogenous marker for proliferating cells. Doublecortin is an endogenous microtubule- associated protein that is expressed by the migrating and differentiating neuroblasts. Hence, DCX expression is specific to newly generated neurons hence it is a good marker for immature cells (Couillard-Despres *et al.,* 2005). The dividing progenitor cells can be labelled with mitotic markers (Lois and Alvarez- Buylla,1993). For example, Bromodeoxyuridine (BRDU) is a thymidine analogue that gets incorporated into newly synthesized DNA during mitosis. It is not naturally found in the body so using antibodies, newly created cells can be identified.

1.3.5 Effects of VD on adult neurogenesis

Evidence from *in vitro* studies show that VD has a role in cell proliferation and differentiation (Banerjee and Chatterjee, 2003). The following *in vitro* studies reported proapoptotic effects of VD: In human breast cancer cells, VD treatment increased apoptosis significantly compared to non-treated cells through reciprocal modulation of pro-apoptotic Bax/Bak, and anti-apoptotic Bcl-2 (Blutt *et al.,* 2000). *In vivo,* 1 α hydroxylase knock-out mice that are unable to form the active metabolite of VD, $1,25OH_2D_3$, have shown an increase in cell-proliferation in the hippocampal DG, reduction in the survival and increased apoptosis of the new- born hippocampal

neurons in the adult mice (Zhu *et al.,* 2012).VD alters the expression of neurotrophic factors such as *BDNF*, nerve growth factor *(NGF)* and glial cell line derived neurotrophic factor (*GDNF*), which are key regulators of cell proliferation, differentiation, and survival. The expression of *NGF,GDNF, BDNF* were upregulated, and the expression of ciliary neurotrophic factor (*CNTF)* was induced following the *in vitro* administration of $1.25OH_2D_3$ to NSCs. The same study also showed that $1.25OH_2D_3$ enhanced NSCs proliferation and differentiation into oligodendrocytes (Shirazi *et al.,* 2015). Additionally, VD regulates adult neurogenesis via interaction with neurotransmitters and neuromodulators such as dopamine, serotonin which participate in a wide range of neurodevelopmental processes, neuronal migration, circuit formation and dendritic growth (Sabir *et al.,* 2018; Eyles *et al.,* 2005). Dopamine and serotonin receptor in the midbrain and their target neurons downstream were found to express VDR. This was complemented by behavioural studies; VDD animals had altered sensitivity to anti-dopaminergic reagents (O'Loan *et al.,* 2007) and decreased concentrations of brain serotonin levels in autism (Patrick and Ames, 2014). VD and VDR interact with the activity of several neurotransmitters and neuromodulators that act on neurogenesis and cell survival including neuronal growth factor (NGF) and brain-derived neurotrophic factor (BDNF) (Kim, 2015; Hong *et al.,* 2017).

1.4 Epigenetics

In 1942, Conrad Waddington introduced the term 'epigenetics' to describe the interaction between external and internal environment and the genes that leads to a certain phenotype (Waddington,1942). In time, the definition became more advanced after the identification of DNA as a genetic material (Avery *et al.,* 1944).'Epigenetics' is described as the heritable changes in gene expression without anychanges to the underlying DNA sequence. Environmental factors, behaviours and pathological conditions can all affect epigenetic processes, thereby can influence gene expression and phenotype without changing the genotype. Foetal programming is an example to the description of epigenetics, where the foetal development *in utero* reacts to the information/ signals it receives (Tronick and Hunter, 2016).

The main molecular epigenetic mechanisms are DNA methylation, histone modifications and miRNAs.

1.4.1 DNA methylation

DNA methylation is the most studied epigenetic modification mechanism which has important roles for mammals such as genomic stability during mitosis and parent-of-origin imprinting (Xue *et al.,* 2016; Suderman *et al.,* 2016). It is dynamic. DNA methylation is the process where a methyl group (CH_3) is added to a cytosine,without changing the DNA sequence, which in turn modifies the gene expression.This can happen in two ways. Firstly, by recruiting proteins responsible for gene. Secondly, by inhibiting the binding of the transcription factors to the DNA. The most common DNA methylation mechanism is the addition of a methyl group onto the 5^{th} carbon of

the cytosine ring to form 5-methylcytosine (5mC). A family of DNA methyltransferases (Dnmts) catalyse this process; they catalyse, recognise, and bind to CH_3 and modify/erase the CH_3. Maintaining proper control of gene expression is of importance for processes such as differentiation of the neurons to astrocytes and oligodendrocytes and migration to cortical layers during initial stages of nervous system development. The transcription factors transduce the signals of various chemicals into the nucleus and activate their specific gene promoter. This is regulated by epigenetic modification of the cell-type specific genes. CpG methylation: methylation of the genomic DNA at CpG dinucleotides is a major epigenetic modification which has been shown to be regulate cell-type specific gene expression, also in genomic imprinting (Bird and Wolffe, 1999).

High levels of 5mC are unique to the brain and it suggests that DNA methylation has a major role in development (Feng *et al.,* 2005). Dnmts are required for neuronal differentiation. Double knock-out of Dnmt1 and Dnmt3 resulted in reduced DNA methylation hence impaired synaptic plasticity, learning and memory in the adult brain (Feng *et al.,* 2010). Transgenic mice lacking Dnmt expression had decreased learning and memory in the adult brain (Feng et al., 2010). In addition, Martinowich and colleagues demonstrated that increase in membrane depolarization is correlated with a decrease in CpG methylation within the regulatory region of the BDNF gene, thus, increase in BDNF expression (Martinowich *et al.,* 2003). BDNF is a methyl CpG-binding protein target (MeCP2) and encodes a protein that promotes the growth and differentiation of new-born neurons. MeCP2 is enriched in neurons, and it regulates the expression of the genes that are essential for normal cognitive function. MeCP2 mutation leads to neurodegenerative diseases such as Rett syndrome (Amir RE et

al., 1999). Studies have shown that diminished DNA methylation in general is associated withneurodegenerative diseases (Turner *et al.,* 1996; Hwang *et al.,* 2017).

1.4.2 Histone modifications

1.4.2.1 Histone acetylation

The acetylation of the lysine residues is catalysed by either histone acetylases (HATs) or histone deacetylases (HDACs). This process is reversible. In the non- acetylated state, the positive charge of the residues attracts the DNA, the chromatin structure is compact, so this makes it more difficult for the gene expression to occur. When acetylated, the negative charge of the acetyl group neutralises which decreases the DNA attraction, the chromatin structure gets loseand this allows gene expression to occur. Histone acetylation is involved in number of processes including DNA repair and replication, cell cycle and transcription (Tamburini and Tyler, 2005).

Conversely, HDACs remove the acetyl group, and this leads to gene repression (Seto and Yoshida., 2014).

1.4.2.2 Histone phosphorylation

Histone phosphorylation occurs by adding a phosphate group to a serine, tyrosine, or a threonine residue on the histone tails. It is catalysed by several protein kinase enzymes. Dephosphorylation occurs by removal of the phosphate groups from the histone tails by the phosphatase enzymes. Histone phosphorylation is involved in several nuclear processes such as DNA damage repair, transcription and chromatin compaction during cell division and apoptosis (Rossetto *et al.,* 2012).

The most extensively studied role of histone phosphorylation is DNA repair. In mammals, the protein kinases ATM (ataxia-telangiectasia mutated) and ATR(ATM-

Rad3-related) aid the phosphorylation of serine 139 histone 2 (S139H2) which is involved in many DNA damage response pathways (van Attikum and Gasser, 2005). Phosphorylation of the serine residues 10, 28 of histone 3 are associated with regulating gene transcription (Choi *et al.,* 2005; Lau and Cheung,2011). Serine 28 of H3 works synergistically with acetylation of H3K27 for transcriptional activation. Phosphorylation of the histone 3 threonine 11 and 6 also has been shown to regulate gene transcription. Phosphorylation of these residues prevents the removal of the repressive mark on H3K9 by Jumonji C domain- containing protein (JMJD2C/KDM4C), which is a histone demethylase, promoting transcriptional regulation (Metzger *et al.,* 2008).

Phosphorylation of H3 is also associated with chromatin compaction and segregation during meiosis and mitosis (Wei *et al.,* 1999).

1.4.2.3 Histone methylation

Histone methylation is a covalent modification that occurs on all basic residues including arginine, lysine, and histidine by removing 1 to 3 methyl groups. Lysine residues such as lysine 4, 9, 27 and 36 of H3 and lysine 20 of H4 are common sites of histone methylation. Histone methylation is associated either with transcriptional gene activity or gene silencing. Three of the main and best characterised histone methylation sites up to date areH3K4me (methylation of lysine 4 on histone 3) which is instructive for transcriptional activation, H3K9me (methylation of lysine 9 on histone 3) and H3K27me (methylation of lysine 27 on histone 3) which mostly prevents transcriptional activity.H3K4 methylation is known to have a role related to embryonic development and cognition (Gupta *et al.,*2010). For example, in an *in vivo* study, 1 hour

of associative learning leads to elevated levels of H3K4 trimethylation at the promoter of BDNF in hippocampus (Collins *et al*., 2019).

Histone methylation process is reversible: Histone methyltransferases (H/KMTs) and histone demethylases (H/KDMs) catalyses the addition and the removal of themethyl group to a target lysine residue on histones respectively. There are two known groups of histone demethylases: Lysine specific demethylase 1 (LSD1), also known as KDM1 (Shi *et al.*, 2004) and Jumonji domain containing histone demethylases (Tsukada *et al.,* 2006). LSD1 was the first known histone demethylase. KDM6B and UTX both belong to the same subfamily (KDM6) of histone demethylases, and they specifically target histone 3 lysine 27 me3 (H3K27me3), associated with the transcriptional repression. Normally, H3K27me3 keeps the promoters in a repressive chromatin state, removal of the methyl group promotes gene activation. On the other hand, KDM5A belongs to the KDM5 subfamily of histone demethylases which targets H3K4me3, which is associated with transcriptional activation. Binding of KDM5A to H3K4me3 mark enables the recruitment of the demethylases to its substrate (Longbotham *et al.,* 2019). This regulatory mechanism mediated by KDM5 subfamily members allow the removal of H3K4 trimethylation in the early embryonic development activity (Klein BJ et al., 2014). These mechanisms are important in mediating developmental gene regulation and differentiation (Meier *et al.,* 2013). In other words, the balance between these two epigenetic marks is crucial to keep the developmental genes in a poised state to be activated upon differentiation (Covic *et al.,* 2010). The enhancer of zeste protein 2 (Ezh2) is the only known histone methyltransferase to methylate H3K27 (Müller *et al.,* 2002). H3K27 trimethylation is associated with transcriptional repression. Ezh2 is a part of Polycomb repressive

complex 2 (PRC2), which has a role in cellular differentiation and proliferation (Kuzmichev A et al., 2004). Ezh2 represses genes associated with cell differentiation (Oliviero *et al.,* 2016). Ezh2 also regulates H3K27 by increasing its methylation therefore increasing transcriptional repression (Donaldson-Collier *et al.,* 2019). Zhang and colleagues showed that Ezh2 is expressed actively in dividing NPCs and mature neurons and the deletion resulted in reduction in proliferation in adult mice (Zhang *et al.,* 2014). The dual methylation of H3K27 and H3K4 in the poised state of NPCs and occurs typically in proliferation - differentiation stage of neurogenesis.

1.4.3 Molecular regulation of adult neurogenesis

Adult neurogenesis is widely believed to be regulated via two pathways: Wnt/β-catenin signalling pathway, also known as the canonical Wnt pathway (Varela- Nallar and Inestrosa, 2013) and the Notch signalling pathway (Artavanis-Tsakonas *et al.,*1999). The function of the *Wnt* signalling pathway is rather complex. Studieshave shown the Wnt pathway directly regulates the promoter of Neurogenin1, a pro-neural, basic helix-loop-helix gene that it's involved in maturation of radial glial cells into basal progenitors thus is involved in neuronal differentiation (Viti *et al.,*2003). Ngn1 has proliferative activity mediated by Emx2 (Muzio *et al.,* 2005). There are other studies which suggest that it also promotes neuronal differentiationin the developing neocortex (Hirabayashi *et al.,* 2009; Muroyama *et al.,* 2004). The signalling pathway activity needs to be controlled in a strict timely manner: the availability of its effectors during the stage of the development, the complex interactions with other signalling pathways and transcriptional factors. The cell fate transition at the beginning of neurogenesis

was found to be regulatedby Notch signalling (Artavanis-Tsakonas *et al.,*1999). The balance between maintenance of the NPC pool and neuronal differentiation is highly crucial. (Imayoshi *et al.,* 2008). Evidence suggests that Notch signalling pathway contributes to the maintenance of this balance in the adult brain (Imayoshi and Kageyama, 2011).

1.4.3.1 Extrinsic and intrinsic factors

In addition to the canonical intracellular signalling pathways, a variety of other intrinsic factors and extracellular factors affect adult neurogenesis (Figure 1.4). The extracellular niche regulates the fate determination of adult NPCs (Ma, 2005). The way intrinsic mechanisms regulate the functions of adult NPCs is not fully understood (Cheng *et al.,* 2005). The understanding of the critical role of epigenetic mechanisms regulating and linking extrinsic and intrinsic environment is relatively recent.

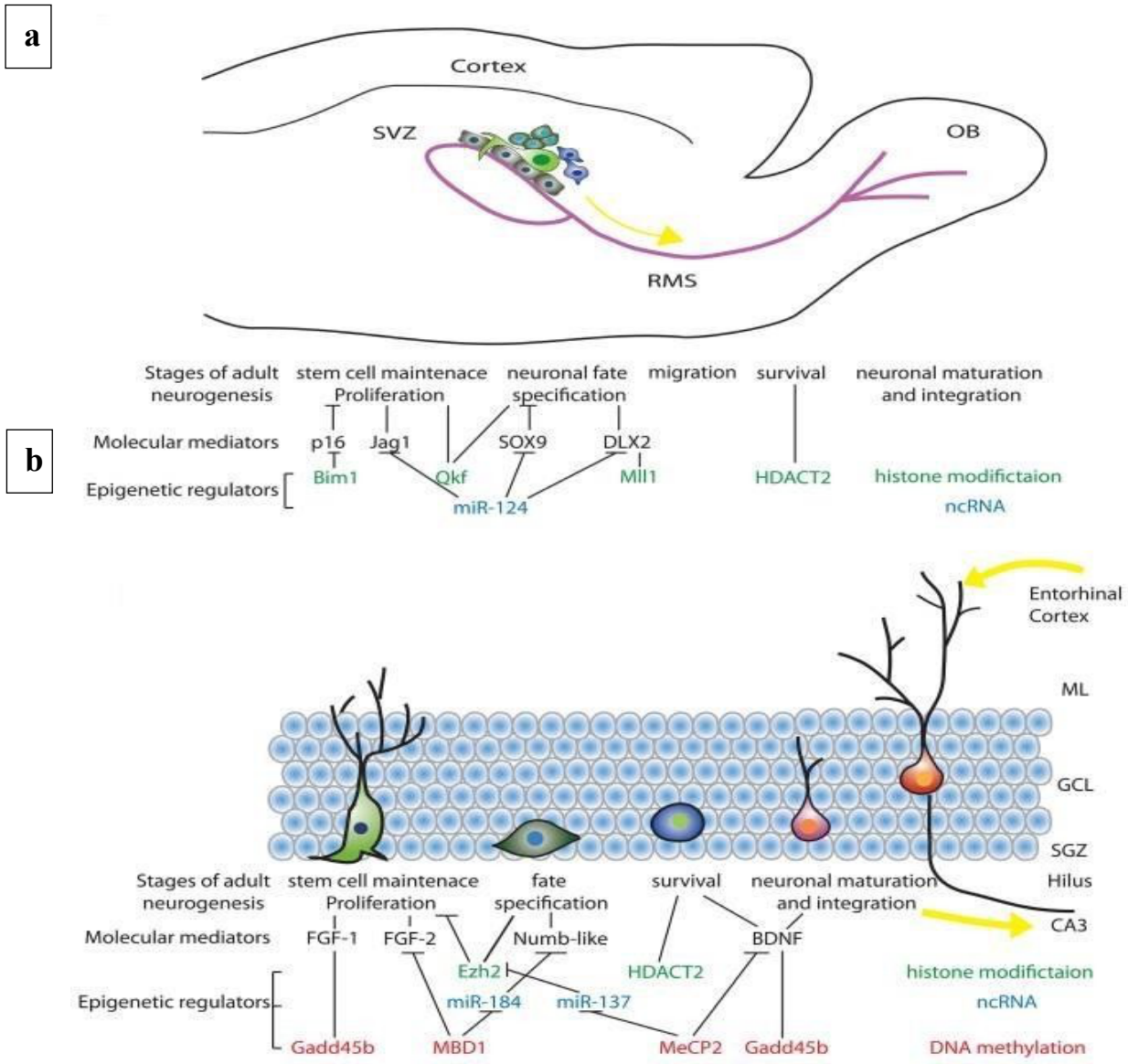


Figure 1.4 Figure shows schematic description of the different stages of adult neurogenesis and series of molecular and epigenetic mediators regulating gene expression and neurogenesis process. a. in the SVZ/OB system and b. in the SGZ/DG system. SVZ: sub ventricular zone; OB: olfactory bulb; RMS: rostral migratory stream; RGL: radial glia-like cell; TA cell: transient amplifying cell; ncRNA: non-coding RNA; ML: molecular layer; GCL: granule cell layer; SGZ: sub-granular zone. *Adapted from Sun et al., 2011*

Intrinsic factors: Epigenetic mechanisms were reported to control the expression of key regulators which in turn influence adult neurogenesis. These factors include Methyl CpG-binding domain protein 1 (MBD1), MYST family histone acetyltransferase Querkopf (Qkf), mixed-lineage leukaemia 1 (Mll1), polycomb complex protein (Bmi-1), histone deacetylase 2 (HDACT2), and microRNAs (miR124, 137, 184, 185, and 491-3p) (Faigle and Sons, 2013; Fitzsimons *et al.,* 2014: Jaenisch and Bird, 2003). Each of these, in their own ways, are critical for adult hippocampal neurogenesis. MBD1 regulates neurogenesis by inhibiting proliferation and promoting differentiation of neural progenitor stem cells by binding to methylated DNA. Qkf, a histone acetyltransferase, has a role in maintaining the population of olfactory bulb interneurons in adult neurogenesis (Merson *et al.,* 2006). During neurogenic development, Qkf is expressed both in the ventricular zone and in the cortical plate, however, it is expressed mainly in SVZ in the adult brain. During prenatal development, Qkf-mutant mice had reduced levels of proliferation in SVZ and fewer neurons in the cortical plate (Thomas *et al.,* 2000).Bmi1 regulate self-renewal and maintain other developmental processes. It is a part of the silencing PcG complex of proteins. Bmi1 is a part of a PRC Bmi1- knockout depletes the number of SVZ-derived adult NSCs. Although underlying mechanisms are unclear, its' overexpression increases proliferation and maintains self-renewal of adult NSCs (Fasano *et al.,* 2009; He *et al.,* 2009). Mll1 is a part of the chromatin activating TrxG proteins that encodes a histone tail H3K4 methyltransferase. It is believed that MII1 controls neuronal differentiation acting synergistically with a lysine-specific histone demethylase, KDM6B. KDM6B is known to be regulated by VD (Pereira *et al.,* 2011). Through

methylation of H3K4 by MII1 and the demethylation of H3K27 by KDM6B, the neurogenic genes start to be expressed therefore neuronal differentiation starts.

Extrinsic factors:

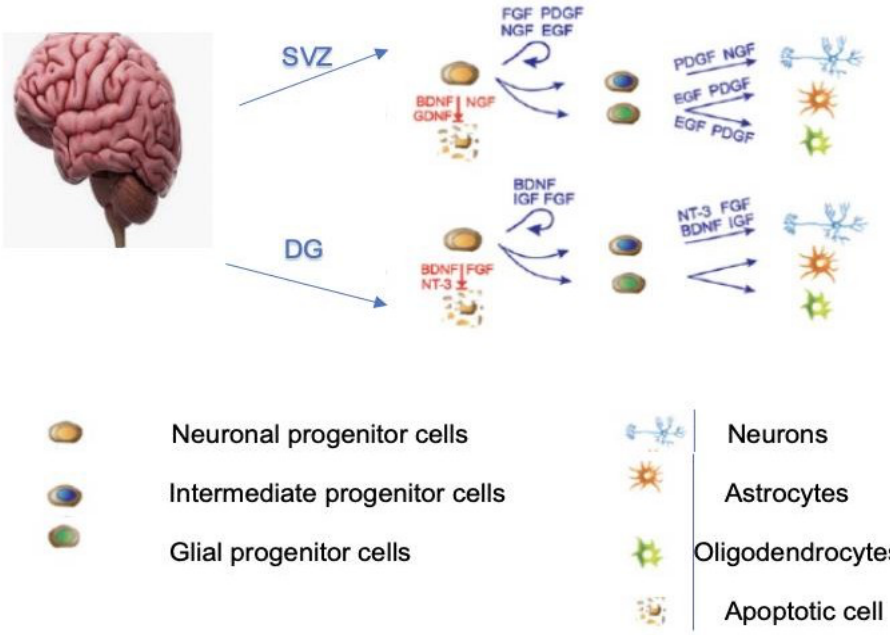


Figure 1.5 Effects of growth factors and neurotrophic factors on progenitor cells in the adult brain. Adapted from Oliveira *et al.,* 2013

Neurogenesis is regulated by growth factors and neurotransmitters. Growth factors such as *BDNF, GDNF*, and *FGF* act to increase the proliferation, whereas neurotransmitters glutamate, GABA and opioid peptides act to down-regulate proliferation of the NSCs (Berg, 2013). One of the earliest *in vitro* studies regarding BDNF function in the adult brain found that it promoted the long- term survival of the SVZ-derived neuroblasts (Kirschenbaum and Goldman, 1995). Another study suggested that it also enhanced neuronal differentiation in the postnatal hippocampus

(Shetty *et al.,* 1998). BDNF exerts its effects via an activation of two receptors, TrkB and p75NTR. BDNF-induced neurogenesis can occur independent of TrkB, via p75NTR alone. An *in vivo* study showed that lack of the p75 gene in mice leads to a decrease in OB volume as well as a decrease of the bio-markers indicative of proliferation and migration of adult NPCs (Young *et al.,* 2007). Several other *in vivo* studies have shown a decrease in postnatal neurogenesis with either trkB or p75NTR deletion in hippocampal DG (von Bohlenund Halbach *et al.,* 2003; Bergami *et al.,* 2009). Collectively, these studies confirm that BDNF is a key regulator of adult neurogenesis.

1.4.4 VD and the epigenome

VD interacts with the epigenome in several ways. Firstly, by methylation of DNA, secondly by interacting with coactivators, corepressors, and chromatin modifiers such as histone acetylates, histone demethylases (Fetahu *et al.,* 2014).

VDR and the CYP enzymes can be silenced by DNA methylation. The expression of the *CYP24A1* gene has been shown to be decreased by DNA methylation in endothelial cells of mice *a*nd rat osteoblastic ROS17/2.8 cells (Novakovic *etal.,* 2012). Furthermore, Zhou and colleagues showed significant changes in the methylation levels in the promoter region of the CYP2R1 and CYP24A1 gene extracted from circulating serum genomic DNA of VDD adolescents compared to controls (Zhou *et al.,* 2014), reinforcing VD's effects on the DNA methylation of important genes. VDR physically forms complexes with chromatin modifiers and chromatin re- modelers. VDR/RXR complex interacts with these to induce transcriptional activation or repression (Karlic and Varga, 2011). VD has also been

shown to affect the expression of certain subgroups of histone demethylases (Pereira *et al.,*2012).

1.5 Pregnancy, foetal programming, and influence of maternal VDD

1.5.1 Long-term impact of early life conditions on health via foetal programming

Foetal periods are critical for the development where interactions between fetoplacental circulation and external environment are crucial. The foetus can sense, receive, and respond to the environmental cues that occur *in utero* during pregnancy. Early life stressors such as abuse, and maternal depression also have a major rolein healthy development of the offspring and can alter long-term physiology and psychology, behaviour, and susceptibility to disease. It can cause premature birthdue to intra-uterine growth restriction, lower birth weight for gestational age. Thereis a correlation between maternal psychological stress and malnourished offspring. Neuroplasticity requires proper modulation of gene expression, which is regulated interactively by epigenomic mechanisms (Hanson and Gluckman, 2008). The rat offspring which were exposed to reduced licking and grooming (LG) and arched-back nursing (ARN) compared to high licking/grooming arched-back nursing (LG-ARN) postnatally showed excessive behavioural and stress responses later in life. (Caldji *et al.,* 1998). These cross-fostering studies showed that when offspring from the low LG-ARN mothers fostered by high LG-ARN mothers, they behaved like normal offspring

of high LG-ARN mothers (Francis *et al.,* 1999). To answer the question of how these altered responses persists into adulthood, the researchers investigated the DNA methylation of the glucocorticoid receptor exon 1_7 promoter from the hippocampal tissue. They found significant differences in site-specific DNA methylation of the GR exon 1_7 promoter. The change in methylation resulted in a change in the expression levels of certain genes affected the behaviour. These differences in methylation developed during the first 6 postnatal days, where the maternal care has the most impact (Liu *et al.,*1997). Early-life stress causes a decrease in hippocampal volume, cognitive dysfunctionthat persists to adulthood, has an impact on long-term integrity of the hippocampus. (Youssef *et al.,* 2019). BDNF is a key regulator of neural plasticity in the hippocampus and an important target of glucocorticoids. Early-life stress influences the methylation status of the BDNF exon IV (Chen *et al.,* 2017). Suri and colleagues subjected rat pups to maternal separation and observed effects on hippocampal neurogenesis, epigenetics, and behaviour from postnatal to adolescence. The rats subjected to maternal separation had significantly lower spatial and object memory in adulthood. They also showed significantly enhanced BDNF exon IV mRNA expression and decreased repressive $H3K9me_2$ at the age of P21 and 2 months. Strikingly, when the rats were 15 months old, BDNF exon IV mRNA expression significantly decreased (Suri *et al.,* 2013). Early-life stress may allow adaptive changes to occur to cope with stressful situations better, however, this comes with long-term adverse effects later in life.

1.5.2 Long-term impact of early life nutrition on health via epigeneticmodifications

Maternal malnutrition during pregnancy has serious adverse consequences for the mental and physical health of the offspring that persist in adulthood. Apart from the quantity, dietary quality is crucial for healthy development. An optimal supply of essential macro and micro-nutrients are necessary for the foetal development. Severe malnutrition during pregnancy impacts offspring health in number of ways:reduced cognitive abilities, delay in speech, neural tube defects, mental and neurodevelopmental disorders. Pre-clinical and human studies identified key macro- and micro-nutrients which deficiencies have significant impact on long-term dysfunction during critical periodsof development. Protein, fatty acids, folate, iron, zinc, thyroid hormones, choline, and B-vitamins are extensively studied for maternal impact of early-life nutrition on the offspring health. Maternal supplementation of specific nutrients such as folatehas been found to be associated with reduced risk of psychopathologies inadulthood (Marques *et al.,* 2013). An extensive study that has been done in two Guatemalan villages during the years 1970s and 1980s revealed the importance of nutrition, especially protein (Pollitt *et al.,* 1996) to achieve full development. Despite many variabilities in the field study mentioned above, they found that the 3 years and 4 years old pre-school children scored higher in psychological test scores when they were supplementedprotein (Freeman *et al.,* 1980). Adequate intake of protein is also essential for normal growth. Dietary ratio of micronutrient intake contributes to regulation of epigenetic mechanisms in various ways (Dominguez-Salas *et al.,* 2012). Pre- clinical models demonstrated smaller brains (Katz HB et al,

1982), reduced hippocampal neurogenesis, disruptive brain plasticity and neuronal architecture and altered epigenetic landscape (Winick, 1985; Ke *et al.,* 2006). Long-chain polyunsaturated fatty acids (PUFAs) have a key role in neuronal production, migration, and synaptogenesis (Innis, 2008). Fatty acids are required for the synthesis of membrane phospholipids for hippocampal neurogenesis, thus important for the development of the brain. The effects of PUFA deficiency during pregnancy and lactation could not be reversed with repletion after weaning, they make lasting impact on the brain. Methylation status of BDNF exon IV might have contributed to this effect. The animals exposed to Western diet, low in omega-3 PUFAs showed significantly increased DNA methylation at the BDNF exon IV promoter in adulthood (Fan *et al.,* 2016). As mentioned before, MeCP2 targets BDNF at the promoter VI and recruits transcriptional repressors. In addition, the low-omega 3 fed animals showed anxiety-related behaviour compared to the animals reared with high omega-3 diet which might suggest a correlation between BDNF methylation and anxiety-like behaviour (Tyagi *et al.,* 2015). Although mixed findings are reported. Limitations include differences in socio-economic status affecting depression-anxiety levels and gender differences (Simmer, 2001; Delgado-Noguera *et al.,* 2010). In humans, the positive impact of PUFAs in cognition may not be apparent until 3 to 6 years of age according to a study (Scholtz *et al.,* 2013).

In addition to PUFAs, essential amino acids and micronutrients are also important for brain development. Folate and choline deficiencies are known to be associated with neural tube defects (Molloy *et al.,* 2020). Moreover, Vitamin B6, B12, choline and folate deficiencies during gestation and lactation lead to impaired learning and memory

coupled with increased apoptosis of hippocampal cells (Blaise *et al.,* 2007).Dietary ratio of micronutrient intake contributes to regulation of epigenetic mechanisms in various ways (Dominguez-Salas *et al.,* 2012). Inadequate intake of these nutrients in early-life influence DNA methylation and histone acetylation because the folate mediated one-carbon metabolism is dependent on these dietary donors and co-factors (Gicquel *et al.,* 2008; MacLennan *et al.,* 2004). Folate, for example, was found to promote the S- adenosyl methionine reactions which are necessary to form glutathione, a primary defense molecule to protect the body against oxidative stress. (Abbasi *et al.,* 2018). These nutrients can also contribute to chromatin restructuring (Tiffon, 2018). Another example is a high intake of unsaturated fatty acids that causes a rise in glucocorticoid release whichin turn results in impaired learning and memory (Hennebelle *et al.,* 2014).

Additionally, maternal malnutrition may also result in offspring that has increased susceptibility to develop hypertension and obesity in adulthood (Vickers *et al.,* 2000), altered endothelial function, altered appetite regulation and activity and overall neuroendocrine control (Vickers *et al.,* 2003).

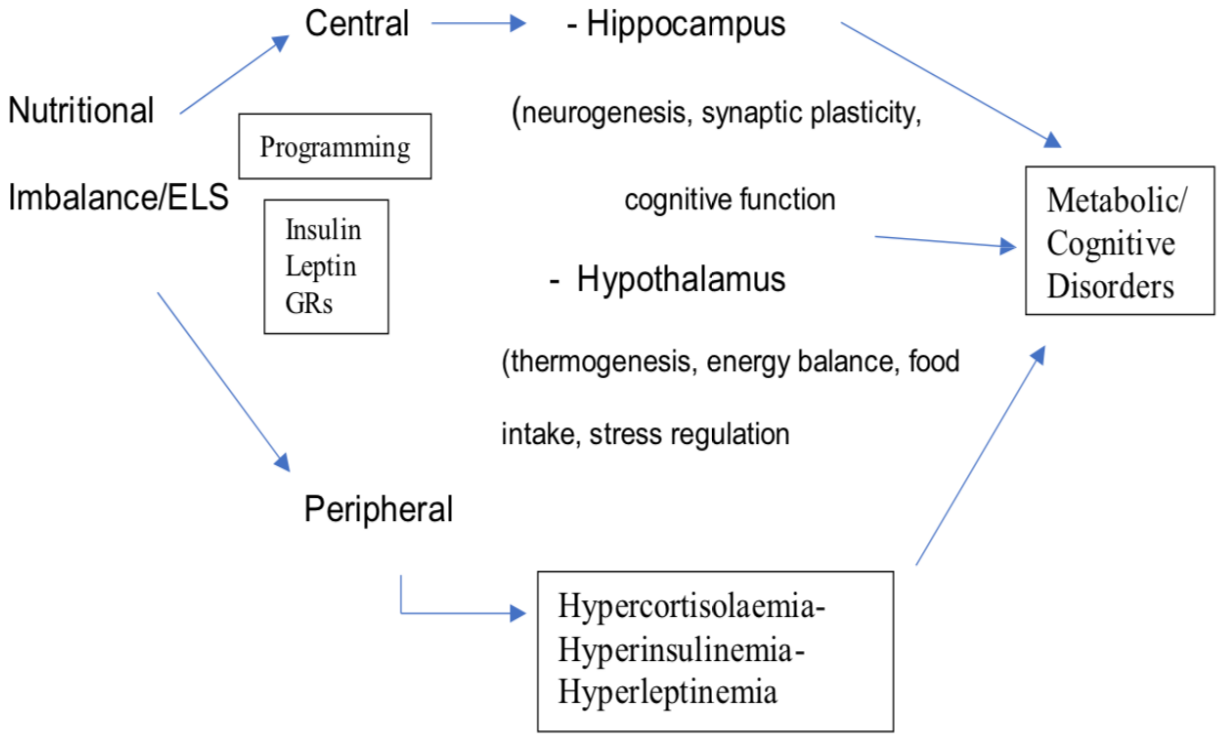


Figure 1.6 Figure summarizing the central and peripheral systems and consequences of early life-stress leading to metabolic and cognitive disorderslater in life.

A study reported that the administration of exogenous leptin to the neonatal offspring of the malnourished dams corrected the changes induced by early-life stress including the associated changes in PPAR-alpha gene methylation (Vickers *et al.,* 2005). Maternal high-fat diet during gestation/lactation increased maternal leptin content therefore, increased the offspring's adipose levels (Walker *et al.,* 2008), coupled with anxiety behaviour in the offspring later in adult age (Sasaki *etal.,* 2014). The adult offspring of the high-fat diet fed dams showed increased food intake, glucocorticoid and leptin production, fat deposition, which are some of the characteristics of obesity and MetSyn. Moreover, the children whose mothers had low levels of Vitamin B12

and high levels of folate in their diets during pregnancy had increased insulin resistance, had a higher body fat percentage and visceral fatat the age of 6, therefore, more prone to develop type 2 diabetes (Roberfroid *et al.,* 2012). Insulin and leptin are important regulators of brain function and metabolism, and their receptors are present in hippocampus as well as hypothalamus (McNay, 2007). Early neonatal leptin treatment increased the expression of the synaptic protein synaptophysin expression in the adult offspring that were fed high-fat milk compared to control-milk, indicating the nutritional imbalance influences the synaptic plasticity, in hippocampus (Walker *et al.,* 2007). Leptin has a 3-kb promoter region on the CpG islands which contains transcriptionfactor binding sites where glucocorticoid receptors can bind too so leptin gene expression can be modulated by DNA methylation. Indeed, after being fed with a high-fat diet, the methylation thus the gene expression of the dopamine and opioid-related genes were decreased suggesting a mechanism for the changed appetite and the food preference of the offspring in postnatal life (Vucetic *et al,* 2010). Furthermore, Heijmans and colleagues showed that the insulin growth factor (IGF-2) gene, which is a maternally imprinted, epigenetically regulated loci important indevelopment, was less methylated in humans conceived during the Dutch hungerwinter compared to their siblings conceived before and after the hunger winter (Heijmans *et al.,* 2008) demonstrating that the epigenetic mechanism are involved and that optimal nutrition and quality/quantity of the early life stressors early in life is crucial for healthy physiological and psychological state for the rest of life.

1.6 Aims and Objectives

The purpose of this study is to gain further understanding of the long-term impact of VDD on offspring metabolic condition, behaviour, and adult neurogenesis and to investigate epigenetics as a potential underlying mechanism based on the previous studies.

The aim of this study was:

1. to investigate relationship between VD and MetSyn using systematic review and meta-analysis

- We hypothesized a negative correlation between the serum levels of VDand the risk of developing MetSyn.

2. to investigate the metabolic effects of VDD during pregnancy *in utero* using metabolic tests and to test the impact of VDD on mouse behaviour by conducting anxiety and memory tests by comparing maternally deficient VDD mice to control male mice at 8 weeks of age.

- We hypothesized that the maternally VDD mice would have alteredmetabolic state, increased levels of anxiety and decreased memory.

3. to investigate the effects of VDD on adult neurogenesis in 3 weeks female and 8 weeks old male mice by

assessing the number of $Ki67^+$ and DCX^+ expression in DG and SVZ.

by measuring the gene expression of two neurotrophic factors, VGF and BDNF in hippocampus and hypothalamus.

- We hypothesized that the maternally VDD mice will have increased count of $Ki67^+$ and decreased count of DCX^+ cells in both DG and SVZ, suggesting decreased neuronal proliferation and differentiation rates.

- We hypothesize that maternally VDD mice will have significantly decreased VGF and BDNF expression

4. To examine the expression of key enzymes associated with neurogenesis and believed to be under the influence of VD regulation, namely *KDM6B, UTX, KDM5A*, and Ezh2, in the hippocampus and hypothalamus of 3-week-old female and 8-week-old male mice

- We hypothesized that there will be a significantly decreased expressionof all the genes in maternally VDD mice in both hippocampus and hypothalamus.

5. To confirm the impact of VDD on neuronal proliferation and differentiation and gene expression by using *in vitro* cell culture in N2a cell line,

a) by conducting an MTT assay as an assessment of cell proliferation

b) by measuring the axon outgrowth as an assessment of cell differentiation.

c) by looking at the gene expression of two transcription factors, Synaptophysinand NeuroD1.

- We hypothesized that the *in vitro* results would confirm the decrease in the rates of cell proliferation and cell differentiation resulting from VDD that we have seen *in vivo*. We propose that we will see decrease in proliferation, increase in axon outgrowth with the treatment of VD and asignificantly decreased Syp and NeuroD1 expression.

Chapter 2. Exploring the relationship between VD and metabolic health: A Systematic Review and Meta-analysis

2.1 Background

VDD has been linked to effects on long term metabolic health of the offspring such as increased risk of obesity and diabetes, causing MetSyn. MetSyn has become a common health challenge worldwide but the prevalence of MetSyn varies. Age, gender, ethnicity, and diagnosis criteria are some of the possible reasons for this variability (Rochlani *et al.,*2017). MetSyn effects about a quarter of the population in Europe, but areas with lower prevalence are moving towards similar rates (Beltrán-Sánchez *et al.,* 2013). MetSyn is not a disease but a cluster of individual risk factors which are used to dentify those with increased risk of developing CVD and DMT2. As described in an earlier chapter(Chapter 1.3.3), the associated risk factors include hypertension, hyperlipidaemia, hyperglycaemia, and insulin resistance. However, the most important risk factors for the development of MetSyn is excess adipose tissue deposition, particularly in the visceral area. Palaniappan and colleagues found that just increasing the waist circumference by 11cm increased the risk of developing MetSyn by 80% (Palaniappan *et al.,* 2004). Mentioned in section 1.3.3, MetSyn is a constellation of physiological, biochemical, and metabolic abnormal factors that are intertwined and in turn increase the risk of developing CVDs and therefore, mortality.

It is diagnosed by anthropometric measurements (birthweight, BMI and laboratory analysis of blood glucose levels, insulin measures, triglycerides, and total cholesterol. Sedentary lifestyle, excessive consumption of caloriedense foods and smoking are contributing to the escalating numbers of MetSyn worldwide. Although this does depend on the MetSyn criteria used, diagnosis of MetSyn may help convince patients about lifestyle modification and the importance of consuming a balance diet. Several dietary factors, particularly micronutrients, are known to reduce the risk of developing MetSyn (Goncalves and Amiot,2017). These micronutrients can affect inflammation and oxidative stress, both of which are related to the development of MetSyn(Damms-Machado A. et al., 2012). The prevalence of micronutrient deficiency has been reported in obese individuals due to inadequate intake and/or alterations in nutrient absorption or metabolism. However, very little is known about the adverse relationships between micronutrient intake and MetSyn. Thus, studying the role of these nutrients mayprovide intervention strategies that could reduce the morbidity of MetSyn and CVD. Recently evidence has suggested that optimal VD levels could be important in reducing the risk of developing MetSyn (Wang *et al.,* 2018).

One billion individuals worldwide are thought to be VDD, suggesting that this may have dire consequences to the health of the individual. Indeed, epidemiological evidence suggests that VDD may be associated with the development of the MetSyn (Wieder- Huszla S et al., 2019). Abnormal energy balance results in hypertrophy and hyperplasia in the adipose tissue. As the adipocytes enlarge with the progressed obesity, the blood supply to the adipocytes reduced through hypoxia, in turn, leads to

macrophage infiltration and overproduction of adipocytes such as TNF-alpha, CRP, IL-6 and FFA (Halberg N et al., 2008). Many studies have shown that in adequate VD can lead to the alterations of differentiation of pre-adipocyte (Kong J et al,2006: Sergeev I., 2009, Ding C. et al., 2012), inhibition of pancreatic β-cells, insulin resistance, and systemic inflammation and elevated blood pressure induced by activating the renin-angiotensin-aldosterone system (Grimnes G et al., 2010), suggesting that VD may play a role in reducing the deposition of adipose tissue. In addition, there is a strong link between season, altitude and latitude and cardiovascular risk. A population-based study in older adults investigating the effects of sun exposure reported that sun exposure alone does not explain the inverse relationship between %body fat and lower serum VD (Harris *et al.,* 2007). Further studiesare needed to clarify the relationship between the environmental factors, VD, and susceptibility to metabolic conditions.

VD can regulate calcium dependent processes via the expression of calbindin, which is a cytosolic calcium binding protein in β-cells. Through mediating intracellular calcium influx for depolarization stimulated insulin release (Johnson *et al.,* 1994). In support, a meta-analysis conducted by Pittas and colleagues concluded that insufficient VD and calcium appear to hinder glycaemic control and a combined daily intake of VD and calcium reduced the risk of DMT2 by 33% (Pittas *et al.,* 2006). Interestingly, many have suggested that low levels of VD could be predictive for the development of DMT2 (Forouhi*et al.,* 2008), as a study conducted by Parker and colleagues revealed that increasing VDserum levels to normal reduced the risk of developing DMT2 by 55% (Parker *et al*., 2010). In addition, MetSyn is also associated

with increase inflammation and oxidative stress as both insulin resistance and obesity cause low grade inflammation and oxidative stress intissues. Studies have shown that adequate dietary VD can ameliorate oxidative stress and inflammation by reducing the levels of pro-inflammatory cytokines (e.g., interleukin 1β, tumour necrosis factor alpha) as well as reducing nitric oxide (Rapa *et al.,* 2019). Thus, further suggesting that inadequate VDD can highly increase the susceptibility for developing MetSyn.

2.2 Aims and Objectives

VDD is increasing in both developing and underdeveloped countries (Khan *et al.,* 2014) and the only diagnostic approach is to prescribe optimal VD supplementation. However,not only are there different cut off points associated with VDD, but there are also differences in suggested dietary guidelines between different authoritative bodies such as Institute of Medicine (Ross *et al.,* 2011) and Endocrine Society Clinical Practice Guidelines (Holick *et al.,* 2011). Many studies reviewed the dose-response relationship of VD and supplementations in different populations. The lack of unambiguous view on the cut-off points of VD status leads to diagnostic difficulties of associated disorders including MetSyn.

The aim of this study is to determine the connection between VDD and MetSyn and its components using a systematic review and meta-analysis.

2.3 Materials and Methods

A systematic review to investigate the relationship between VDD and the development of MetSyn in adults was performed based on the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines which provide a checklist for items which should be reported in a systematic review (Moher *et al.,* 2010). We defined MetSynas elevated cardiovascular risk factors, glucose metabolism and insulin measures.

2.3.1 Search Strategy

A computerized, systematic search of the literature was performed between 31\10\2016 to 16\12\2016 using the databases Pub Med, Web of Knowledge and SCOPUS. A computerized, systematic search of the literature was performed between 31\10\2016 to16\12\2016 using the databases PubMed, Web of Knowledge and SCOPUS. Cross- sectional, case-control and cohort studies measuring vitamin D status and metabolic syndrome were included. Reviews, conference proceedings and editorials and any article not written in English were deleted prior to study selection. The search strategy encompassed three exclusive searchers of comparable terms. Search terms included 'vitamin D OR vitD OR dihydroxyvitaminD*', 'metabolic syndrome*, obesity, diabetes, and cardiovascular*'. Studies were included if all the following criteria were met: (1) VD levels were measured and (2) VD levels was defined as deficient if having less than 25 nmol/L serum levels, (3) provided a definition of the metabolic syndrome, (4) provided anthropometric measurements, such as blood pressure, insulin sensitivity, body

mass index, weight to height ratio etc. and (5) were prospective, cross-sectional, case-control and cohort studies. Studies were excluded if (1) participants were younger than 18 years old, pregnant, or presented diseases not within the definition of metabolic syndrome such as cancer; (2) were published before 2010; or (3) were randomized control trials or conducted in animals or cells. Three independent reviewers (GM, MT, and PJ) assessed the titles, abstracts and full articles based on the above inclusion andexclusion criteria. Any disagreements with the selection of the article were resolved through discussion. Full articles of the selected titles were retrieved, and the reference lists of these articles were searched to identify any additional publications.

2.3.2 Selected Articles Criteria

Inclusion criteria:

- Measurement of VD status (deficiency was defined serum levels as less than 20 nmol/L)
- Definition of metabolic syndrome as elevated cardiovascular risk factors and diabetes, glucose metabolism or insulin measures.
- Provided anthropometric measurements such as glycaemic control, blood pressure, insulin sensitivity, body mass index and weight to height ratio.
- Fully published articles
- Human studies (Prospective, cross-sectional, case-control and cohort studies)

Exclusion criteria:

- Published prior to 2010
- Participants under the age of 18
- Participants who were pregnant or have disease not within the definition of metabolic syndrome such as cancer
- Animal or cell trials
- Randomised control trials (RCTs) due to practice misalignment and/or loss of follow-up data
- Not in English

Outcome measurements

The primary outcome measure in this review was the correlation between low serum VD levels and risk of developing MetSyn. The secondary outcomes were waist circumference, HOMA-IR, cholesterol, fasting glucose levels, blood pressure and triglycerides.

2.3.3 Data extraction

The following variables were extracted from the studies:

(1) Authors and year of publications,

(2) Country of origin,

(3) Detail of population studied including anthropometric data, age, and gender

(4) Study design and methodology, including definitions of VDD and MetSyn

(5) Outcomes for homeostatic model assessment of insulin resistance (HOMA-IR), High density cholesterol (HDL-C), low-density cholesterol (LDL), triglycerides (TG), fasting glucose, systolic and diastolic blood pressures (SBP-DBP). SEM/SDs were extracted from the included articles.

2.3.4 Quality assessment and risk of Bias

Quality assessment was conducted by reviewer GM for the methodological quality of the included studies using the modified Newcastle-Ottawa scale. The Newcastle-Ottawa scale for assessing the quality of studies in meta-analysis and systematic review includes series of questions about studies, selection, comparability, and exposure. Each includedstudy was given a score from 0-8 for each question and the overall score is calculated. The studies were classified as low quality if 0–4 points. The cut off score of 7 was considered a high quality (QA scores included in **Table 2.1**). We conducted sensitivity analyses by excluding trials with high or unknown risk of bias, excluding trials with high risk or unknown risk of bias. We assessed heterogeneity using the I^2 test.

2.4 Results

2.4.1 Study selection

PubMed, Web of Science and SOPUS were used to identify relevant articles and a totalof 8776 studies were identified with 8334 being excluded following the title and

abstract screen, leaving 34 articles to which the inclusion/exclusion criteria were applied. Of these, 24 were identified and accepted for full-text screening. After full text screening and discussion among authors, six case-control studies, five cohort studies, and eight cross- sectional studies, two prospective and two observational and one longitudinal study met eligibility criteria and was included for further analysis. 10 were excluded based on the exclusion criteria of minimum accepted year, conducted animals/cells and unhealthy population and no definite VD cut-off points. leaving 24 articles eligible for inclusion (**Figure2.1**).

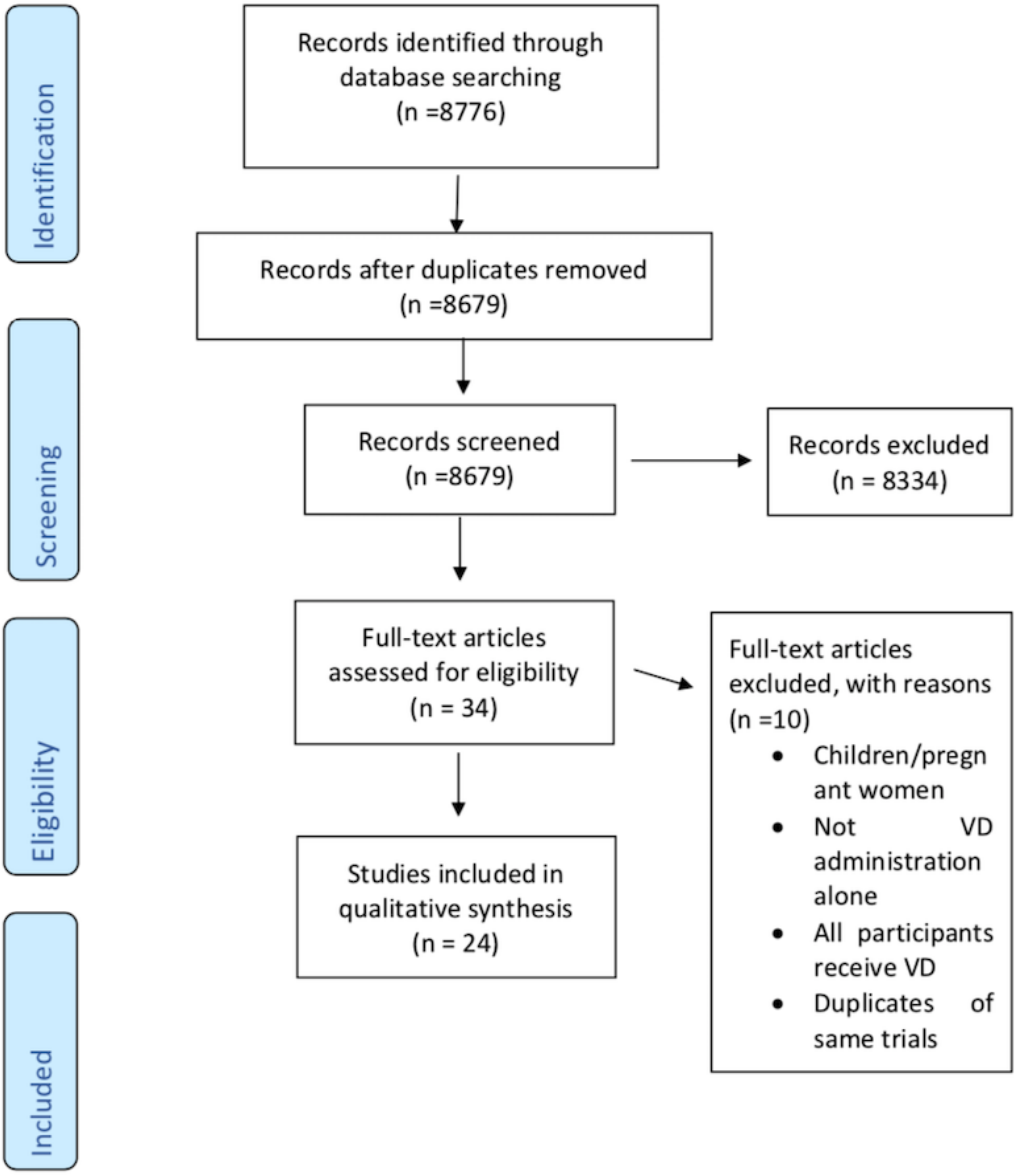


Figure 2.1 PRISMA flow diagram summarizing the extraction process.

Study characteristics

Twenty-four studies were articles met eligibility criteria and was included for further analysis, this includes six case–control studies, five cohort studies, and eight cross-sectional studies, two prospective, two observational and one longitudinal and a total of 27974 patients (**Table 2.1**). Although most of the studies were conducted in different countries (USA, Canada, Asia, and Europe), and the ethnicity of the participants varied indicating heterogeneity among the studies. All studies defined VDD as having less than 20 nmol/L serum $25D_3$, which is based on our inclusion criteria, however the definition of MetSyn was different. All studies except 2 defined MetSyn according to National Cholesterol Education Program Adult treatment panel III. This defined MetSyn as presentation of three or more of the following components: i) waist circumference ≥90 cmfor men or ≥80 cm for women; ii) triglyceride ≥1.7 mmol/L; iii) HDL cholesterol <1.03 mmol/L for men or <1.30 mmol/L for women; iv) blood pressure ≥130/85 mmHg; and v) fasting glucose ≥5.6 mmol/L. The remaining two articles defined MetSyn according to International Diabetes Federation. This defines MetSyn as central obesity (BMI>30 kg/m_2) plus two of the following: a triglyceride level >150 mg/dl, an HDL cholesterol level <40 mg/dl in men or <50 mg/dL in women, systolic blood pressure >130 mmHg and/or diastolicblood pressure >85 mmHg, and fasting glucose >100 mg/dL.

Twenty-two of the studies included MetSyn and\or the analysis of the components of MetSyn. The relationship found between the MetSyn components and the 25OHD3 levelsis the following: increasing $25D_3$ levels were significantly negatively correlated with waist circumference, systolic blood pressure, diastolic blood pressure,

triglycerides, fasting glucose levels and is associated with higher values of HDL-C. In two of the case-control studies conducted on DMT2 patients, systolic blood pressure, waist circumference, insulin, glucose, HOMA-IR, $25D_3$ levels, and HDL-C were significantly increased in Diabetes Mellitus patients compared with controls. There are other studies that were conducted on obese patients which confirmed a similar relationship, a negative correlation, between $25D_3$ levels and obesity parameters. Several studies showed a high prevalence of VDD inextremely obese individuals. In addition, all the cohort studies show a higher risk of MetSyn with increasing quartiles of $25D_3$ levels. After data extraction, meta-analysis was performed. The baseline characteristics of the studies which are included in the meta- analysis are shown in **Table 2**

Paper no	Year of publication	Authors	Country of Origin	No of Participants	Male/Female	Type of study	Measures	QA score
1	2014	Patchaya Boonchaya-anant	America	191 adults (≥18 yo)	51 male/99 female	Prospective	Height, weight-HC,mean SBP-DBP, BMI, FPG, FPI, serum 25D3 levels	7

2	2013	Nasser M. Al-Daghri et al.	Saudi Arabia	266 adults(26-80yrs)	142 male /124 female (26-80 yr)	Case-control	Height, weight-HC,mean SBP-DBP,BMI, FPG, lipid profile, serum25D3 levels, leptin, insulin, TNF-alpha,adiponectin, resistin	8
3	2011	Darren R Brenner et al.	Caucasians, Asians (Koreans, Filipinos, Japanese, Chinese, South Asians, Southeast Asians, Arabs and West Asians) , and others (Blacks , Latin Americans and mixed)	1818(16-79 yrs)	869 male/949 female	Cohort	WC, BMI, HDL-C, FPG, SBP-DBP, TG, serum25D3 levels	6

4	2013	Kai-Hung Cheng et al.	Taiwa nese	694 (44-77 yrs)	all male	Cross-sectional	A complete medical, surgical, and psychosex ual history,weight-height, blood pressure, BMI, FPG, FPI	6
5	2010	S. Devaraj et al.	Northern American	81 adults (21-70 yrs)	17 male/64 female	Cross-sectional	Fasting blood count, plasma lipid and lipoprotein profile, urea nitrogen, creatinine, aspartate aminotransferase, alanine aminotransferase, glucose, calcium, phosphate,and TSH, Insulin, adiponecti n and leptin	9
6	2012	Grace J Fung et al.	African American & white man and women	4727 adults (24.7-25.12 yrs)	2600 male/2127 female	Longitudinal	BMI, WC, SBP, DBP,HDL, TG, FPG, smoking, physical activity alcohol and exposure to sunlight	9

7	2014	Sheena Kayaniyil et al.	Caucasians, hispanic, South Asian, other	489 adults	132 male/357 female	Prospective	BMI, WC, physical activity, smoking,VD supplement use, 25D3, SBP-DBP,TG, HDL- C, FPG-FPI (HOMA-IR)	7
8	2011	Sheena Kayaniyil et al.	London, Toronto, Ontario, Canada, aged 30 yr	654 adults	461 female/193 male	Cohort	BMI, WC, 25D3, PTH, SBP-DBP, TGs,HDL-C, ALT, Albumin to creatinine ratio, eGFR, WBC, CRP, FPG, 2-h glucose, FPI	9
9	2015	M. Kramkowska et al.	Caucasian	70 adults	34 female/36 male	Cohort	BMI, height, weight,WC, WtHR, Concentrations of 25D3 (25-hydroxyergocalciferol and VD	7

10	2015	Yanhui Lu et al.	China	3275 adults	2801 male/474 female	Cross-sectional	BMI, WC, SBP, DBP, TG, Total Cholesterol, HDL- LDL, FPG, serum25D3 levels(ng/ml)	6
11	2014	Amal M.H. Mackawy et al.	Egypt	190 adults	85 female/105 male	Case-control	BMI, FPG, HbA1c, Lipid profile (TG,HDL-LDL),FPI, HOMA-IR, plas ma IL-6 levels, serum 25D3 levels	8
12	2014	Maija E. Miettinen et al.	Finland	2822 adults	1348 male/1479 female	Cohort	WC, TG, HDL-C, SBP-DBP, FPG, 25D3 levels	9
13	2011	Foong-Ming Moy et al.	Malaysia	380 adults	220 female/160 male	Cross-sectional	FPG, SBP-DBP, weight, height andWC-HC, LDL-HDL-C, TG, VD levels	6

14	2015	Poonam K Pannu et al.	Australi a & overseas	3404 (18-75 yrs)	1237 male/ 1565 female	Case-control	Ca intake, total energy intake, alcohol, dietary fibre, Mg and retinol,WC, FPG, HDL, TG, SBP-DBP, 25D3 levels	7
15	2011	Karolina Rogal et al.	Poland	52 women(20-24yrs)	52 females	Case-control	WC, fasting TG,HDL, high SBP-DBP, elevated FPG, 25D3 levels	9
16	2011	Shabnam Salekzamani et al.	Iran	100 adults	100 females	Case-control	BMI, BSA, ; DBP, FM, fat mass; HDL-C,HOMA-IR, hs-CRP, intact PTH,LDL-C, malondialdehyde; SBP, total antioxidant capacity, TG, WC ; WtHR, 25D3 levels.	6
17	2013	Natielen Jacques Schuch et al.	Sao Paulo , Brazil	243 adults	95 male/ 148 female	Cross-sectional	Height, WC, BMI, SBP-DBP, LDL-HDL- C, TG, FPG, HOMA IR / HOMA β, intact PTH and 25D3 levels.	9

							Genotypin g of BsmI (VDR 1544410 A > G) and FokI (VDR2228570 C > T) VDR gene polymorphisms	
18	2012	Manuj Sharma, V.K. Sharmaet al.	India	50 partici pants (18-65 yrs)	_	Cro ss-secti onal	WC, weight, height, SBP-DBP,TG, HDL- C, FPG, 25D3 levels	5
19	2015	Guler Tosunbay raktar et al.	Turkey	90 partici pants (18-63 yrs)	_	C as e-co nt rol	Height, weight, WC-HC, BMI, SBP- DBP, FPG,FPI, HDL-LDL, TG, calcium, phosphor, HbA1C, PTH, and25D3 levels	6
20	2016	Azam Ahmadi Vasmehja n et al.	Iran	156 adults (28-76 yrs)	156 females	Cro ss-secti onal	height, weight, WC, BMI,FPG, TG,(HDL-C), 25D3 levels IGF-1	8
21	2012	Xiao Yin, Qiang Sun et al.	China	601 adults (35-60 yrs)	152 female/ 476 male	Cro ss-secti onal	BMI, WC, FPG, FPI (HOMA-IR), TG, HDL-C-LDL SBP- DBP, 25D3 levels	8

22	2015	Hyun Yoon et al.	Korea	5,483 adults (≥20 yrs)	2330 male / 3,153 female	Cohort	25D3 levels, height, weight, BMI, WC,TG, HDL-C, FPG, SBP-DBP	9
23	20jm k	Guo-Tao Pan et al.	China	270 elderly	78 male/ 192 female	Observational	Weight, Height, WCC- SBP-DBP,TG, HDL-C-LDL, FPG, (HOMA-IR), glycosylated haemoglobin and CRP	9
24	2014	Joanna Mitri et al.	U.S (White, African American, Other (Hispan ic, Asian)	2000 adults	1337 female/663 male	Observational	Calcium intake, SBP-DBP,FPG, FPI, insulin sensitivity-HDL-C, TG, CRP, 25D3 levels	11
Abbreviations: BMI: Body mass index; WC-HC: waist to hip circumference: SBP-DBP: systolic-diastolic blood pressure; TG: triglycerides; FPG: fasting plasma glucose; FPI:								

Table 2.1 Baseline information on the articles eligible for inclusion after the initial full screening.

2.4.3. Data Extraction for Meta-analysis

As mentioned in the study characteristics (see 2.4.2), 24 papers in total were included in this study after the initial full screening, according to our inclusion/exclusion criteria.

The selected 11 were included for the meta-analysis due to having the same parameters for the analysis. 13 articles were excluded because they did not include the subject data for MetSyn + AND MetSyn – onset. 6 of these does not provide SD/SEM values for all the individual MetSyn parameters (Table 2.2): 2 of these are investigating VD gene polymorphisms, 3 of these are investigating the effects of dietary VD supplementation and lifestyle factors, 1 of these is using participants for dry eye syndrome, 1 for artery risk development, 4 of these for only insulinr4esistance and beta-cell dysfunction. And 7 articles. In total do not have the similar MetSyn parameters for accurate comparison (Table 2.3).

Paper no	Year of publication	Authors	Incldued in Meta-Analysis	Parameters for Metabolically Healhty and Unhealthy participants
1	2014	Patchaya Boonchaya-anant et al.	YES	+
2	2013	Nasser M. Al-Daghri et al.	NO	-
3	2011	Darren R Brenner et al.	NO	-
4	2013	Kai-Hung Cheng et al.	YES	+
5	2010	S. Devaraj et al.	NO	-
6	2012	Grace J Fung et al.	NO	-
7	2014	Sheena Kayaniyil et al.	YES	+
8	2011	Sheena Kayaniyil et al.	NO	-
9	2015	M. Kramkowska et al.	YES	+
10	2015	Yanhui Lu et al.	YES	+
11	2014	Amal M.H. Mackawy et al.	NO	-
12	2014	Maija E. Miettinen et al.	NO	-
13	2011	Foong-Ming Moy et al.	NO	-
14	2015	Poonam K Pannu et al.	YES	+
15	2011	Karolina Rogal et al.	YES	+
16	2011	Shabnam Salekzamani et al.	YES	+
17	2013	Natielen Jacques Schuch et al.	NO	-
18	2012	Manuj Sharma, V.K. Sharmaet al.	YES	+
19	2015	Guler Tosunbayraktar et al.	NO	-
20	2016	Azam Ahmadi Vasmehjan et al.	NO	-
21	2012	Xiao Yin, Qiang Sun et al.	NO	-
22	2015	Hyun Yoon et al.	NO	-
23	2016	Guo-Tao Pan et al.	YES	+
24	2014	Joanna Mitri et al.	YES	+

Table 2.2 Comparison between the included and excluded papers for the Metanalysis.

Paper no	Year of publication	Authors	Included in Meta-Analysis	Parameters for Metabolically Healthy and Unhealthy participants	No of participants	Age	BMI(kg/m2)	VD levels measured	SBP(mm/Hg)	DBP(mm/Hg)	Glucose (mmol/L)	TG(mmol/L)	Total Cholesterol	LDL-C(mmol/L)	HDL-C(mmol/L)	Other measures
2	2013	Nasser M. Al-Daghri et al.	NO	-	120	36.7 ± 3.6/ 25.9 ± 16.1	28.2 ± 6.8/ 26.4 ±6.1	33.4 ± 1.6/28.1±1.4	-	-	5.2 ± 0.63/ 13.8 ± 7.7	1.5 ± 0.74/ 1.4 ± 0.86	-	3.4 ± 0.95/ 3.5±0.91	-	Calcium, Dietary components
3	2011	Darren R Brenner et al.	NO	-	1818	16-65 y	26.56	67.80	110.74	70.70	4,00	1,3	-	-	1,34	No of MetSyn components
5	2010	S. Devaraj et al.	NO	-	50	31 ± 11	25 ± 5	≥20mmol/L	-	-	4,8 ± 0.6	0,9 ± 0.4	4,53 ± 0,67	-	-	hs-CRP, TLR2, TLR4 monocytes, NFkB activity
6	2012	Grace J Fung et al.	NO	-	4727	25- 36. y	-	measured at 5 quartiles	"	"	"	"	"	"	"	Artery risk development
8	2011	Shiena Kayaniyil et al.	NO	-	712	-	-	-	-	-	-	-	-	-	-	insulin resistance and beta-cell dysfunction
11	2014	Amal M.H. Mackawy et al.	NO	-	109 subjects w/diabetics + MetSyn/ -MetSyn	Gr1: 47.96 ± 5.61 Gr2: 47.39 ± 6.01 Gr3: 48.16 ± 8.26	Gr1: 21.36 ± 2.60 Gr2: 30.04 ± 2.94 Gr3: 33.84 ± 3.71	Gr1: 35.25 ± 6.06 Gr2: 19.21 ± 3.20 Gr3: 14.74 ± 4.01	Gr1: 110.95 ± 12.3 Gr2: 118.73 ± 11.46 Gr3: 138.21	Gr1: 77.33 ± 8.04 Gr2: 81.34 ± 9.51 Gr3: 94.92 ± 9.27	Gr1: 100.35 ± 9.89 Gr2: 138.86 ± 7.91 Gr3: 139.15 ± 8.70	Gr1: 142.96 ± 16.38 Gr2: 177.80 ± 32.44 Gr3: 222.97 ± 19.13	Gr1: 199.5 / ± 20.34 Gr2: 288.55 ± 69.04 Gr3:	Gr1: 106.49 ± 11.45 Gr2: 228.22 ± 22.03 Gr3:	Gr1: 54.47 ± 9.86 Gr2: 43.00 ± 4.43 Gr3: 40.80 ± 6.26	IL-6, PCR for FokI and BsmI gene polymorphisms
12	2014	Maija E. Miettinen et al.	NO	-	2868	45-74 y	men: 4.2 women: 5.3	< 50mmol/l=deficiency	men: 18.7 women: 18.8	men: 9.9 women: 9.0	men: 1.3 women: 1.1	men: 0.99 women: 0.61	-	-	men: 0.32 women: 0.34	Lifestyle factors; menstruation, smoking, VD supplements, hormone therapy
13	2011	Foong-Ming Moy et al.	NO	-	380	48.5 ± 5.2	-	sufficient:n:122 insufficient:n:258	-	-	-	-	-	-	-	Hypertension,smoking
17	2013	Nathalen Jacques Schuch et al.	NO	-	601	49.36 ± 7.10	25.21	26.91	132	85	5,20	1,58	-	3,32	1,2	Insulin, HOMA-IR
19	2015	Guler Tosunbayraktar et al.	NO	-	90	18-63 y	Sufficient: 4.5 VDD: 5.2	Sufficient: ≥20μg/L VDD:<20μg/L	Sufficient: 10.8 VDD: 14.3	Sufficient: 8.8 VDD: 9.2	Sufficient: 9.2 VDD: 8.8	Sufficient: 60.4 VDD: 66.9	Sufficient: 34.7 VDD: 38.6	Sufficient: 31.2 VDD: 34.3	Sufficient: 11.2 VDD: 10.6	HbA1C (%), Calcium, PTH, insulin
20	2016	Azam Ahmadi Vasmehjani et al.	NO	-	-	-	-	-	-	-	-	-	-	-	-	
21	2012	Xiao Yin, Qiang Sun et al.	NO	-	601	49.36 ± 7.10	25.21		132	85	5,20	1,58	.	3,32	1,2	HOMA-IR, Insulin
22	2015	Hyun Yoon et al.	NO	-	17,542	≥19y	Adequate (≥20), Inadequate (12-20) Deficient (<12)	-	-	-	-	-	-	-	-	Dry eye syndrome, diabetes, thyroid disorder, depression, RA, surgery

Table 2.3. Baseline characteristics of the 13 studies excluded in meta-analysis.

Year	Authors	Study Type	No of participants	Age (years)	BMI (kg/m2)	VD levels	SBP (mm/Hg)	DBP (mm/Hg)	Glucose (mmol/L)	TG (mmol/L)	Total Cholesterol	LDL-C (mmol/L)	HDL-C (mmol/L)
2014	Patchaya Boonchaya-anant et al.	Retrospective	MHO:63	41.5 ±11.2	40.9± 9.1	57.9± 22.9	125± 13	79± 9	4.9± 0.6	1.5±0.89	5.172± 1.2	3.2± 0.93	1.32± 0.36
			MUO:128	39.4±11.5	44.0± 9.1	50.4± 22.0	129± 18	80± 9	5.9± 0.25	1.7± 0.925	4.835± 0.88	2.9± 0.72	1.14± 0.31
2013	Kai-Hung Cheng et al.	Cross-sectional	MetSyn-:412	55.16± 3.6	24.4± 2.3	47.7± 19.2	128.4± 11.8	80.5± 8.2	5.21± 0.78	1.23± 0.95	4.9± 0.88	–	1.33± 0.28
			MetSyn+:243	56.7± 5.8	27.6± 9.1	40.6± 16.1	136.5± 10.8	86.3± 8.5	10.5± 1.37	2.00± 1.05	4.9± 0.82	–	1.08± 0.22
2014	Sheena Kayaniyil et al.	Cross-sectional	MetSyn-: 225	48.31±9.41	31.42± 5.69	61.7± 22.5	120.7± 15.23	77.72± 10.37	4.73± 0.25	1.06± 0.25	–	–	1.52± 0.23
			MetSyn+:76	51.74±8.54	28.48± 5.31	55.0± 20.3	129.0± 18.1	81.49± 10.94	5	1.39	–	–	1.42
2015	M. KRAMKOWSKA et al.	Observational	MetSyn-: 19	49.0± 3.46	13.68± 1.46	56.23± 7.0	25 (117;144)	87 (77;92)	72 (4.30;5.5)	1.35± 0.25	–	3.31 (2.65;3.87)	1.45 (1.28;1.89)
			MetSyn+:51	45.0 (38;50)	26.10 (32.35;42.90)	39.0± 8.8	40 (140;150)	90(89.5)	82 (5.32;6.4)	29 (0.94;1.43)	5.20± 0.21	3.51 (2.82;4.04)	1.11 (0.93;1.28)
2015	Yanhui Lu et al.	Cross-sectional	MetSyn-:3091	60.96± 0.36	24.49± 0.05	48.7± 21.6	128.2± 0.29	76.02± 0.20	5.49± 0.02	1.39± 0.02	4.72± 0.02	2.91± 0.05	1.35± 0.006
			MetSyn+:184	47.0±0.93	28.24± 0.21	38.3± 15.6	140.51± 1.30	85.85± 0.71	5.8± 0.12	2.47± 0.13	5.14	3.13± 0.06	1.11± 0.02
2015	JOANNA MITRI et al.	Randomised-control clinical trial	MetSyn-:578	51.8±11.5	31.3± 6.1	58.25± 25.7	118.5± 13.3	75.2± 7.8	5.8± 0.5	1.28± 0.55	5.28± 0.93	3.29±0.84	1.40±0.32
			MetSyn+:1422	50.7± 10.6	35.1± 6.7	52.25± 23.5	126.3±14.5	79.7± 9.3	6.0± 0.4	2.11± 1.14	5.28± 0.91	3.23± 0.85	1.09± 0.26
2016	Guo-Tao PAN et al.	Survey-design	MetSyn-:100	63.97± 0.96	22.92± 0.40	62.3± 21.9	130.2± 14.14	76.82± 7.87	7.71± 0.84	1.09± 0.25	4.98± 0.42	2.91± 0.39	1.56± 0.23
			MetSyn+:170	66.90±0.66	25.78± 0.26	54.3± 18.5	144.10±15.09	82.44± 8.52	7.65± 0.85	1.85± 0.38	5.27± 0.47	3.02± 0.40	1.17± 0.23
2016	Poonam K Pannu et al.	Cross-sectional	MetSyn-:2669	41± 0.9	–	57.5± 2.1	122± 07	71± 0.5	4.9± 0.02	1.1± 0.02	–	–	1.5± 0.02
			MetSyn+:735	52± 1.0	–	49.6± 2.1	136± 0.9	81±0.4	5.5± 0.04	2.1± 0.06	–	–	1.2± 0.03
2011	Karolina Rogal et al.	Observational	MetSyn-:25	–	29.0± 3.6	49.7±23.7	120.6± 3.3	75.9±3.9	4.8± 0.4	0.82± 0.23	4.08± 0.46	2.35± 0.35	1.35± 0.25
			MetSyn+:27	–	36.1± 6.2	68.1± 28.5	140± 5.8	93± 4.62	5.3±0.8	1.32±0.28	5.12± 0.63	3.02± 0.39	1.15± 0.22
2011	Shabnam Salekzamani et al.	Case-control	MetSyn-:100	42.3± 5.6	30.4± 4.5	13.9± 14.1	112.6± 12.5	71.4±9.7	5.1± 0.6	1.18± 0.49	4.40± 1.04	2.37± 0.56	1.32± 0.24
			MetSyn+:100	42.0±8.5	33.1± 15.4	16.7± 16.4	122.5±16.2	76.7± 11.3	5.6± 0.8	2.07± 0.83	5.03± 0.19	2.73± 0.63	1.07± 0.26
2012	Manuj Sharma, V.K. Sharmaet al.	Cross-sectional	MetSyn-:25	41.6± 8.7	21.4± 5.1	68.1± 26.2	115.6± 5.1	78.4± 3.7	5.0± 0.3	1.14± 0.16	–	–	1.34± 0.15
			MetSyn+:25	42.7+14.8	33.0± 16.4	45.2± 22.5	147.2+ 16.4	88.8±7.5	5.9± 1.3	1.79±0.23	–	–	1.23±0.21

Table 2.4. Baseline characteristics of the 11 studies included in meta-analysis. Values are represented with as mean ± SEM/SD.

2.4.5 Main result

2.4.5.1 MetSyn and VD status

In this meta-analysis of 11 studies exploring the relationship between low VD status and MetSyn compared to healthy individuals, a random effects model was employed to calculate the pooled effect size, considering both random error, and real differences between studies. The results were reported alongside a confidence interval (CI). To gauge the extent of variation among these studies, the researchers used the I^2 index, which indicated high heterogeneity (83%) highlighting substantial differences between the studies, potentially stemming from varying methodologies or populations.

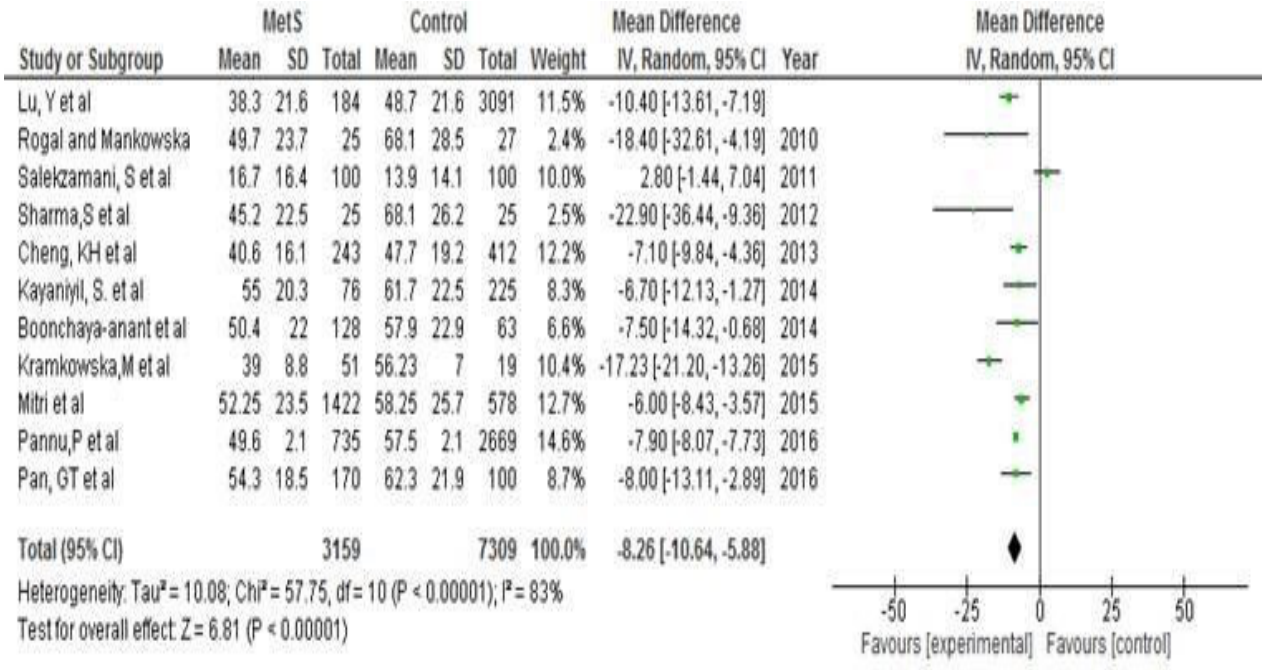


Figure 2.2 Meta-analysis assessing the relationship between VDD status and MetSyn.

2.4.5.2 Relationship between Vitamin D and the components of Metabolic Syndrome

Overall, we have looked for a significant negative correlation between serum VD levels with MetSyn components including fasting glucose levels, waist circumference, systolic blood pressure, diastolic blood pressure, triglycerides, but with higher values of HDL-C (**Table 2.2**). In two of the case-control studies conducted on DMT2 patients, systolic blood pressure, waist circumference, insulin, glucose, HOMA-IR, 25D3 levels, and HDL-C were significantly increased in DM patients compared with controls. Thus, the significant increase of MetSyn components was confirmed. There are other studies that were conducted on obese patients which confirmed a similar relationship, a negative correlation between 25D3 levels and obesity parameters (Foss YJ *et al.,* 2009; Vranić L *et al.,* 2019). Several studies showed a high prevalence of VDD in extremely obese individuals. Our findings are consistent with the systematic review and meta-analysis study by Pereira-Santos et al. (2015), as they also reported a higher prevalence of VDD in obese subjects, with a 35 %increase compared to the eutrophic group. They observed 24% higher prevalence of VDD deficiency in the overweight group. In addition, all the cohort studies show a higher risk of MetSyn with increasing quartiles of 25D3 levels. Obesity is characterised by excess body weight and abdominal fat. An observational study, Rogal and Mankowska., 2011, found that VD insufficiency is more common in women with excessive body weight and MetSyn than women without MetSyn.Same study also found a significant negative correlation between serum VD levels and

components of glucose metabolism. There is accumulated data suggesting that people with lower VD levels have impaired glucose tolerance (Liu *et al.,* 2005).

We performed a meta-analysis of studies that fulfilled our inclusion criteria to assess the relationship between the 25D3 serum levels and the intake of VD in relation to MetSyn incidence. 11 studies are accepted for meta-analysis that provided similar measures and included SD/SEM measures of VD. All the studies looked at systolic and diastolic blood pressure, glucose levels, and triglyceride levels as MetSyn components. Total cholesteroland LDL has been looked at in all except three of the cross-sectional studies; Kayaniyil Set al 2014, Pannu P et al, 2016 and Sharma S et al, 2012. Except one case-control study,meta-analysis results confirmed a positive effect. Salekzamani S et al, 2011, found no significant difference between control and MetSyn components. However, upon adjustingthe participants for VD status, he found a significant increase in glucose levels with VDD ($p < 0.001$).

In conclusion, there is a link between MetSyn and low levels of VD.

2.5 Discussion

Many have suggested that the risk of MetSyn could be potentially determined by VD status. This is supported by the fact that the prevalence rates of MetSyn in male subjects was higher in the winter than in the summer with higher levels of fasting insulin, HOMA- insulin resistance, and triglycerides (Chen *et al.,* 2008: Kamezaki *et al.,* 2010: Rintamaki *et al.,* 2008). This prevalence remained even after controlling for BMI. Therefore, we systematically searched the literature to determine whether those with MetSyn were morelikely to be VDD. Papers included in the systematic review have

shown that higher serum VD is positively associated with specific MetSyn components; body mass index, waist circumference, plasma glucose levels and blood pressure, and negatively associated with high density lipoprotein cholesterol. This systematic review emphasizes the scientific importance of the relationship between VDD and the prevalence of MetSyn. Despite the high heterogeneity between the studies, the pooled analyses of this current study summarize the correlation between the components of the MetSyn and VDD. Thus, suggesting that an inadequate intake of VD may be a risk factor for disorders such as MetSyn.

As stated earlier, the main risk factor for MetSyn is obesity, and VD insufficiency was negatively correlated with BMI, waist circumference and body weight. Our study, in contrast to the findings of Saneet et al. (2013), revealed a significant positive association between low serum 25D3 levels and BMI. Specifically, our data. Demonstrated. That as BMI increase, this is correlated with a decreased VD level. Interestingly, it is unknown whether VDD results in obesity or whether the accumulation of fat leads to VDDdue to sequestering of serum VD into the adipocytes causing reduced bioavailability (Migliaccio *et al.,* 2019). Although a 12-week RCT revealed that VD supplementation significantly decreased body fat mass and improve insulin sensitivity in healthy and obesecompared with the control group (Salehpour *et al.,* 2012, Nagpal et al., 2009). While numerous studies have revealed that VDD causes adipogenesis, fat accumulation, leptin,and changes in cytokine expression through its genomic actions via the interruption of theNf-kB signalling pathway (Abbas, 2017). This is further supported by *in vitro* studies in which treatment of 3T3-L1 pre-adipocytes cells with VD reduces triglyceride accumulationthrough increased activity

of the NAD-SIRT1 pathway, suppression of peroxisome proliferator-activated receptor γ (*PPAR-γ*) and CCAAT/enhancer binding protein (C/EBP)through maintenance of WNT/beta-catenin pathway and unliganded VDR sharing a heterodimer RXR to block PPARγ activity (Chang and Kim, 2016: Sakuma *et al.,* 2012: Sun and Zemel, 2008). Thus, suggesting the possibility that VDD may in part be responsible for the increased adiposity observed in obesity. Interestingly adipocyte differentiation is controlled by epigenetic regulation of several genes such as the transcription factors such as C/EBP gene family and *PPAR-γ* (Yang *etal.,* 2011). In turn, this increases lipoprotein lipase (LPL) activity, an enzyme known to orchestrate the expression of the adipocyte-phenotype markers and lipid metabolism. Thus, it is proposed that VD can influence the adipose stem cell differentiation to an osteogenic phenotype (Song *et al.,* 2011), and therefore linking the genetic influencefor *familial* clustering of MetSyn related traits, particularly obesity (Tang *et al.,* 2006).

Insulin sensitivity is a characteristic of the metabolic disorders like obesity and diabetes mellitus. Defective glucose production and metabolism due to trace amounts or no insulinproduction results in significant constant rise in serum glucose levels leads to accumulation in the tissues and cause metabolic disorders. Studies have shown a negative correlation between VD and BMI, also fat mass (Zhu et al., 2013). Among obesitypatients, excess adipose tissue causes an increase in lipolysis, FFA from lipids, hormones, and pro-inflammatory cytokines (Peterson *et al.,* 2014). The increase of pro- inflammatory cytokines such as TNF-alpha, impair insulin signalling and the crosstalk between the insulin signalling molecules resulting in translocation of the GLUT-4 to the cell surface. The active metabolite of VD, $1.25OH_2D_3$, binding to

VDR in pancreatic beta cells, increases insulin secretion. A more indirect way is through the Ca+ channels. It was found that VD reduce the L-type Ca+ channel expression thus decreasing intracellular Ca+ levels. In turn, increase in the cytoplasmic Ca+ level activates the exocytosis of the insulin from pancreatic β-cells (Glion *et al.,* 2014). In adipose tissue, liver and skeletal muscle, VD has been shown to increase hepatic and peripheral uptake of glucose through upregulation of SIRT1/IRS1/GLUT-4 signalling cascade. Evidence shows that this occurs in the skeletal muscle cells by upregulating the GLUT-4 transporter expression (Tamilselvan *et al.,* 2013), and in adipocytes by promoting GLUT-4 translocation, a key piece in glucose metabolism (Manna *et al.,* 2012). Therefore, VD decreases insulin resistance and maintains glucose metabolism via Ca+- dependent signalling cascade and GLUT-4 translocation.

Many studies support the hypothesis that the VDD is a risk factor for diabetes mellitus andmany metabolic components such as glucose and insulin sensitivity. (Dodie *et al.,* 2015; Zhang *et al.,* 2014). In addition, a recent study in children and adolescents suggests a positive correlation between serum VD levels and blood levels of total cholesterol, apolipoprotein A1, apolipoprotein B, and TG (Delvin et al.,2010). Two systematic review and meta-analysis have been conducted on the effects of VD supplementation on insulin and glucose homeostasis, failing to find a significant correlation. However, these studies did not include enough RCTs in their studies, therefore their results are likely be uncertain (Seida *et al.,* 2014: Pulsop *et al.,* 2016). However, many other studies suggest an inverse association between MetSyn components such as BMI, waist circumference, insulin sensitivity, glucose levels, TG,

and blood pressure with serum VD levels, which is in line with the findings of this present systematic review. The findings of He et al., 2018, also align with the broader body of research that suggests an inverse association between MetSyn components with serum VD levels. In the study, He et al. reported that VD supplementation had no significant effect on controlling fasting plasma glucose, but when stratified by BMI, individuals with a. BMI less than 25 showed a decrease in fasting plasma glucose levels after VD supplementation. These results are consistent with the collective. Evidence presented in this present systematic review, supporting the notion of the inverse relationship between MetSyn components and serum VD levels. One mechanism for the elevated blood glucose and insulin is that VDD impairs proper functioning of the pancreatic beta cells and cause insulin resistance (Chiu *et al.,* 2004). Regarding TG and cholesterol, Oh J and colleagues might underline the relation: VD protects against foam cell formation and suppresses macrophage uptake of cholesterol in DMT2 patients (Oh *et al.,* 2009). In addition, VD administration lowers SBP and DBP in older adults with existing hypertension (Margolis *et al.,* 2008). Low levels of VD can lead to hypertension through upregulating the renin-angiotensin-aldosterone system (Li *et al.,* 2004). In line of these, we can conclude that VDD might result in long term changes to the homeostatic mechanisms.

VD interacts with the epigenome, thus, the life-long effects of VDD on health might arise from epigenetic changes. (Fetahu *et al.,* 2014). As mentioned, much of the VD signalling occurs upon binding of the $1,25OH_2D_3$ to the VDR which is a ligand-dependent transcription factor that has been shown to modify the activity of VD-VDR complex as wellas the polymorphisms in the VDR gene. One of the included cross-

sectional studies (Schuch.; *et al.,* 2013) show that there is a relationship between VDR polymorphisms andMetSyn components. Therefore, the impact of VD in deregulation of the epigenetic mechanisms may suggest that deficiency may lead to certain pathological conditions or health outcomes.

CHAPTER 3. Long term effects of VDD on offspring energy metabolism and behaviour

3.1 Background

VD is involved in crucial actions throughout the brain development including cell differentiation, proliferation and apoptosis, neuroprotection, and anti-inflammation to modulating dopamine (DA) and serotonin (5HT) signaling pathways (Anjum *et al.,* 2018).DVD-deficient rodent models have altered brain structure and composition: fetuses exposed to insufficient maternal VD levels have low birth weights and are small for gestational age (Wang *et al,* 2018), have reduced bone growth, poor skeletal mineralization, and lower bone mineral content (Laird *et al.,* 2010).

Rodent studies have indicated that embryos exposed to VDD during pregnancy is associated with a range of neuropsychiatric and neurological, as well as metabolic disorders (Hawes *et al.,* 2015). However, the mechanism is unknown. In line with this, maternal VDD in offspring shows impaired learning, memory, and behaviour (Becker *et al.,* 2005). Collectively, evidence indicating an increased volume of the lateral ventricle, change in expression of neurotrophic factors, altered expression of genes

involved in dopamine and glucocorticoid signalling pathways (Yates *et al.,* 2018), altered spatial learning and hyperlocomotion which are collectively associated with neurological diseases such as autism and schizophrenia (McGrath *et al.,* 2010). We addressed this by investigating the effects of maternal VDD *in utero* using metabolic and behavioural tests.

To follow our hypothesis, we further investigated the effects of VDD environment by conducting behavioural tests to assess anxiety and memory. As previously mentioned VDD during pregnancy is associated with adverse effects in mothers such as pre-eclampsia. VDD during pregnancy is also associated with gestational diabetes, which can affect offspring's development and health (Senti *et al.,* 2012). Evidence from human and rodent studies suggest that foetal programming and *in utero* environment can determine the offspring's health and susceptibility to metabolic diseases later in life (Saffery and Novakovic, 2014). Data from 977 pregnant women revealed that lower maternal $25D_3$ concentrations were associated with higher fat mass in their offspring at the age of 6 (Crozier *et al.,* 2012). Amsterdam Born Children and their Development (ABCD) study on 1887 women and their offspring reported a similar result; maternal $25D_3$ concentrations were inversely correlated to the percentage fat mass at the age of 5 in addition to higher insulin resistance markers in children whose mothers were overweight during pregnancy (Hrudey *et al.,* 2015). The inverse correlation between low VD concentrations and insulin resistance markers were confirmed in another study when they were assessed at 9.5 years old (Krishnaveni *et al.,* 2011). Several studies using rodent models subjected to *in utero* VDD has reported the adverse effects of maternal VDD on offspring metabolic health. Among these

adverse metabolic outcomes are commonly an abnormal fat metabolism and increased fat mass, insulin resistance and hypertension (Reichetzeder *et al.,* 2014). F1 generation of the VDD damsshowed increased insulin secretion and higher body and epididymal fat mass compared to controls (Nascimento *et al,* 2013). In line with the findings above, a Sprague-Dawley rat model of *in utero* VDD reported increased serum inflammatory markers and increased HOMA-IR in 16-weeks old adult offspring (Zhang *et al.,* 2014). Furthermore, smaller pancreatic and islet structure with less beta cell mass coupled with reduced expression of PDK-1, a mediator of insulin-signalling was reported in the offspring of VDD C57BL/6 dams which might lead to reduced insulin secretion (Maia-Ceciliano *et al.,* 2016). Thus, explaining the positive correlation between insulin sensitivity and maternal VDD. Regarding the correlation between maternal VDD and adipogenesis, many studies demonstrated clear adverse consequences using VDR-knockout models and offspring of VDD dams. Narvaez and colleagues found that the level of uncoupling protein 1 which mediates the dissociation of cellular respiration from energy production, was more than 25-fold elevated in VDR-knockout white adipose tissue (Narvaez *et al,* 2013). The effects of VDD-diet on the human body are rather complex because of the involvement of both genomic and non-genomic effects of VD. Therefore, VDR-knockout models might not be the best to outline the consequences.

VD's role in the brain was first discovered in 1984, where Balabanova and colleagues found VD metabolites in the cerebrospinal fluid of healthy adults (Balabanova *et al.,* 1948). Later, $1.25OH_2D_3$ binding was mapped in rodent brains by radiography (Stumpf WE and O'Brian LP, 1987). Then, VDR was discovered in multiple brain regions of

neonatal and adult rats (Veenstra *et al.,* 1998: Prufer et al., 1999) supporting the hypothesis that VD is involved in brain development and function. Significant changes due to VD levels have been demonstrated throughout life in the brain (Evatt et al., 2008). VD has been under spotlight for its role during pregnancy and foetal development. Moreover, the foetal development is thoroughly dependent on the maternal nutrient status and circulation of VD levels. This raises concerns about maternal VDD and its long-term effects on foetal development during pregnancy and the offspring's long-term health, including offspring cognitive health. In rats, VDR emerges in the developing brain at embryonic day 12. It is present in several different regions of the brain and preferentially localized in differentiating areas (Veenstra *et al,* 1998). The experimental animal model of developmentally VDD requires that the dams receive a diet lack of VD and are housed under incandescent lightening free of VD spectrum of light. All the dams and litters then return to their normal diet after birth. The offspring born from VDD mothers had an abnormal anatomical brain development compared to controls. The deficient offspring had enlarged brains; enlarged lateral ventricles, decreased width of the neocortex along with the changes in the amounts of neurotrophic factors (Eyles *et al.,* 2003). Due to the structural changes occurring in hippocampus of the developing brain, alterations in motor function, memory and sensory motor abilities and perceptions of the VDD animals has been observed. These findings can be due to VD's ability to control cell cycle, proliferationand differentiation and apoptosis through the expression of genes involved in the cell cycle which possess VDR response elements. Maternal VD plays a role in cell cycle andapoptosis (Tabasi *et al.,* 2015). The studies have shown that VDD led to spontaneous

hyperlocomotion in rats in various behavioural tests including elevated plus maze, forced swim test, open field (Burne *et al.,* 2004: Kesby *et al.,* 2006) however, these results proved to be inconsistent (Becker *et al.,*2005: Burne *et al.,* 2015). Moreover, data from human studies showed that the expectant mother with insufficient levels of VD has a higher risk of having children with reduced social development, cognitive and motor skills in childhood (Darling *et al.,* 2017).

3.2 Aims and objectives

Prenatal VDD has been linked to effects on long-term metabolic health of the offspring such as increased risk to develop obesity and diabetes possibly leading to MetSyn (Chapter 2). The purpose of this study is to investigate the effects of VDD during pregnancy *in utero* on metabolic and cognitive outcomes in the offspring.

The objectives are:

1. To assess the effects of a VDD environment during development on metabolism and adiposity
2. To assess the effects of a VDD environment during development on anxiety and memory

3.3 Materials and Methods

3.3.1 EXPERIMENTAL ANIMALS

All animal procedures were approved by the University of Nottingham Animal Welfare and Ethical Review Board and were carried out in accordance with the UK Animals (Scientific Procedures) Act 1986 (project licence PPL 40⁄3598). Virgin female C57BL/6J mice and male studs (Charles River, Margate, UK) at 3 weeks old were maintained under controlledtemperature (21 ± 1ºC) and light (12h light/dark cycle, lights off at 19:00h) with *ad libitum* access to food and water unless described.

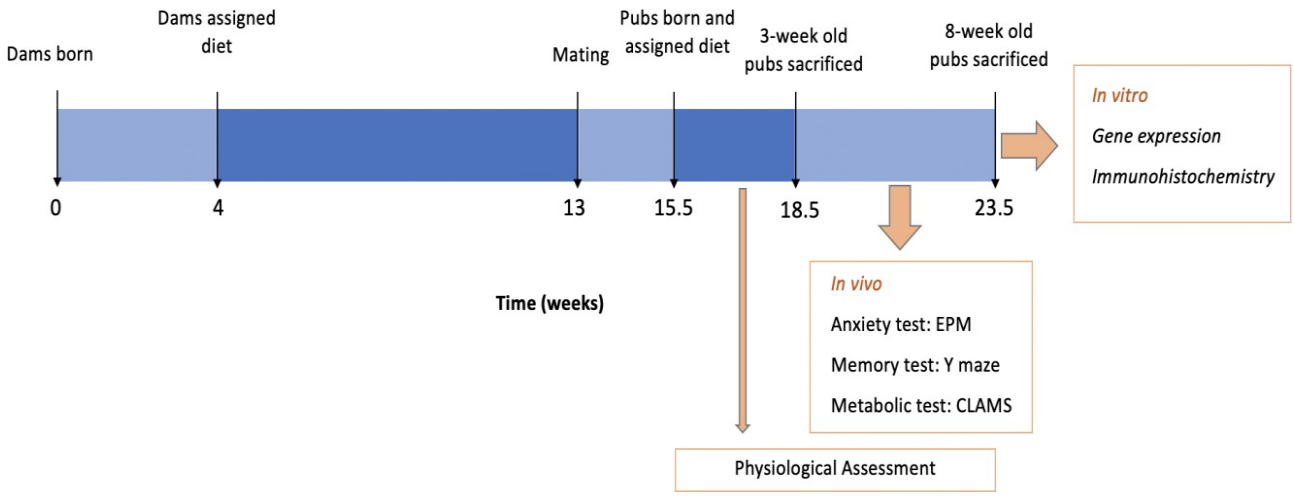


Figure 3.1 Experimental timeline for animal handling. Female mice sacrificed at 3weeks and males at 8 weeks of age.

3.3.2 Experimental Plan

Virgin female C57BL/6J mice (4 weeks) (N=6) were obtained (Charles-River, UK) and housed with ad libitum access to food and water under incandescent lighting free UVB radiation prior to mating. From 4 weeks of age, they were fed a Vitamin d control diet (CONTROL: 2.2 IU D/g; TD 89124, Tekland Research Diets, Harlan UK) or Vitamin D deficient diet. (DEFICIENT: 0.0IU D/g; TD 89123, Tekland Research Diets, Harlan UK). Females were mated with control males and pregnancy was confirmed by the presence of a virginal plug. Female dams remained on their diet throughout gestation and lactation. Control and deficient female mice were housed in this environment, and they mated twice, at 5 weeks and 9 weeks respectively, to give two litters. A pilot was formed on a subset of dams who were sacrificed alongside the 8-week-old pubs at approximately 23.5 weeks of age (N:4 control, N=2 deficient). Following this, the 6 dams were euthanized by injection of pentobarbital sodium (Euthatal; Rhone Merieux, Harlow, UK).

Offspring

Offspring remained on the maternal diet for the duration of the study. Bodyweight of the offspring was measured weekly from postnatal day 3 to postnatal day 68. At postnatal day 12, offspring were housed individually and remained on their maternal diet. At 6 weeks (P42-45) of age, offspring were placed into the CLAMS to measure energy metabolism and underwent a battery of behavioural tests to measure anxiety (elevated plus maze) and memory and learning (Y maze). 8 weeks old male mice were used to conduct the behavioural tests.

At 3 weeks and 8 weeks respectively, they were euthanized by injection of pentobarbital sodium (Euthatal; Rhone Merieux, Harlow, UK), brains were cut down the midsagittal line. Half was used for immunostaining and the other half for gene analysis, alongside the collection of white and brown adipose tissue (see Chapters 4 and 5). The blood was taken from the tail vein. Blood samples were obtained to measure circulating levels of VD while the animals were under terminal anesthesia.

Sample size was a key limiting factor in our data analysis. There were N=2 6-week-old controls due to breeding issues during the first pregnancy, so 6-week-old mice couldn't be statistically analysed. See below for the details of the animals used in this study. Groups sizes vary for the different outcome measures, due to mice being culled during the course of the study or capacity limits of equipment. The reason for the differences is (1) the clams have 8 cages, so we had a group for this (4 controls and 4 treated) and (2) we culled some animals at 3 weeks and then at 6 weeks so there were two different litter groups which we did not use these mice for behaviour.

Gender	Age/ weeks	Dam diet onset/ weeks before mating	Deficient N=	Control N=
Female	3	9	4	8
Male	6	5	7	1
Female	6	5	0	1
Male	8	9	6	12

Table 3.1. showing the animals categorised into different experimental groups based on gender, litter, and the diet.

3.3.2 Assessment of metabolism

CLAMS apparatus: Ingestive behaviour, metabolic gases (oxygen consumption (VO_2) and carbon dioxide production (VCO_2) and activity were measured using a Comprehensive Lab Animal Monitoring System (CLAMS; Linton Instrumentation, Linton,UK, and Columbus Instruments, Columbus, OH, USA) as previously described (Jethwa *et al.,* 2007). The CLAMS is a modified open calorimetry consisting of eight individual chambers with feeders in the middle and constant supply of water provided by dropper bottles (water intake was not recorded). The system operated with air intake of 0.6litres/minute per chamber and an extracted outflow of 0.4liter/min. About of food intake (a meal) was defined as an intake of greater than 0.02g while activity (linear and vertical) was recorded when two or more consecutive infrared beams traversing each cage were broken. The oxygen consumption (VO_2) and carbon dioxide production (VCO_2) measured normalised over the estimated lean mass ($BW^{0.75}$) due to the change in body composition, such that the energy expenditure (EE) and respiratory exchange ratio (RER). The air flow per min was set for each cage in the circuit and the room temperature was 21 °C throughout the measurements.

3.3.3 Behavioural apparatus setup and tests

3.3.2.1 Elevated Plus Maze (EPM)

The apparatus constructed accordingly (**Figure 3.2**). The EPM consisted of two opposing open arms and two wall-closed arms with a 7.5cm to 35cm dimension, extending from a centre forming a cross shape. Foam blocks were places under the

apparatus to protect the mice if they fell during the test. The apparatus was placed in the middle of the behavioural testing room with a dim light illumination of 2-5 lux. The animals were broughtto the behavioural testing room shortly before the test in their home cages. To prevent stress caused by scent trails and minimise odour cues, the maze was cleaned by 20% EtOH between each trial. The mice were handled according to the regular protocol and in the same way to minimise stress or anxiety before testing which can alter test results. The mice explored the maze for 5 minutes. The distance travelled over this course and the amount of time spent in the open and closed arms were measured. The mice were watched with a video-tracking system which was by a camera hanging from the ceiling and the behaviour at the maze recorded by a video analysis software Ethovision (XT V10Copyright © 2013 Noldus Information Technology), which tracked the position of the mouse throughout the trials.

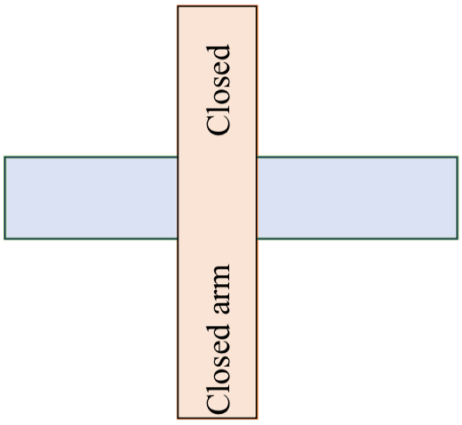


Figure 3.2. Diagram of EPM apparatus layout showing the open and closed arms.

3.3.2.2 Spontaneous Alternation Y maze (SA)

~~Each~~ arm of the Y-maze was 45 cm long, 7.5 cm wide and 14 cm high (**Figure 3.3).** To reduce anxiety in the animals, light in the testing area was dimmed to 3 lux. The mice were placed into the center of the Y shaped maze (arms 45cm x 7.5cm x 14cm). The test consisted of a 5-minute trial where mouse was exploring the three arms of the Y-maze. The number of entries to each arm and the number of triads were recorded manually and scored for the calculation of the percentage alternation. Each arm entry was given a score of 1 or 0. 1 was awarded if the arm entered was different from the previous entries. Otherwise, the score would be 0. These were added up to give the alternation score. The total number of alterations made was also added up.

The alternation index is calculated as: $a_{(n-1)}$

Between each individual test, the arms were cleaned with 20% EtOH to remove the scentof the previous animal. Mouse location and movement was tracked by Ethovision video analysis software.

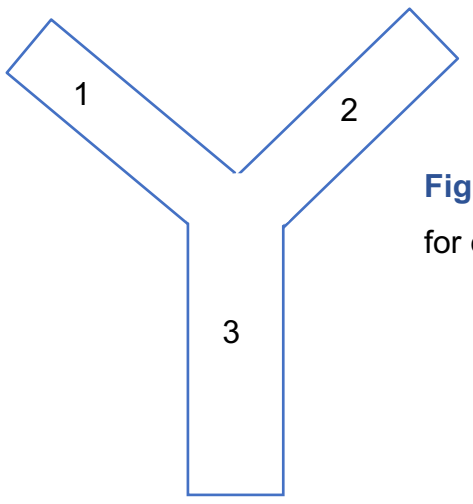


Figure 3.3. Diagram of the SA Y maze. Arms labelled for entry recording.

3.3.3 Statistics

The statistical software used was Prism (Graph pad version 7, USA) for Windows. Data for bodyweight at birth was analysed by a student t-test. Bodyweight treatment over P56 and all Clams data was analysed using a two-way ANOVA. All data is represented as mean ±S.E.M. The significance threshold was set at $p < 0.05$. Mann-Whitney test was used where necessary.

3.4 Results

3.4.1 Lower birth weight and further increase in weight in offspring born fromVDD dams

Offspring born to dams on a VDD diet had significantly lower bodyweight at post-natal day3 (**Fig 3.4A** t (5) =3.599 p=0.016). However, continued exposure to a VDD diet led to an increase in weight and at P56 days, mice exposed to VDD during development were 11%heavier (group x time interaction **Fig 3.4B** F=1.704 p=0.0461). This was even though there was no difference in food intake (**Fig 3.4C** F= 0.8734 p=0.5535), meal duration(**Fig 3.4D** F= 2.692 p=0.1519) or frequency **(Fig 3.4E** F= 2.438 p= 1694

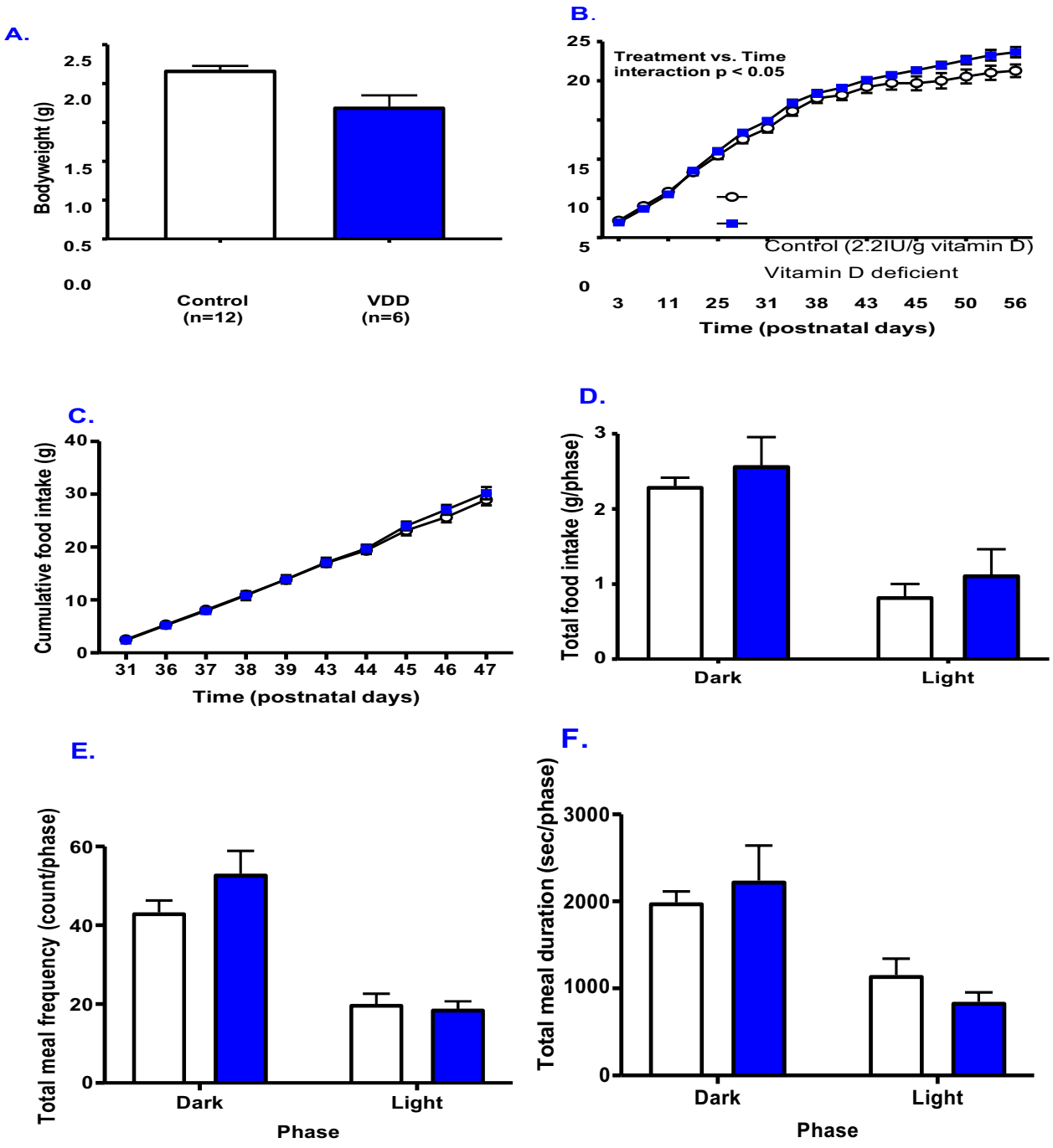


Figure 3.4. Offspring exposure to a VDD environment *in utero* **A.** The birthweights of VDD offspring compared to controls B. Bodyweight measurement over postnatal 56 days (n=18). **C.** Cumulative food intake over postnatal 47 days (. **D.**Total food intake (avg/day) during dark and light phases. **E.** Total meal frequency during dark and light phases. **F.** Total meal duration during dark andlight phases (N=18). Values represented as mean ± SEM * $p<0.05$

3.4.2 Reduced energy metabolism in offspring born from VDD dams

At 5 weeks, male mice born to mothers consuming a VDD diet had an overall decrease in oxygen consumption (group x time interaction (**Fig 3.5A** F = 5.104, p=0.0017) suggesting that the effect of VDD is different across the light/dark cycle, with significantly lower levels in the dark phase (**Fig 3.5B** F = 8.605, p=0.026).

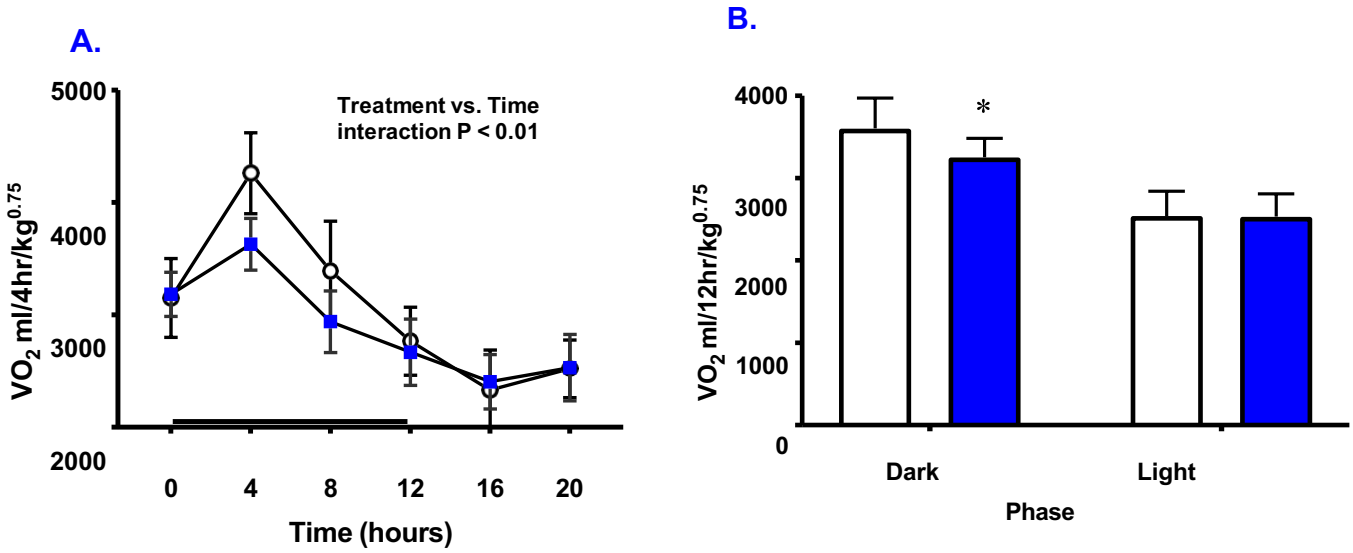
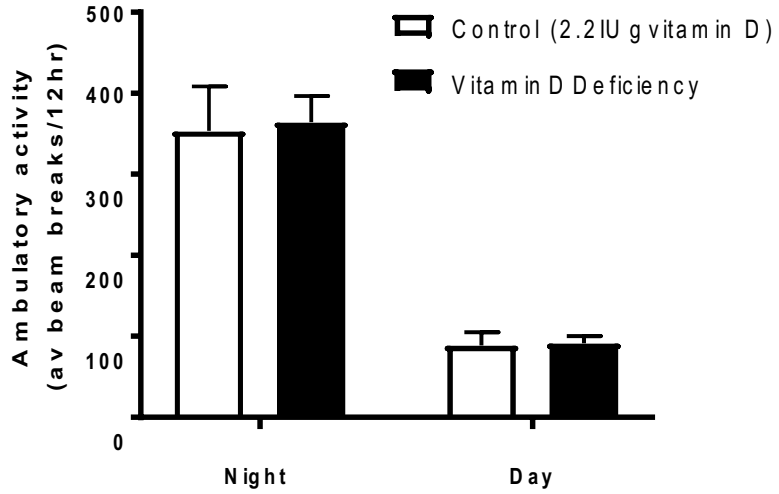


Figure 3.5. A. CLAMS analysis showing changes in energy metabolism **B.** Oxygen consumption during light and dark phases between VDD mice compared to controls. Values represented as mean ± SEM

3.4.3 No significant change in the locomotor activity of the offspring born from VDD dams compared to the controls

At 5 weeks, male mice born to mothers consuming a VDD diet had no significant change in locomotor activity levels measured by average beam breaks compared to the control group, during night and day (group x interaction **Fig 3.6A** p=0.8728). The levels of ambulatory activity between VDD and controls group per hour shows that there is no significant difference between the groups (group x interaction **Fig 3.6B** p=0.6313).

A.



B.

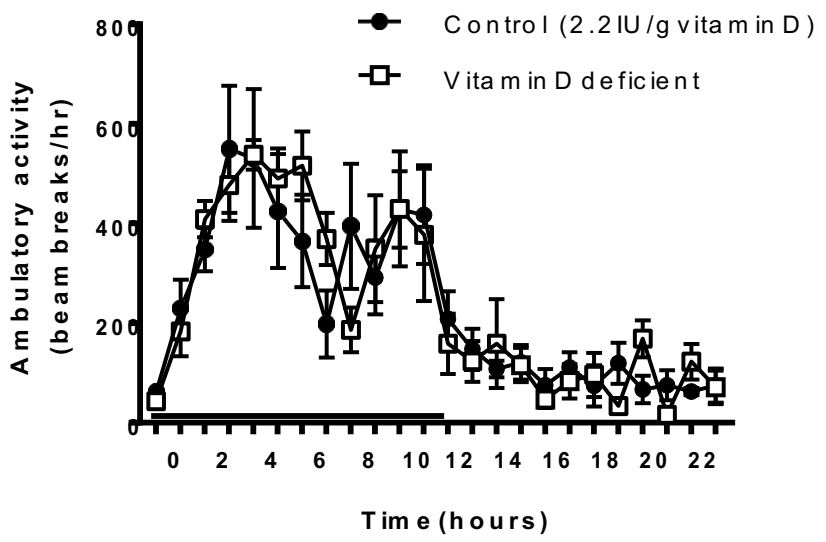


Figure 3.6 A. CLAMS analysis showing changes in average ambulatory activity levels per 12 hours during night and day between VDD mice compared to controls.

B. Data showing the ambulatory activity levels per hour. Values represented as mean ± SEM.

3.4.4 The effect of pre-and postnatal VDD on anxiety and memory

We observed that mice exposed to VDD during development a significant increase in anxiety compared to those exposed to control diet (**Fig 3.6A** t (21) =2.342 p= 0.029). However, although there was a reduction in memory, as shown by a decrease in alternation in the Y maze, this failed to reach significance (**Fig 3.6B** t (25) =1.724 p=0.097).

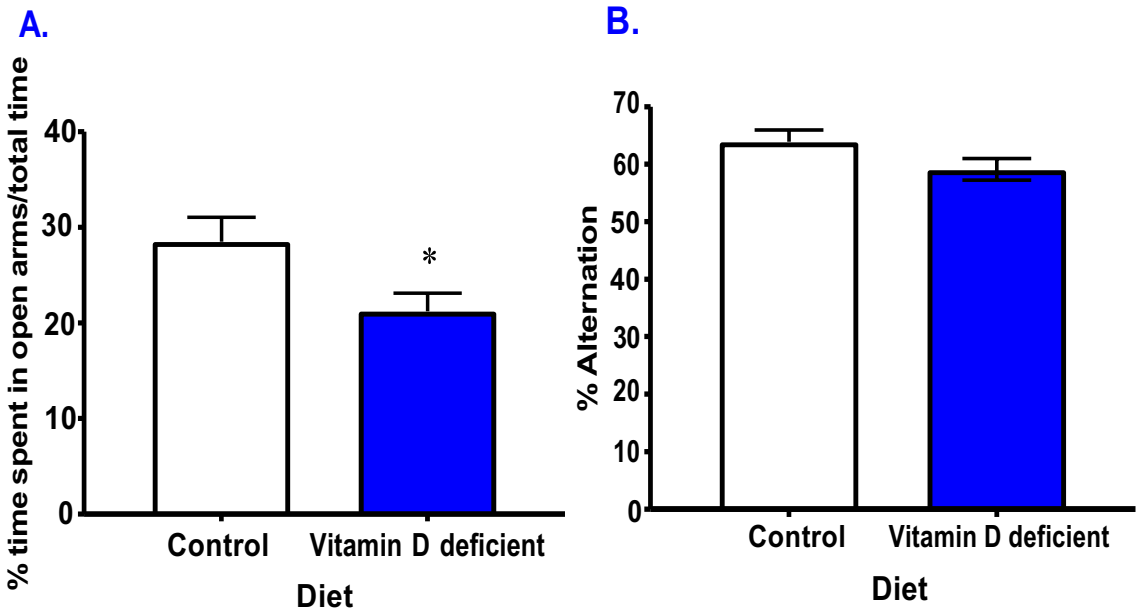


Figure 3.7 A. EPM data showing percentage time spent in open arms. **B.** SA Y maze showing percentage alternation among groups arms. Values represented as mean ± SEM * p<0.

3.5 Discussion

3.5.1 Energy metabolism

We have developed a mouse model to mimic the clinical findings in humans. Maternal VDD has been shown by numerous studies to influence birth weight (Crozier *et al.,* 2012) as we have shown in our model. Furthermore, studies have shown that neonates to mothers with a VDD status show accelerate growth in weight during the first year (Viljakainen *et al.,* 2011). Interestingly, the increase in bodyweight was associated with a decrease in energy expenditure and not an increase in food intake. The data derived from the cage monitoring has offered additional insights into the phenomenon of reduced oxygen consumption observed in the VDD group. Importantly, the evidence suggests that the observed reduction in oxygen consumption is not primarily attributable to diminished physical activity levels among the experimental animals. Specifically, the analysis of the ambulatory activity data revealed that there was no statistically significant reduction in the level of physical activity exhibited by VDD group compared to the control group. This observation serves to discount the hypothesis that decreased activity levels are the principal driver behind the observed decrease in energy expenditure within the VDD group. These findings, taken together, provide compelling evidence that factors other than physical activity levels are likely contributing to the reduction in energy expenditure observed in the VDD group. Further investigation is warranted to elucidate the underlying mechanism. Responsible for this intriguing metabolic response.

A possible reason for this reduction in bodyweight at birth could be a reduction in general organ development *in utero* (Hart *et al.,* 2015). There are several possible mechanisms connecting maternal VDD to foetal somatic growth (O'loan *et al.,* 2007). VDR and CYP27B1 are present in the lungs and in the placenta. In the lungs, VD play a role in lung development and lung maturation (Edelson JD et al, 1993: Nguyen M et al, 2004: Zosky *et al.,* 2011). Two of the known mechanisms by which it does are: regulating transforming growth factor- β1, which is known for airway remodelling (Foong *et al.,* 2014) and through stimulating pulmonary artery endothelial cell growth (Mandell *et al.,* 2015). In placenta, VD is known to be critical to foetal growth by stimulating the secretion of placental hormones and through mediating circulating essential nutrients such as calcium (Lapillonne *et al.,* 2010). Although VDR null mice have a lean phenotype due to increased energy expenditure associated with increased fatty acid oxidation and up-regulation of uncoupling protein 1 (UCP-1) (Narvaez *et al.,* 2008), numerous studies have shown an inverse association between VD status and total body fat or visceral adiposity (Narvaez *et al.,* 2013). Thus, we propose that the increase in bodyweight could be attributed to increased adiposity. In support, Kong and Li showed a significant increase in proliferation rate and number of lipid droplets for pre-adipocytes in VDD offspring while $1,25(OH)_2D_3$ (10^{-11}-10^{-8} M) has been shown to inhibit adipogenesis in 3T3-L1 cells, a cell line derived from mouse for using in adiposity research, inhibiting the expression of early and late markers of differentiation (Kong and Li, 2006). Thus, suggesting that VDD during pregnancy may promote the proliferation and differentiation of pre-adipocytes over skeletal tissue (Wen *et al.,* 2018). In line with these *in vitro* studies in the mouse C2C12 muscle cell

line have shown that $1,25OH_2D_3$ could regulate the trans differentiation of myoblasts into adipocytes. They showed that very low physiological concentrations (10^{-13} & 10^{-11}M) of $1,25OH_2D_3$ (equivalent to a vitamin D deficient state) increased formation of lipid dropletsin C2C12 cells were cultured in adipogenic media. This was associated with up-regulation of PPAR□ and Fatty Acid Binding Protein-4 (FABP4) mRNA expression, which are key genes in the formation of adipocytes (Ryan *et al.,* 2013). Further evidence comes from VDR-null mice, which have smaller muscle fibres, with increased expression of embryonic and neonatal isoforms of myosin heavy chain (MyHC), indicative of relatively immature muscle (Endo *et al.,* 2003). Increase in adiposity and decrease in muscle fibers is associated with decrease energy expenditure possibly via altered mitochondrial activity (Toledo *et al.,* 2018: Dzik and Kaczor, 2019). In support, Ryan and colleagues revealed an oxygen consumption rate through increased ATP generation in human skeletal muscle cells, hSkMCs, following VD treatment, a mechanism that is VDR-dependent (Ryan *et al.,* 2016). Furthermore, Sinha and colleagues demonstrated that cholecalciferol treatment for VDD human improves the maximal mitochondrial oxidative phosphorylation rate, which reflects mitochondrial numbers, oxidative enzyme content and mitochondrial components in skeletal muscle (Sinha *et al.,* 2013). Interestingly along with cellular metabolism, mitochondrial dysfunction is associated to the accumulation of reactive oxygen species (ROS) via an increase in NF-κB pathway and thought to be the underlining mechanism leading to muscle atrophy (Berridge *et al.,* 2016: Wimalawansa *et al.,* 2019: Wang *et al.,* 2015). Thus, suggesting a potential link between VDD and increased risk of metabolic diseases in adulthood. Indeed, mitochondrial dysfunction

has been associated with components of MetSyn (Sivitz *et al.,* 2010: Hulsmans *et al.,* 2012: Montezano *et al.,* 2015: Ahmad *et al.,*2017: Ghosh *et al.,* 2011: Spahis *et al.,* 2017). The findings here underscore the need for further research to better understand the intricate connections between VD, energy expenditure, adiposity, and related mechanisms to metabolic diseases.

3.5.2 Anxiety and memory

We conducted behavioural tests to observe changes in cognition and behaviour in VDD mice that has been VDD pre- and postnatally. Our study showed a significantly increased anxiety and impaired memory in VDD mice compared to controls. The EPM test showed a significant increase in anxiety in VDD group compared to controls: VDD mice spent more time in closed arms compared to controls indicating that they were more anxious and have less will to explore open spaces. It has been shown by studies that developmentally VDD rats exhibit behavioural changes in adulthood, which includes increased locomotor activity (Burne TH et al., 2004). This behaviour can also be due to changes in rat brain morphology and/or might be regulated by neurotransmitter systems such as dopamine (Kesby JP et al., 2010). In these studies, the Sprague-Dawley rats were the experimental animals and were put on a control diet at birth, contrary to our study. A negative emotional state might have led to less EPM activity in open arms, however, no significant changes in the ambulatory activity data of the VDD group suggests that the animals chose to spend time in the closed arms due to anxious behaviour. SA test confirmed a tendency towards impairment in memory and learning ability. Although not significant, VDD mice scored less

alternations (p.0.09) compared to controls. The results obtained from EPM, and a spontaneous alternation test favours our hypothesis on cognitive impairment in VDD. VD is essential for brain development and neurological health functioning as autocrine and paracrine neuro steroid hormone, through induction of many CNS genes, modulating neuroinflammation, neuroprotection and metabolism of the key neurotransmitters and enzymes. Behavioural alterations in animals and mood-elevating effects of DVD - deficiency are documented in the literature (O`Loan *et al.,* 2007). VDR-null mutation in mice demonstrated anxiety-like behaviour (Kalueff *et al.,* 2004). The presence of VDR in the limbic system and its associated functions suggests a potential mechanism for emotional changes seen in rodents. Our results are in line with these previous studies. Numerous studies have linked VDD to dementia and cognitive decline, however its' direct link to memory is largely unknown. Population-based studies demonstrated decline in visual memory in adults (Darwish *et al.,* 2015: Nagel *et al.,* 2015). Also, low levels of VD are correlated with faster memory loss and learning ability in older adults from various ethnic backgrounds (Miller *et al.,* 2015).

In addition to cognitive decline and behavioural changes, VDD causes brain structural abnormalities. Rodent studies have indicated that embryos from VDD pregnancies have enlarged lateral ventricles (Hawes *et al.,* 2015) possibly due to decreased levels of apoptosis, increase meiosis and decrease in the levels of the neurotrophic factors (Ko P *et al.,* 2004). Hippocampus has considerable importance for cognition and mood: In humans, posterior hippocampus is larger in individuals who require large capacity for spatial processing and memory such as taxi drivers (Maguire *et al.,* 2000).

Also, lesions of the ventral hippocampus alter emotional behaviour and stress resilience in rodents (Felix-Ortis and Tye, 2014). The mechanism behind the increased anxiety and impaired memory observed in our study can possibly be due to modified proliferation and differentiation of neurons within the hippocampus. This may be associated with changes in the methylation of genes, ultimately leading to perturbed anxiety and memory.

In this study serum VD levels were not measured; this means we cannot confirm that the dams were truly vitamin D deficient. There is evidence in humans that VD levels can be elevated for some time after supplementation (Martinaityte I et al., 2017), and therefore to validate these studies ELISA should have been performed to check VDD. However, this was not possible.

CHAPTER 4 Vitamin D deficiency at pre and postnatal stages combined alters adult neurogenesis in the offspring hippocampus

4.1 Background

Adult neurogenesis can be described as generation of new functional neuronal cells in the adult brain. Prior to 1960s, neurogenesis was believed to be exclusiveto only embryonic stages. Altman and Das in 1965, suggested the presence of neurogenesis in the adult brain. They used autoradiography to record a 6-fold increase in the incorporation of thymidine-H^3 into dividing cells of a rat DG in the sub granular zone (SGZ), from birth to 3 months (Altman and Das, 1965). Adult neurogenesis has been identified in these two main locations: In the DG, where differentiating cells migrate outwards into the granular layer and in the SVZ of lateral ventricles where neurogenesis provides constant turnover of interneurons in the olfactory bulb. In this study, KI67 and DCX (see 1.3.4.4 for more details) markers were used to label the cells at different stages of neurogenesis (active proliferation and differentiation) in the DG and SVZ of a VDD and control groups (**Figure 4.1**)

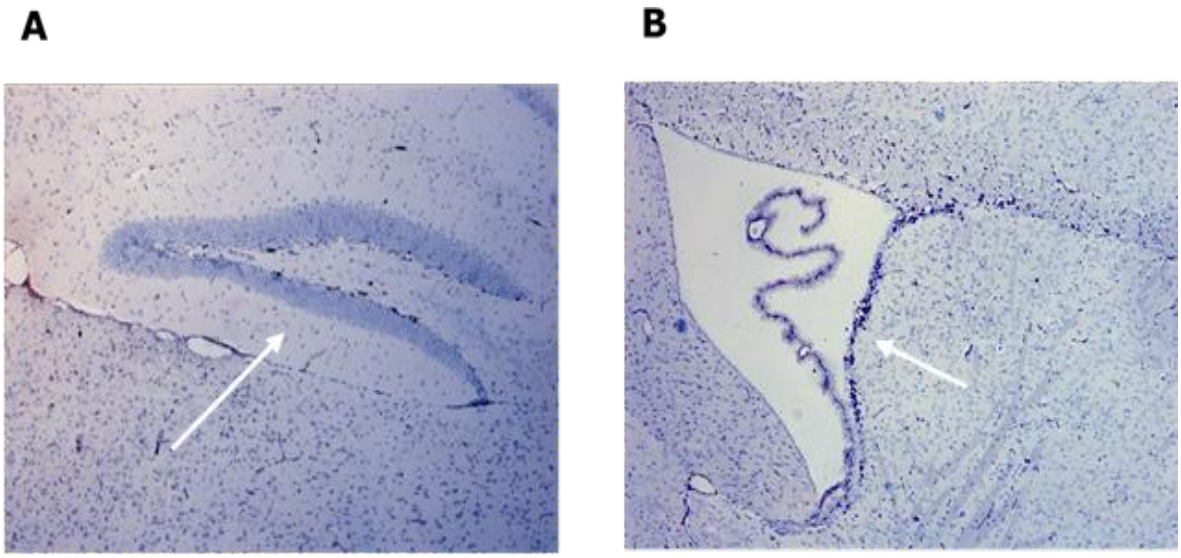


Figure 4.1 Figure showing staining in the Sub granular zone of the DG and SVZ. Dark brown cells are actively proliferating. Blue background from counterstainingwith Harris Haematoxylin. **A.** The sub granular zone of DG **B.** The SVZ of the striatum.

Functional outcomes of VDD and knock-out studies provide increasing evidence that VD has a role in regulating proliferation, differentiation, survival, and growth of the neurons in the developing and in adult brain (Groves *et al.,* 2014). Adult hypovitaminosis D has been shown to increase proliferation of neuroblasts in the SGZ of the hippocampus and alters neuronal differentiation, while supplementation with VD enhanced proliferation in cell cultures (Zhu *et al.,* 2012). Furthermore, Morello and colleagues (Morello *et al.,* 2018) showed improvements in neurogenesis following VD supplementation in mouse model of Alzheimer's disease, however, Groves and colleagues showed that adult VDD did not influence neurogenesis per se but changed the function of the newly formed neurones in the hippocampus leading to poor

cognitive outcomes (Groves *et al.,* 2013). Another study from Groves and colleagues demonstrated an increased proliferation and decreased level of survival of the adult-born neurons as well as alterations in differentiating ability of the immature neurons, using an adult VDD mice model (Groves *et al.,* 2016).

The following studies indicate that the apoptotic and proliferative effects of VDD in mice appear late in development. Ko and colleagues demonstrated that apoptosis significantly reduced at E21, but not at E19 or P7, suggesting that the effects of VDD is dependent on specific developmental stages (Ko *et al.,* 2004). In line with this finding, Zhu and colleagues reported an increase in apoptotic cell numbers inthe DG of 8 weeks old VDD mice compared to controls (Zhu *et al.,* 2012).

VDD's impact on neurogenesis leads to long-lasting alterations in brain morphology and behaviour in the adult offspring (Pet *et al.,* 2016). DevelopmentalVDD rodent (DVD) models showed impaired learning, memory, and behaviour (Byrne *et al.,* 2013). Offspring were introduced to VD in their diet at birth and theyreached their normal VD levels prior to weaning. They had altered brain structure:Longer, thinner cortex, enlarged lateral ventricles along with changes in the amounts of neurotrophic factors such as NGF and glial cell-derived neurotrophic factor (GDNF) was observed. There was a significant increase in the number of the proliferating cells at birth, more mitotic cells throughout the brain and cell differentiation were dysregulated (Eyles *et al.,* 2003). They confirmed that the timing of the mRNA and protein expression of VDR coincides with the terminationof mitosis and initiation of apoptosis (Ko P et al., 2004). The behavioural phenotype of DVD-deficient animals had altered hyperlocomotion

(Kesby *et al.,* 2006), deficitsin pre-pulse inhibition (the normal sensory response to a loud sound was attenuated) (Burne *et al.,* 2014), sensitivity to NMDA antagonists and altered sensitivity to anti-dopaminergic agents (O'Loan et al., 2007). The studies that showed spontaneous hyperlocomotion in rats in various behavioural tests including elevated plus maze, forced swim test, open field (Burne *et al.,* 2004; Kesby *et al.,* 2006) however, proved to be inconsistent (Becker A et al., 2005; CuiX et al., 2015). VDD *in utero* had significantly altered the expression of GABA, MAP-2 (microtubule-associated protein), NF-L (neurofilament light) and NGF at different stages of development (Feron *et al.,* 2005). NF-L and MAP-2 are indicators of neural function and structure. NF-L gene is the key component of nerves responsible for signal transmission and are the by-product of nerve cell degeneration. MAP-2 gene, on the other hand, are neuron-specific protein responsible in determining and maintaining neuronal morphology. The decrease in NF-L and MAP-2 gene expressions at birth and at weaning compared to controls may suggest that early neural differentiation and maturation processes are altered upon maternal VDD.

VDD is a plausible risk factor for adverse brain outcomes in humans (Ginde *et al.,* 2009) as well as rodents (Groves *et al.,* 2014) as validated by the growing body of evidence from epidemiological studies which associated VDD to several neuropsychiatric and neurodegenerative disorders. Brain structure and function is altered in schizophrenia. The behavioural phenotype of DVD- deficient rats share similar phenotype to schizophrenia. Low maternal VD has been proposed as a potential risk factor for increased risk for the diseases and has been considered in schizophrenia research (McGrath *et al.,* 1999). In addition, impaired metabolism of

neurotransmitters and altered GABAergic function (Li *et al.,* 2016) are implicatedin mental disorders thus, involvement of VD in these suggests that VD can be beneficial in the pathology of the neuropsychiatric and neurodegenerative disorders (see 1.1.3.2 for more detail).

Our previous results showed that VD affects the behaviour and cognition of the offspring born from the VDD dams. We hypothesized that this might be due to the changes in proliferation and differentiation rates in the brain. This current chapter investigate the changes in adult neurogenesis of VDD mice.

4.2 Aims and objectives

The aim of this study was to investigate the impact of VDD on adult neurogenesis and gene expression of the offspring that has been on a VDD diet since conception. The two markers of neurogenesis, Ki-67 and DCX were used to assess cell proliferation and differentiation respectively. Gene expression analysis was also conducted to assess the expression levels of two neurotrophic factors crucial for neurogenesis: VGF and BDNF in the hippocampus and hypothalamus. Hippocampus and hypothalamus are the two areas where adult neurogenesis occurs.

We hypothesized that the VDD mice will have decreased Ki-67 and DCX immunoreactivity as an indication of decreased cell proliferation and differentiationin both SVZ and DG of hippocampus, decreased gene expression of VGF and BDNF in both tissues.

4.3 Methods and Materials

4.3.1 *Ex vivo* tissue analysis

4.3.1.1 Dissection and tissue processing

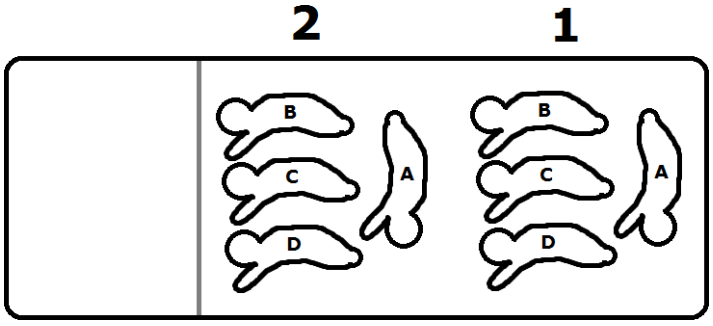
A total of 30 offspring C57BL/6J mice were used for the experiments in this chapter (6 dams and 6-week-old mice were not used here due to limitations). The 3-week-old female VDD mice (N=4) and control (N=8) and the 8-week-old male VDD mice (N=6) and control (N=12) mice are used for this experiment.

It is important to note the gender difference between the study groups throughout the study.

The brain was dissected, collected and snap frozen and stored at -80□C for *ex vivo* analysis. The brain was cut in half; one half was fixed in 4% Paraformaldehyde (PFA) for immunostaining and the other half was dissected into frontal cortex, striatum, hypothalamus, pituitary, hippocampus, amygdala, cortex, and cerebellum for further gene expression analysis. The brain tissues collected, hypothalamus and half of the hippocampus were snap frozen on dry ice to be used in gene expression analysis. Other half of the hippocampus was transferred to 4% PFA solution for 24 hours, the next day transferred and preserved in Phosphate-buffered saline (PBS) supplemented with 0.1% Sodium Azide (*NaN3)*.

4.3.1.2 Immunostaining procedure

The part of the brain which had been fixed in 4% PFA solution was processed in a Leica tissue processor for paraffin embedding. The procedure was as follows: Incubation in 70%, 80% and 96% alcohol for 90 minutes each, followed by three 60-minute incubations in 100% alcohol. Then, two incubations of 3 hours in Chloroform before two incubations of 2 hours in molten paraffin wax. Each paraffin wax block contained up to four tissues of the same treatment group (VDD or control). The ID of the animals was recorded. Tissues were sectioned using a microtome (Leica Microsystems RM2245, UK) at 7 µm thickness. Brain sections were mounted on 3-Aminopropyltriethoxysilane (APES)-coated glass slides and let dry on heat blocks. Each slide contained two sections of each brain, for example tissue samples 1A and 2A from the same brain on one glass slide as shown below.



Immunostaining was conducted according to the following protocol: The tissues were deparaffinised and re-hydrated by incubating with Xylene, 100% ethanol, 70% ethanol, 50% ethanol and rinsed in dH2O twice for 5 minutes respectively. The slides were placed in buffer (citric acid pH 6, 0.05% triton) in the water bath at 98C for 20 minutes for antigen retrieval. Then, cooled at RT around ~15 minutes. The slides were

washed 2 times with dH_2O. Once cooled, the area around the tissues were delineated using a liquid blocker pen and slides were covered with PBS until further process. Once all the glass slides were delineated endogenous peroxidase activity was removed by incubating with 0.5% Hydrogen Peroxide (H2O2) in PBS for 10 minutes. Afterwards, the slides were washed 2 times in PBS (5 min each). Sections were incubated in blocking solution (2% of secondary antibody animal serum (rabbit/donkey) PBS + 1% bovine serum albumin (BSA) (100mg) + 0.3% triton 300ul 10% triton) for an hour at room temperature, then incubated in 1st antibody diluted in day1 buffer **(Table 4.1)** at 4°C overnight. The next day, sections were washed twice for 5 minutes in 0.3% BSA, 0.1% triton X-100 in PBS and incubated with 2nd antibody diluted in day 2 buffer **(table 4.1)** for an hour at RT. Afterwards, they were washed with PBS + 0.3% BSA (30mg) + 0.1% triton 100ul 10% triton repeated twice for 5 minutes. Sectioned were then incubated in Avidin-biotin-horseradish pre oxidase reagent (ABC) (Thermofischer UK) for an hour at RT. Then washed with 10 ml PBS + 0.3% BSA (30mg) + 0.1% triton 100ul 10% triton 1 time for 5 minutes and rinsed with PBS twice for 5 minutes. The staining was developed using DAB solution (3,3'-diaminobenzidine) (1 ml DAB (Thermofischer UK) +19ml PBS and 6.6ul 30% H2O2). Counterstaining was performed in following order; 1 minute in Haematoxylin, 5-7 seconds in Acetic Alcohol, 10-15 seconds in Scotts Water then 10 seconds followed by 50%, 70%, 90%, and 100% (x2) alcohol respectively for dehydration. The sections were rinsed twice for 2 minutes in Xylene. Slides were cover-slipped by DPX-mounting media.

Buffers and antibodies:

Citrate buffer – 10mM sodium citrate buffer solution with a pH of 6 made up with 10mM citric acid.

Day 1 buffer - 10 ml PBS (phosphate buffered saline) + 1% BSA (bovine serum albumin) (100mg) + 0.3% triton 300ul 10% triton

Day 2 buffer- 1 in 3 dilution of day 1 buffer with PBS DAB (Sigma, UK) solution: 19ml PBS and 6.6ul 30% H_2O_2 in 1 ml DAB aliquots

	Doublecortin	**Ki-67**
Serum	2% Rabbit serum	2% Donkey serum
Primary Antibody	Sheep anti DCX (1:200) (Santa Cruz **#** 8066)	Mouse anti Ki67 (1:100) Invitrogen **Cat #** VPK 452)
Secondary Antibody	Rabbit anti sheep (1:200, Invitrogen **Cat #** 31480)	Horse anti mouse (1:200, Invitrogen **Cat #** 31181)

Table 4.1. Table showing the antibodies used for immunostaining with DCX andKi-67 neurogenesis markers.

4.3.1.3 Cell counting and Imaging

DCX+ and Ki-67+ cells in the DG were counted using Axioplan light microscope with Zeiss LSM 880 confocal system. The people counting the slides were blindedto the identity of the treatment. Four sections per animal were used for quantification. Images were analysed using ImageJ 1.43 (National Institutes of Health, Bethesda, Maryland, USA). The DG and SVZ were identified and traced using Image J software a 5X lens. The area was then calculated. The positive stained cells were counted using 40x objective.

Cell number and the area (mm^2) was used to determine cells/mm^2 using

$$Cells/mm^2 = (Cell\ count \div area\ (nm^2)) \times 1{,}000{,}000$$

The cell count for all brain sections were averaged for each slide to obtain the results for each animal.

4.3.2 Nucleic acid extractions and Procedures

4.3.2.1 Primer Design

The gene sequences of the primers of interest were obtained from NCBI. Afterwards, the primers were designed by using the Primer 3 and Serial Cloner software. Amplifx software was used to check *in silico* if the primers have any dimers in order to select the intron-spanning amplicons so that mRNA can be differentiated from gDNA amplifications. Then, they were checked whether the sequence has introns by blasting the sequence (http://blast.ncbi.nlm.nih.gov/). The desired primer sequences were chosen depending on length, nucleotide composition and the primer-template match.

Gene sequences of genes of interest:

BDNF	**F**	5′ ggtatccaaaggccaactga3′
	R	5’ cttatgaatcgccagccaat3′
VGF	**F**	5′ catccgtcctcttctgcttc3′
	R	5’ctgaggggaggaggaaaaac3′

4.3.2.2 Nucleic acid extraction

RNA extraction from the hippocampal and hypothalamic brain tissues were performed with TRIzol Reagent (Invitrogen, Carlsbad, California, USA)., following the manufacturers protocol, as follows: the tissues were weighted and the amount of TRIzol used was adjusted accordingly. As tissues weighed less then 25 mg, we used 0.8ml of TRIzol reagent and they were left at room temperature for 5 minutes. 320µl of 1-bromo-3-chloropropane was added and the tubes were shaken for 15 minutes then left at room temperature for further 10-15 minutes. Then, they were centrifuged at 4°C and 12,000g for 15 minutes for phase separation. The clear aqueous phase was transferred into a new tube. To each tube, 0.5ml isopropanol and 1µl glycogen was added and mixed. After 10 minutes at room temperature, they were centrifuged again at 4°C and 12,000g for 15 minutes. The supernatant was discarded. The RNA pellet was collected and washed with 0.8ml ice cold 75% ethanol followed by centrifuging at 4°C and 12,000g for 10 more minutes. Finally, the supernatant was discarded, and the pellet left to air dry. After air-drying, 50µl of RNAse-free water was added to dissolve the RNA pellet. The samples were lastly incubated for about 10 minutes at 50-60°C to help dissolve the RNA. The concentration and purity of the RNA samples were determined using Nanodrop UV-Vis Spectrophotometer 2000 (Thermofisher, UK). Further ethanol precipitation was performed in order to increase the quality of the RNA if needed. In order to do this, solutions were added 1/10 volume of Sodium Acetate (NaAct) with 2 volumes of 100% Ethanol and kept at -20 °C overnight for precipitation. They were further washed with 75% Ethanol the next day, supernatants were discarded, and the precipitate were kept in EB/RNA (Thermofisher,

UK 10 mM Tris-HCl, pH 7.5) buffer. The concentration and purity ofthe RNA samples were checked again. Meanwhile, RNA was stored at -80C.

4.3.2.3 Reverse Transcription, PCR

Firstly, to ensure any contaminating DNA was destroyed, DNase treatment was conducted. For every sample, 1µl DNase buffer and 1µl DNase I enzyme from the Invitrogen DNase I kit were added and DPEC treated water (Thermofisher, UK, Cat: AM9906) to make the total volume of 10µl. Then samples were incubated for 15 minutes at room temperature. Afterwards, 1µl EDTA was added to each sample and the samples were heated in the PCR thermocycler for 10 minutes until to 65°C and immediately cooled by placing onto ice for deactivating the enzyme. 1 ng of the target RNA was reverse transcribed using the Superscript III reverse transcriptase enzyme (Life Technologies, UK) and primers (Sigma, UK) according to the manufacturer's instructions: First, 1.25µl of dNTP (0.5mM) and 3.75µl N15 oligonucleotides (0.52µg) to each sample and the total volume of 17.25µl by adding DPEC-treated H_2O.Samples were heated to 65°C for 5 minutes before being snap cooled on ice for 1 minute. They were then spun down and kept on ice. 5µl 1x first strand buffer, 1.25µl DTT (5µM), 0.5µl RnaseIn (20U) and 1µl Superscript™ III (200U) from the Invitrogen Superscript™ III reverse transcriptase kit was added per each sample. The total volume reached to 25 µl. Each sample was mixed by shaking gently and they were placed into PCR machine (Corbett research): 25°C for 5 minutes without the lid on, 60 minutes with the lid at 95°C and finally 70°C for 15 minutes.

For regular PCR amplification, the protocol consisted of 35 cycles of 94°C for 2minutes 20 s (denaturation), 57°C for 30s (annealing), and 72°C for 2 minutes 30s (extension). cDNA samples were stored at -20° until use.

4.3.2.4 Agarose gel electrophoresis

After PCR amplifications, samples were separated in gel electrophoresis on a %2 Agarose gel (0.9 g of agarose, 45 ml of 1xTBE, 4.5 µL of SYBR safe SYBR® Safe DNAGel Stain, Thermofisher, UK), electrophoresis was performed at 90W for 20 minutes in 1x Tris-Borate-EDTA (TBE) at room temperature. The gel was visualized under the short-wavelength UV light for detection. Primers giving bands indicated the presence orabsence of the knock in\knock/out.

4.3.2.5 QPCR and analysis

qPCR reactions were performed using Rotor Gene 6000 cycler (Corbett Research) and in triplicates. Reactions were performed on ice and used immediately.

qPCR was performed by using 7.5µl enzyme by SYBR® Green JumpStart™ Taq ReadyMix™ and mixed with RNAse-free water to a total of 15µl per reaction. qPCR reactions performed using SYBR® Green JumpStart™ Taq ReadyMix™ for Quantitative PCR by Sigma-Aldrich. After primer efficiency was calculated, relative expression was calculated. Q-PCR amplification: initial denaturation at 95C for 10 minutes, 40 cycles starting with denaturation at 95C for 15 seconds, annealing temperature (***Table 4.2***) for 20 seconds, and elongation at 72C for 30 seconds. Melt curve analysis were used with ramp rate of 72°C to 95°C with 1°C per step. The details of the genes can be found in ***Table 4.2.***

	Primer Sequence/ Gene bank no	Concentration	T_m	Dilution
Genes of interest				
BDNF	**F** 5′ ggtatccaaaggccaactga3′ **R** 5’ cttatgaatcgccagccaat3′	500 mM	63°C	1:16
VGF	**F** 5′ catccgtcctcttctgcttc3′ **R** 5’ctgaggggaggaggaaaaac3′	200 mM	62°C	1:64
Housekeeping genes				
Hprt1	**F** 5′ cgaggagtcctgttgatgttgc 3′ **R** 5′ ctggcctataggctcatagtgc 3′	500 mM	63°C	1:64
Pgk1	**F** 5′ tagtggctgagatgtggcacag3′ **R** 5′ gctcacttcctttctcaggcag 3′	500 mM	63°C	1:64
CyclA	**F** 5′ ggcaagttcaatggcacagt 3′ **R** 5′ tggtgaagacgccagtagactc3′	500 mM	63°C	1:16

Table 4.2 The information of the genes that were used to analyse the mRNA expression level in hippocampus and hypothalamus in this study. Each row shows the gene name, the information of the primer sequence used, the primer concentration and the cycling temperature used.

4.3.2.6 RNA quality assessment and quantification

A pool was created using all samples for primer calibration by using 1µl from each sample and mixing them in one main tube, creating further dilutions of 1:4 (1:4 to 1:1024). For dilutions used for each primer see ***Table 4.2.***

Primer's efficiency and specificity were verified using melting curve analysis (single-melt curve peak). CT for each sample and the primer efficiency was analysed using Rotor-Gene software analysis. The relative expression levels of each mRNA were calculated by $CT\text{^}((Group\ average) - (Efficiency + 1))$ (Pfaffl, 2001). Gene expression was normalized to the geometric average of three control genes (*Hprt1, Pgk1 and CyclA*) according to the GeNorm normalization: Normalisation factors were calculated for each sample by

$$\frac{Geometric\ mean\ of\ HKG\ for\ sample}{Geometric\ mean\ of\ all\ samples\ \&\ HKGs}$$

(Vandesompele *et al.*, 2002). Finally, the normalised expression of each sample was calculated as $\frac{Relative\ expression}{Normalisation\ factor}$

4.3.3 Statistics

The statistical software used was Prism (Graph pad version 7, USA) for Windows. All data was checked for normality using D`Agostino test. The analysis was done using an unpaired student t-test and two-way ANOVA where necessary.

Unpaired t-tests were used when comparing two groups with parametric data and Mann Whitney for non-parametric data or if not normally distributed. All data was represented as mean ±S.E.M. The significance threshold was setat $p < 0.05$.

4.4 Results

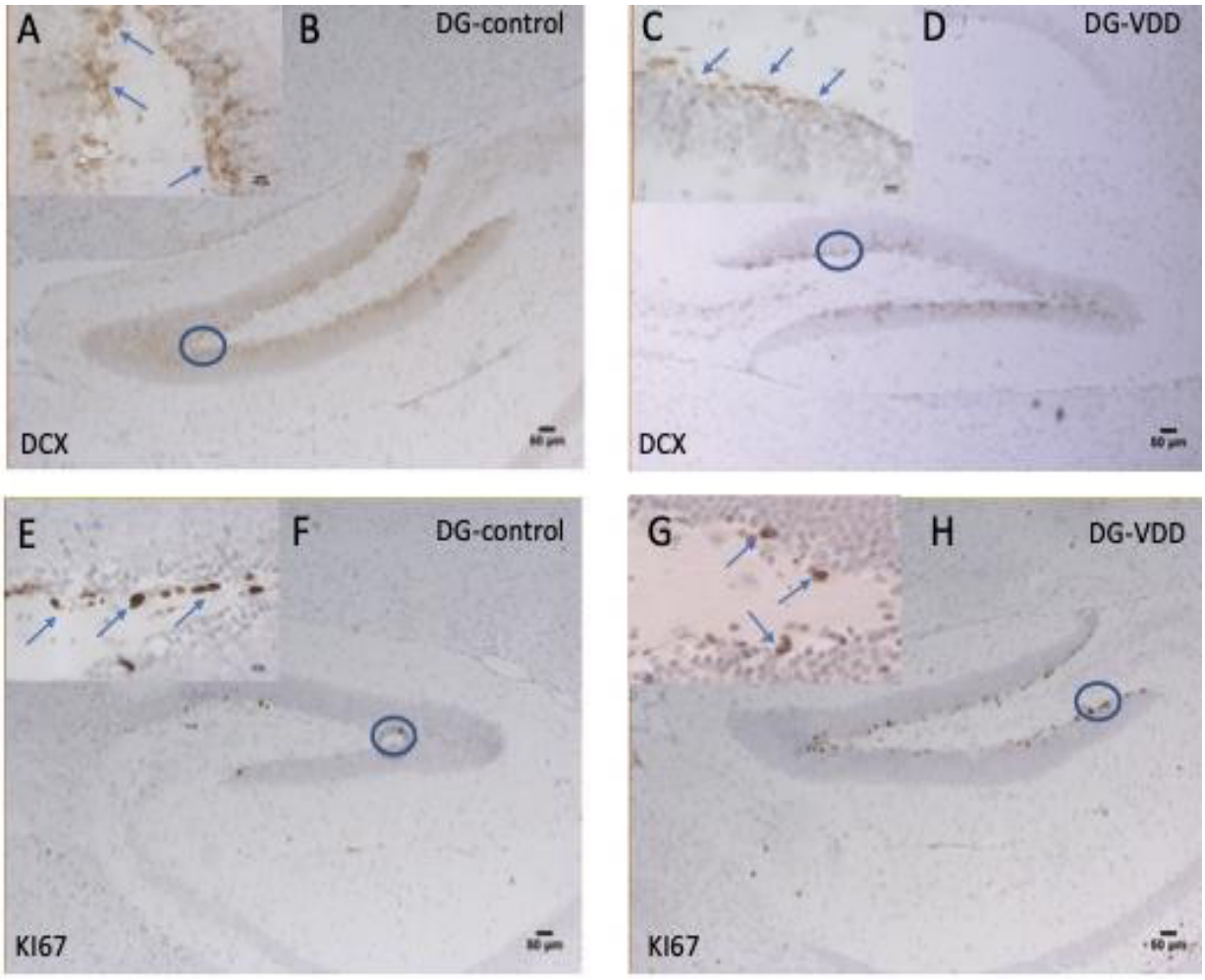


Figure 4.2 Images of neurogenesis staining from VD control (A,B-E,F) and from VDD (C,D-G,H) mice. Circles represent the corresponding magnified area. (A-C) A DCX-stained DG visualized under the microscope at 40x magnification, scale bar 50μm. (B-D) 5x magnification, scale bar 500μm. DCX-stained cells exhibited a brown staining of the cytoplasm and cell membrane. (E-G) A Ki-67-stained DG visualized under the microscope at 40x magnification, scale bar 50μm. (F-H) 5x magnification, scale bar 500μm. Ki-67-stained cells express brown staining in the nuclei. Background staining isthe artefact of the Harris Haematoxylin staining. Blue arrows show the dark brown stained cells.

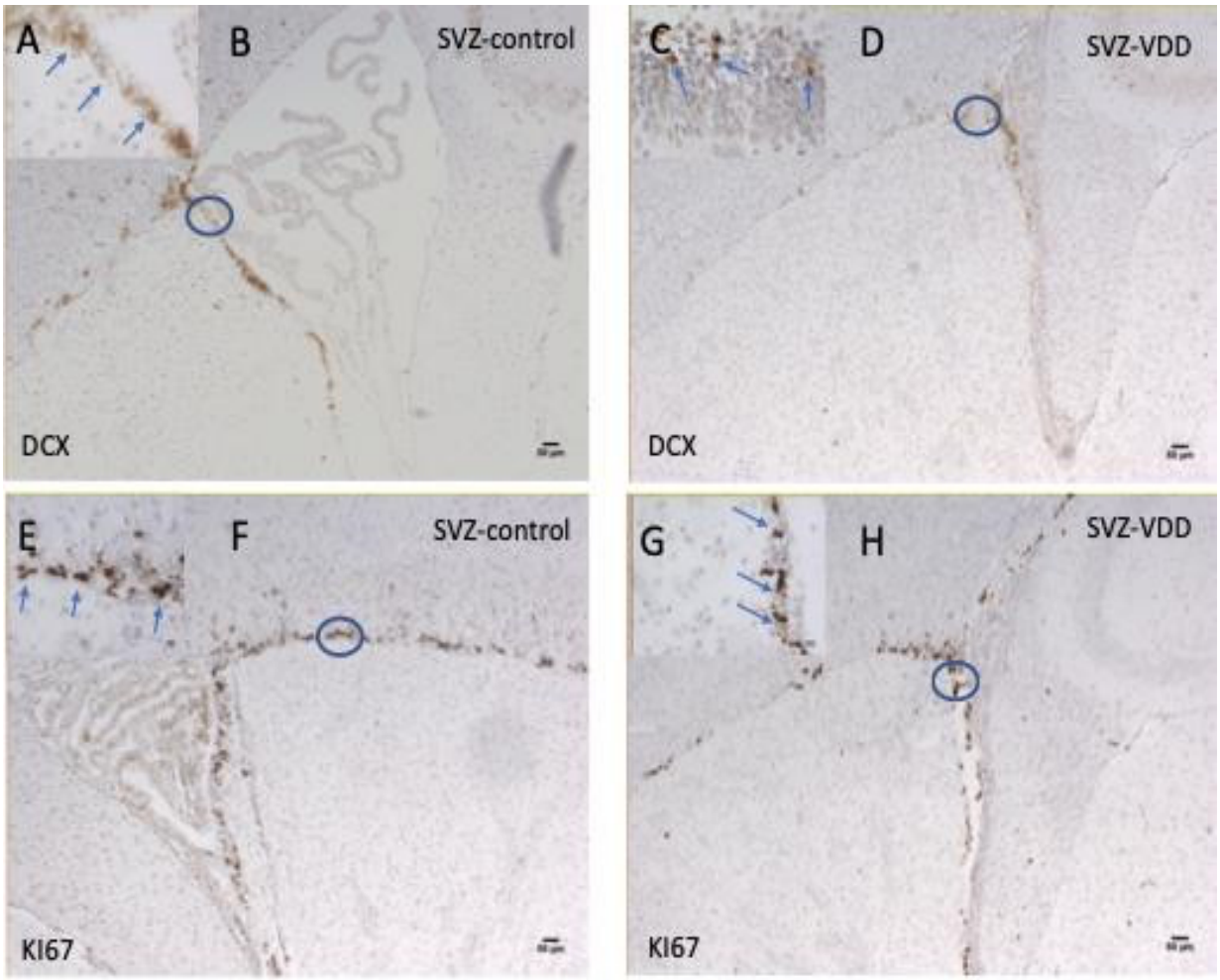


Figure 4.3 Images of neurogenesis staining from VD control (A,B-E,F) and from VDD (C,D-G,H) mice. Circles represent the corresponding magnified area. (A-C) A DCX-stained SVZ visualized under the microscope at 40x magnification, scale bar 50μm. (B-D) 5x magnification, scale bar 500μm. DCX-stained cells exhibited a brown staining of the cytoplasm and cell membrane. (E-G) A Ki-67-stained SVZ visualized under the microscope at 40x magnification, scale bar 50μm. (F-H) 5x magnification, scale bar 500μm. Ki-67-stained cells express brown staining in the nuclei. Background staining isthe artefact of the Harris Haematoxylin staining. Dark brown colour represents the stained cells of which the arrows show.

4.4.1 VDD at PPS affects the neuronal cell proliferation and differentiation in dentate gyrus of the hippocampus and SVZ of lateral ventricles at 3 weeks of age

VDD had no significant effect on the number of proliferating (Ki67 positive) cells/mm^2 quantified in the DG of the 3 weeks old female VDD mice (**Fig4.4 A.**t (10) =0.4663 p=0.6510) compared to controls. However, in SVZ, the number of Ki67 positive cells/mm^2 significantly decreased in 3 weeks old female VDD mice (**Fig4.4 B**. t (10) =3.244 p=0.0088) compared to controls.

The number of immature neuronal (DCX positive) cells/mm^2 significantly decreased in both the DG **(Fig4.4 C**. t (10) =*4*.554 **p=0.0011) and SVZ (**Fig4.4 D**. t (11) =5.275 ***p=0.0003) of the 3 weeks old VDD mice compared to controls. The decrease in the DCX positive cells/mm^2 SVZ was more pronounced than in the DG.

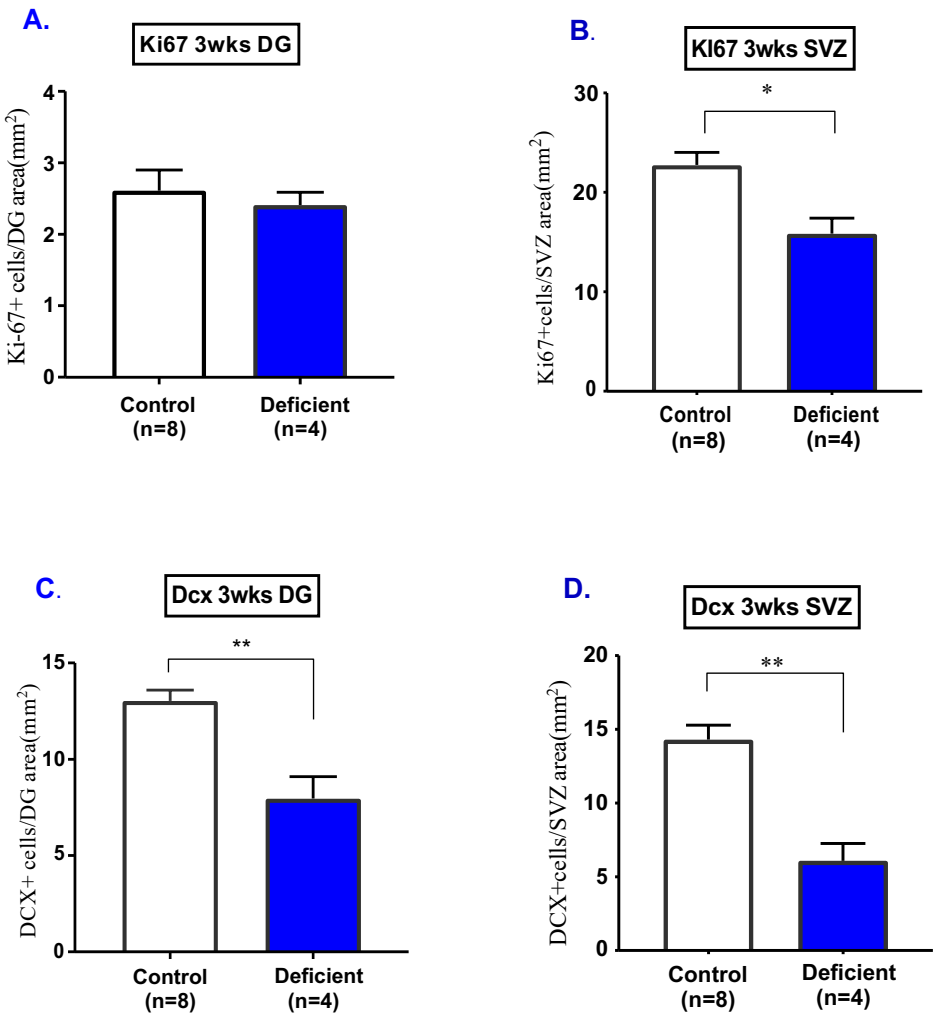


Figure 4.4 Data showing the effect of VDD on the number of Ki67 and DCX stained cells in dentate gyrus and SVZ of 3 weeks old female VDD mice (N=4) compared to controls (N=8). Values represented as mean ± SEM. **A.** Number of Ki67 stained cells/mm^2 in the dentate gyrus **B.** Number of Ki67 stained cells/mm^2in the SVZ (**p=<0.01) **C.** Number of DCX stained cells/mm^2 in the dentate gyrus(**p=<0.01) **D.** Number of DCX stained cells/mm^2 in the SVZ (***p=<0.001)

4.4.2 VDD at PPS affects the neuronal cell proliferation and differentiation in the dentate gyrus of the hippocampus and SVZ of Lateral ventricles at 8weeks of age

The number of proliferating (Ki67 positive) cells/mm^2 quantified in the DG of the 8 weeks old male VDD mice tended to decrease (**Fig 4.5 A.** t (16) =0.6002 p=0.5568) compared to controls. In SVZ, the number of Ki67 positive cells/mm^2 significantly increased in 8 weeks old male VDD mice (**Fig 4.5 B**. t (15) =3.304 **p=0.0048) compared to controls.

VDD had no significant effect on the number of immature neuronal (DCX positive) cells/mm^2 in the DG of the 8 weeks old male VDD mice (**Fig 4.5 C.** t (16) =0.5792 p=0.5705) compared to controls. However, this number significantly increased in the SVZ of 8 weeks old male VDD mice (**Fig 4.5 D**. t (15) =2.485 *p=0.0252) compared to controls.

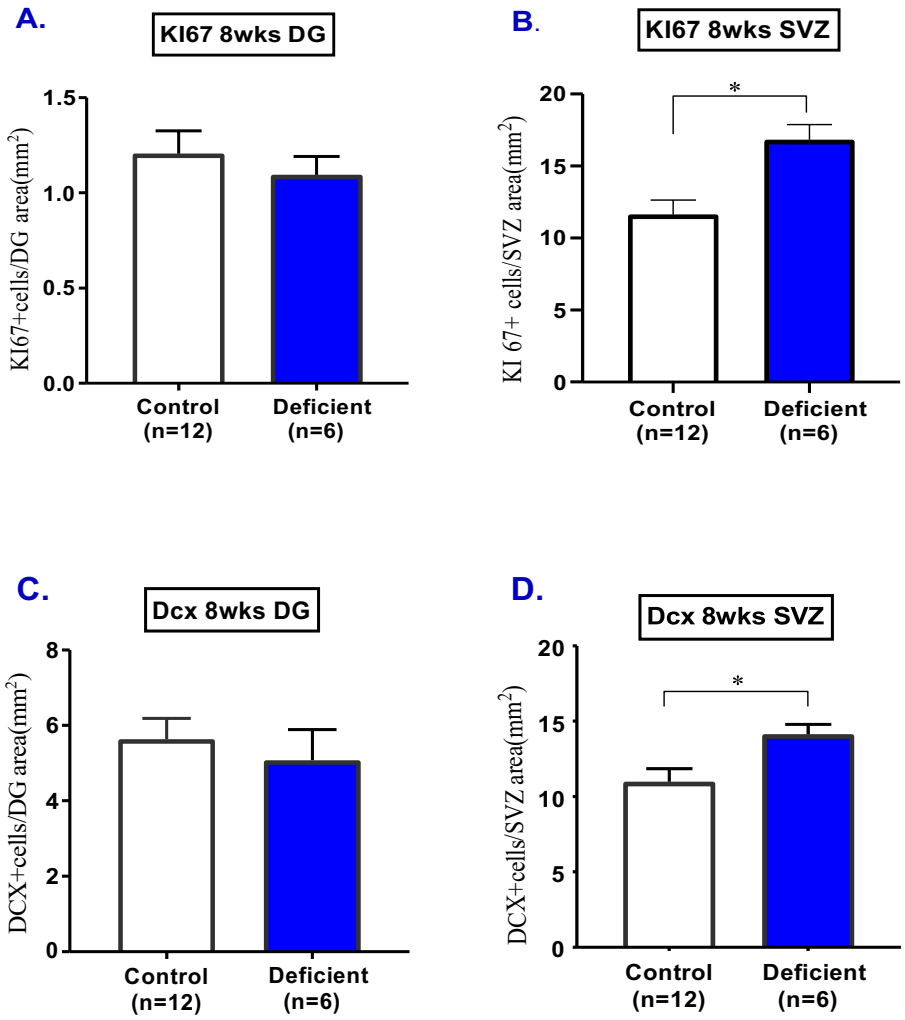


Figure 4.5 Data showing the effect of VDD on the number of Ki67 and DCX stained cells in dentate gyrus and SVZ of 8 weeks old male VDD mice (N=6) compared to controls (N=12). Values represented as mean ± SEM. **A.** Number ofKi67 stained cells/mm^2 in the dentate gyrus **B.** Number of Ki67 stained cells/mm^2 in the SVZ (**p=<0.01) **C.** Number of DCX stained cells/mm^2 in the dentate gyrus **D.** Number of DCX stained cells/mm^2 in the SVZ (*p=<0.05)

4.4.3 VDD at PPS alters the mRNA expression of the neurotrophic factorsVGF and BDNF in the hippocampus and hypothalamus at 3 weeks of age

VDD had no significant effect on the mRNA expression of the VGF in the hippocampus of 3 weeks old female VDD mice (**Fig 4.6 A.** t (1) =0.7444 p=0.4738)compared to controls. However, in the hypothalamus, VDD led to a significant decrease in VGF mRNA expression (**Fig 4.6 B.** t (10) =3.503 p=0.0057) comparedto controls.

VDD had no significant effect on the mRNA expression of the BDNF in both the hippocampus (**Fig 4.6 C.** t (10) =0.6339 p=0.5404) and the hypothalamus (**Fig 4.6 D.** t (10) =0.5599 p=0.5879) of 3 weeks old female VDD mice compared to controls.

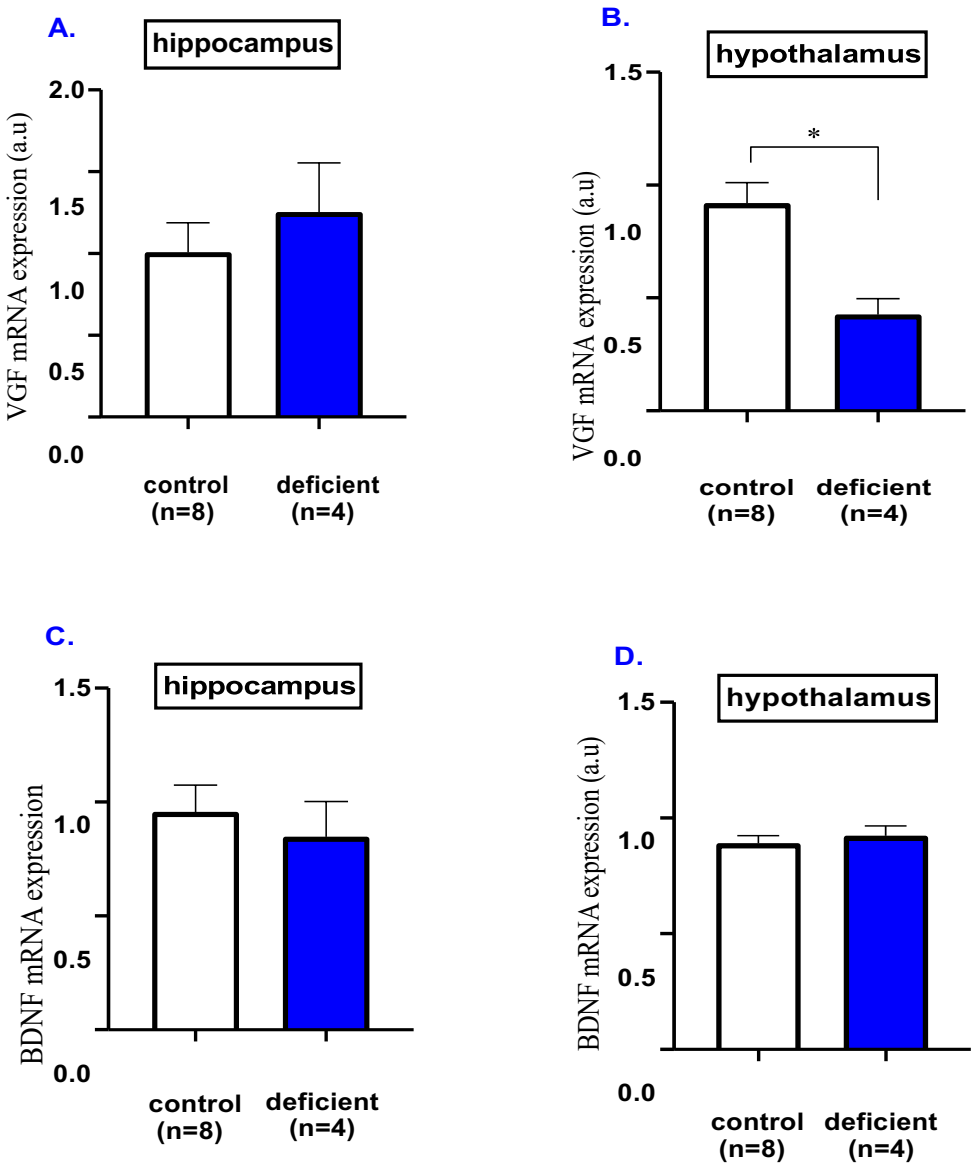


Figure 4.6 Data showing the normalised expression of VGF and BDNF genes in the hippocampus and hypothalamus of 3 weeks old female mice that have been VDD (N=4) and control (N=8) diet. Values represented as mean ± SEM. **A.** VGF mRNA expression levels in the hippocampus at 3 weeks **B.** VGF mRNA expression levels in the hypothalamus at 3 weeks (**p=<0.01) **C.** BDNF mRNA expression levels in the hippocampus at 3 weeks **D**. BDNF mRNA expression levels in the hypothalamus at 3 weeks.

4.4.4 VDD at PPS alters the mRNA expression of the neurotrophic factorsVGF and BDNF in the hippocampus and hypothalamus at 8 weeks of age

The VGF mRNA expression in both the hippocampus (**Fig 4.7 A.** t (15) =8.383 p < 0.0001) and hypothalamus (**Fig 4.7 B.** t (16) =6.327 p < 0.0001) of 3 weeks old female mice had significantly increased compared to controls.

VDD led to a significant increase in BDNF mRNA expression in the hippocampus of 3 weeks old female mice (**Fig 4.7 C.** t (16) =3.304 p=0.0045) compared to controls. VDD also led to a significant increase in BDNF in the hypothalamus of 3 weeks old female mice **(Fig 4.7 D.** t (14) =2.524 *p=0.0243) compared to controls.

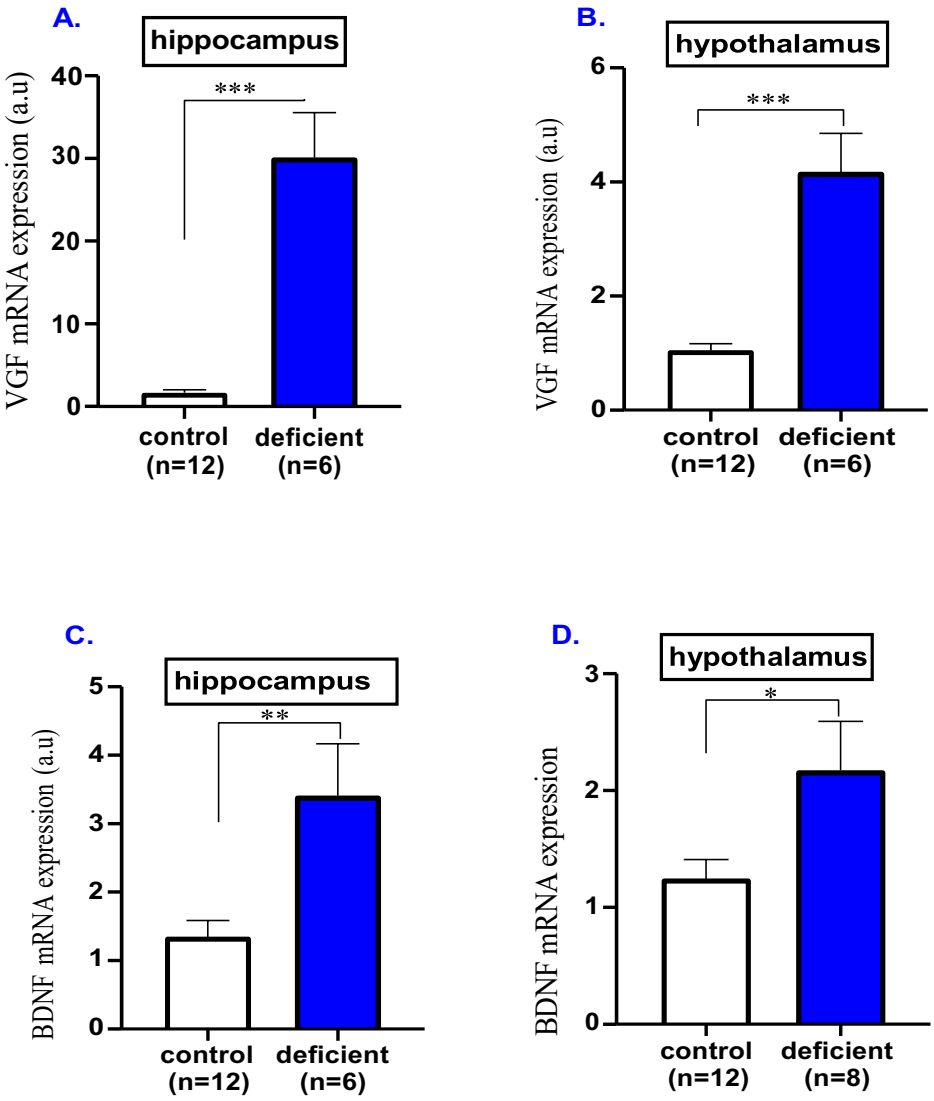


Figure 4.7 Data showing the normalised expression of VGF and BDNF genes in the hippocampus and hypothalamus of 8 weeks old male mice that has been VDD (N=6) and control (N=12) diet. Values represented as mean ± SEM. **A.** VGF mRNA expression levels in the hippocampus at 8 weeks **B.** VGF mRNA expression levels inthe hypothalamus at 8 weeks (**p=<0.01) **C.** BDNF mRNA expression levels in the hippocampus at 8 weeks(***p=<0.001) **D.** BDNF mRNA expression levels in the hypothalamus at 8 weeks (*p=<0.05)

4.5 Discussion

The present study investigated the impact of VDD at PPS on (i) adult neurogenesis by quantifying cells expressing proliferation and differentiation markers, KI 67 and DCX, in SVZ and DG of hippocampus of 3 weeks old female and 8 weeks old male mice (ii) levelsof mRNA expression of neurotrophic factors, VGF and BDNF in the hippocampus and hypothalamus of 3 weeks old female and 8 weeks old male mice. We showed that VDD at PPS alter the rates of neuronal cell proliferation and differentiation in both DG and SVZof the offspring. In addition, we showed that VDD at PPS alters the gene expression of neurotrophic factors, VGF and BDNF in both hippocampus and hypothalamus.

4.5.1 VDD at PPS alters adult neurogenesis in DG and SVZ

Here, we used markers of neurogenesis, Ki-67 and DCX in both SVZ of lateral ventriclesand DG in hippocampus, the two regions where neurogenesis occurs in the brain and VDis abundantly expressed. Ki-67 is an endogenous marker that marks the proliferating cells, expressed during mitosis. Ki-67 immunoreactivity is in the nuclei of the cells. The expression of DCX is developmentally regulated, its expression is specific to newly generated neurons.

Our results showed that there was no difference in the number of Ki-67 positive cells in all the experimental groups in the DG. No change in the number of the Ki-67 marked cells suggests that there was no effect of VDD at PPS on cell proliferation in DG.VD's effect on cell proliferation has been contradictory to this date. Eyles and colleagues

have found that developmentally VDD rats had enhanced number of cell proliferation at birth and Zhu and colleagues showed that adult hypovitaminosis D_3 also enhanced proliferation in hippocampus (Eyles *et al.,* 2003; Zhu *et al.,* 2012) which contradicts our findings. Another study reported inhibited hippocampal cell proliferation in rats (Eyles *et al.,* 2003) which also contradicts to our findings. An *in vivo* study failed to report any impact on adult cell proliferation in mice which agrees with our findings. These mice were 10 weeks old (Groves *et al.,* 2016). It is important to note that both studies listed above which reported an enhanced proliferation rate were in rats rather thanin mice. Regarding the effects of VDD, differences between species and strains of the animal models has been reported (Harms *et al.,* 2008). Furthermore, our animals were VDD at both pre and postnatal stages until the age of 3 and 8 weeks which differs from the studies above. The developing hippocampus may be capable of compensating for thelack of VD postnatally, as the current literature suggests, where VDD increased proliferation prenatally, but not postnatally (Ko *et al.,* 2004). In the SVZ, we saw that VDD at PPS led to a significant decrease at 3 weeks of age of proliferation. At8 weeks, we observed a significant increase in proliferation (Ki-6) immunoreactivity However, we cannot confirm this is an age effect, due to sex differences between the groups. Cui and colleagues showed that VDR is concentrated particularly in SVZ and present in the cultures prepared from the neonatal SVZ (Cui *et al.,* 2007). Although the same study reported an increase in neutrosphere production at postnatal day 1 which contradicts our results, it is clearly shown that VD regulates proliferation in the SVZ. However, these results are based on rats instead of mice and the experiments is on the developing brain, not in adulthood. In maternally VDD mice, the rate of proliferation was increased compared to controls only at E19

and E21, but not E23 and P7. These findings and our results collectively might suggest that the brain might use adaptive mechanisms to adapt and normalise the effect in adulthood. We have shown that DCX positive cell numbers were significantly decreased by VDD at PPS in 3 weeks old offspring. There is no significant effect at 8 weeks in DG. Although this difference may be due to the different sexes of the two groups. In the SVZ, we saw an even more pronounced significant decrease at 3 weeks compared to DG. At 8 weeks,there was a significant increase unlike the effect seen in DG. VD is known to have a pro-differentiation activity (Garcion *et al.,* 2003). DCX marks immature neurons up to the first 28 days after differentiation. It is expected that VDD animals to have a significantly decreased DCX marked cell numbers which agrees with our initial findings at 3 weeks of age. Groves and colleagues reported no significant effect of adult VDD on the number of DCX positive cells, cell differentiation (Groves *et al.,* 2016) which contradicts to our findings. On the other hand, the adult VDD led to a decrease in NGF levels in the DG of hippocampus (Zhu *et al.,* 2012: Becker *et al.,* 2005), which might lead to the apoptosis ofthe new-born cells. The findings of these two studies suggest that the effects of VDD on the DG might predominantly be in the early stages of development as neurogenesis was not interrupted at 10 weeks (Groves *et al.,* 2016) in the DG. This is in line with our results. Additionally, our findings at 8 weeks of age remind the compensatory mechanism of the brain. Our study used C57BL/6 mouse strain which differs from the two studies mentioned above. Indeed, Kempermann and colleagues has shown that mice strains differ in their basal rate of adult DG neurogenesis (Kempermann *et al.,* 1997).

There was a limitation to our study. While the 3 weeks old mice in our study were females,8 weeks old mice were males. VDD's effects on males and females are largely

unknown.However, we know that VDD is more common in women (Calabrese *et al.,* 2006). Women with low serum VD levels were found to have higher risk of developing major depression than man (Bertone-Johnson *et al.,* 2009). These collectively might be due to biological sex differences such as levels of oestrogen. In our study, females appeared to have higher number of DCX positive cells than males, which might be due to higher oestradiol levels. The rodent studies however demonstrated differences among different strains. In rodents, a study showed that there was no significant neurochemical or behaviour changes in adult VDD male Sprague-Dawley rats (Byrne *et al.,* 2013). On the other hand,chronic stress exposure reduced the number of neuronal survival (BrdU+ cells) in the hippocampus in female but not in Wistar rats (Lee KJ et al., 2006). In conclusion, if thereare gender differences upon VDD it is inconclusive up to date and further research is needed.

In addition, we cannot confirm that the dams were truly VDD because the serum 25D3 could not be measured by ELISA. This would further validate these results; however, this was not possible.

4.5.2 VDD at PPS alters the mRNA expression levels of neurotrophic factors, VGFand BDNF

VD regulates the expression of several neurotropic factors and other molecules such as neurotransmitters which is important for the growth and survival of the developing neurons (Brown *et al.,* 2003; Garcion, *et al.,* 2002). Our findings show alterations in the gene expression levels of *BDNF* and *VGF* upon VDD at PPS. These alterations are in consistent with previous studies and suggest a link between VDD and the expression and function of neurotropic factors.

While we saw no significant increase in the VGF mRNA expression in the hippocampus,the VGF mRNA expression significantly decreased in the hypothalamus of 3 weeks old VDD offspring compared to controls. At 8 weeks, we saw a significant increase in the VGF mRNA expression in both tissues. The differences at different ages could be a result of the difference in sex between the groups or age, or a combination of these factors.

VGF is a neurotrophic factor which is expressed abundantly in the brain and is involved in controlling energy homeostasis. The role of hypothalamic VGF in food intake and energy balance has been previously shown (Jethwa *et al.,* 2008; Lewis *et al.,* 2017). Previously, knock-down of VGF signalling in mice resulted in a hypermetabolic phenotypewhich is like the VDR-KO mice phenotype: small, lean coupled with metabolic disturbances (Narvaez *et al.,* 2009). Lewis and colleagues investigated the metabolic effects of VGF over-expression in the hypothalamus of Siberian hamster which resulted in an increased energy expenditure and reduced weight despite increased food intake (Lewis *et al.,* 2017). Moreover, 1.25OH_2D_3 treatment using human neuroblastoma cell line increased endogenous VGF expression and the promoter activity significantly (Lewis *et al.,* 2016). So, the decrease we saw in the brain upon VDD at PPS is expected and is in line with the other studies. Also, it is important to note that the 3-week-old mice are females and 8-week-old are males due to breeding issues in our experiment. The female at this age have no significant sex hormones yet. The males only reach puberty. Given that there are no studies suggesting any sex differences regarding VGF and VD, we might conclude that the significant increase at 8 weeks might indicate a compensation mechanism.

VDD at PPS had no effect on the levels of BDNF mRNA expression at 3 weeks of age neither in the hippocampus or the hypothalamus. At 8 weeks, we saw a significant increase in both tissues. *BDNF*, is a MeCP2 target and encodes a protein that promotes the growth and differentiation of new-born neurons (Numakawa *et al.,* 2010), which expression is highest in the hippocampus and hypothalamus. Again, the differences at different ages could be a result of the difference in sex between the groups or age, or a combination of these factors.

In several studies *in vivo*, *BDNF* concentration has been elevated upon VD administration (Farhangi *et al.,* 2017; Hajiluian *et al.,* 2017). Therefore, we were expecting a significant decrease instead of an increase in the BDNF mRNA expression levels. However, our DCX marked cells show that there was a decrease in cell differentiation of VDD mice compared to controls. So, the BDNF mRNA expression levels might have been increased at 8 weeks of age in order to compensate with these changes in adult neurogenesis. Previously, a study conducted Western blot and ELISA analysis to find that the catalytic isoform of BDNF receptor tyrosine kinase receptor (TrkB) expression decreases in hippocampus and hypothalamus with age (Silhol *et al.,* 2005), which suggests the function of BDNF is altered with ageing. In addition to a decrease in total TrkB mRNA levels, alterations to the cAMP response element-binding activity also have been reported in aging rats (Asanuma M et al, 1996). TrkB expression is known to be regulated by cAMP/CREB pathway and it's signalling involved CREB activation in theneurons therefore, the decrease could be the result of a signalling impairment. This might explain why we saw significant change in BDNF expression at 8 weeks of age and not at3 weeks of age due to VDD. The increase instead of a decrease might be compensatory in the brain

of the offspring that has been on a VDD diet pre and postnatal stages. VDD significantly attenuated the number of immature neurones (DCX^{+ve}) in the DG of 3-week-old pups, but not 8-week-old pups. These findings, combined with current literature, suggest that VDD has age-differential effects. So, this prompted a new area of research for the future studies. However, it needs to be noted that we cannot confirm that it was an age effect in this study, due to sex differences between our groups. In addition, in a study by Keilhoff and colleagues, rats were assigned to a VDD diet at 4 weeks old. Brain samples were taken from pups after 6 weeks. The number of 5-day $BrdU^{+ve}$ cells was significantly lower in the VDD group compared to controls (Keilhoff *et al.,* 2010). So, future studies might consider using BrdU injection to understand the effect of VDD in neuronal survival as well as differentiation and proliferation together with the BDNF expression levels. In cultured hippocampal neurons, VGF synthesis was shown to be stimulated by BDNF (Bozdagi *et al.,* 2008). This might explain why the VGF expression levels we foundwas much higher than the BDNF expression in both tissues.

These results may conclude that VD may regulate the expression of BDNF and VGF andcontribute their function in synaptic plasticity and energy homeostasis within the brain. Further studies might investigate the levels of BDNF and VGF gene expression at different stages of development (at birth, 3 weeks, 8 weeks, and months) to examine howthe change in expression is elevated due to VDD.

The current findings suggest the importance of optimal VD for the prevention of cognitivedecline associated with either dementia or other associated diseases and/or ageing. Our previous results on cognition showed that VDD mice had increased anxiety and impairedmemory. We hypothesized that this might be due to alterations

in cell proliferation and differentiation. This chapter showed that VDD at PPS significantly affect cell proliferation and differentiation, thus adult neurogenesis. A possible mechanism might be epigenetic changes.

Chapter 5 Vitamin D deficiency at pre and postnatal stages combined alters the expression of epigenetic enzymes regulated by VD in hippocampus and hypothalamus

5.1 Background

In the previous study (chapter 4), we looked at the impact of VDD on the cell proliferation and differentiation by quantifying the number of Ki67 and DCX- stained cells as a measure of cell proliferation and cell differentiation, respectively.In addition, we measured the expression levels of neurotrophic factors VGF and BDNF in the adult brain. The number of DCX-stained cells were significantly reduced in DG at 3 weeks in both DG and SVZ, might suggest decreased cell differentiation. The number of Ki-67-stained cells were significantly reduced in SVZ at 8 weeks, however, significantly increased at 8 weeks old male mice, suggesting a decreased proliferation of 3 weeks old female mice and increased cell proliferation at 8 weeks of old male mice in the SVZ. Also, we found that VDD at PPS significantly alters the mRNA expression levels of both VGF and BNDF in the adult hippocampus and hypothalamus. Adult neurogenesis in hippocampus has a role in learning, memory,and mood regulation. DG of hippocampus and SVZ are two areas where adult neurogenesis occurs (Kumar *et al,* 2019). Adult neurogenesis also occurs in hypothalamus where it is essential for the energy homeostasis in the body and adaptive behaviour associated with the diet (Cheng, 2013). Thus, our results in Chapter 4 might suggest that VDD environment at pre- and postnatal stages combined has an impact on adult neurogenesis. Neurogenesis involves cross talk between various signalling pathways, interactions

between various intrinsic and extrinsic factors which involve transcription factors, growth, and neurotrophic factors. Epigenetic mechanisms maintain NPCs and govern their cell fate. The expression of these key regulators is determined by multiple epigenetic mechanisms such as DNA methylation and histone modifications (Yao *et al.,* 2016). For example, a methyl CPG-binding protein, MeCP2, targets the methylated DNA at the promoter IV of BDNF gene andrecruits transcriptional repressors. BDNF knock-down in the mouse DG reduced hippocampal neurogenesis and regulated depressive-like behaviours (Vithlani *et al.,* 2013). VGF is known to be induced by BDNF and increase hippocampal neurogenesis and synaptic activity by enhancing dendritic maturation (Behnke J *et al.,* 2017). Histone methylation is a major histone modification implicated in CNS function (Pattaroni *et al.,* 2013) and is among the epigenetic choreographers that have an essential role in neurogenesis. Recent studies have suggested that VDD can alter the status of histone modifications, resulting in altered gene expression (Sacconе*et al.,* 2015) (details in Chapter 1.3.5). Therefore, epigenetic changes can be a potential mechanism behind the impact of VDD on adult neurogenesis. Dynamic methylation of lysine residues is achieved by the combined effects of histone lysine-specific demethylases (KDMs) and histone lysine-specific methyltransferases (KMTs\0 (see chapter 1.4.3.2 for details). *In vitro* studies on human colon cancer cells have shown that calcitriol increases histone methylation by the transcriptional repression of genes containing JmJC histone demethylase domains or lysine-specific demethylases. The expression of certain subgroups of this family (KDM4A/4C/4D/5A/2B, JMJD5/6, PLA2G4B) are inhibited by $1.25OH_2D_3$, while some (JARID2/KDM5B) are induced (Pereira F et al., 2012). Ezh2 is a histone methyltransferase that catalyses the trimethylation of H3K27 which results

in transcriptional repression. Lin and colleagues found that in colorectal cancer cells, Ezh2 methylates H3K27 at the promoter region of VDR downregulation of VDR expression (Lin Y et al., 2013). In this study, we focused on some of these epigenetic enzymes which have been previously shown to be regulated by VD and neurogenesis. Also, they have been shown to play a role in neurogenesis. Table below summarises the genes of interest and their relationship to VD and their role in neurogenesis.

Genes of Interest	Relationship to VD	Role
KDM5A	Inhibited	Repression of astrocyte differentiation
KDM6A/UTX	Inhibited	Promotes demethylation of neuronal genes including DCX
KDM6B	Induced	Transcriptional activation of NSC/neurogenesis
Ezh2	Regulates the (H3K27me3) in the VDR promoter	Transcriptional repression/regulates progenitor cell proliferation

Table 5.1: Relationship of the genes of interest to VD and their associated role in the brain.

Gene	Role in Neurogenesis	Role in Vitamin D
KDM5A	- Regulates neural differentiation and maturation	- KDM5A has not been directly linked to Vitamin D
KDM6A	- Controls neuronal differentiation and synaptic plasticity	- KDM6A expression can be influenced by Vitamin D levels
KDM6B	- Impacts neural stem cell maintenance and differentiation	- No direct role of KDM6B in Vitamin D metabolism
Ezh2	- Suppresses neurogenesis and promotes gliogenesis	- Ezh2 may indirectly affect Vitamin D-related pathways

Table 5.2: Relationship of the genes of interest to VD and their associated role in neurogene

Indeed, Zhang and colleagues showed that loss of Ezh2 function in NPCs resulted in decreased proliferation and decrease in the number of new neurons in the adult brain. Moreover, the same study showed that conditional KO of Ezh2 impaired spatial learning and memory, contextual fear memory and pattern separation (Zhang J et al., 2014). Furthermore, histone demethylases UTX (KDM6A) and KDM6B have a role in neurogenesis (Shan *et al.,* 2020). In postnatal neurogenesis, KDM6B activates key neurogenic enhancers, DCX and Dlc2, so targeted deletion of KDM6B impaired OB neurogenesis. KDM6B is also required for proper differentiation of SVZ NPCs (Park *et al.,* 2014). In human NPCs, Shang and colleagues showed that both KDM6s are necessary for long-term proliferation (Shang *et al,* 2020). A genome-wide study showed that some of the KDM5A target genes are involvedin differentiation and critical for cell-cycle progression (Lopez-Bigas *et al.,* 2008). In a recent study, knockdown of the KDM5A gene suppressed osteosarcoma cell proliferation and induced apoptosis *in vivo* (Peng *et al.,* 2021). Previously, KDM5Ahas been shown to impact proliferation in several other cancers including lung cancer (Oser *et al.,* 2019), glioblastoma (Romani *et al.,* 2019) and breast cancer (Cao *et al.,* 2014). In addition to above, a recent study showed that KDM5A is also important for astrocyte differentiation NPCs' fate determination as its' mRNA expression is higher when astrocyte differentiation was induced by CNTF (Kong *etal*., 2018).

The studies mentioned above are mostly *in vitro* studies or VD supplementation rather than VDD. Our study fills this gap by focusing on VDD *in vivo*. If VDD has long-term impact on histone modifications at sites associated with regulation of genes that are involved in neurogenesis, VDD may have lasting effects on the offspring born from the VDD mothers.

5.2 Aims and Objectives

The aim of this study is to explore the impact of VDD at PPS on the gene expression of epigenetic enzymes that are orchestrating adult neurogenesis. Thus,we examined the mRNA expression levels of the histone demethylases KDM6B, KDM5A, UTX, a histone methyltransferase Ezh2, in the hippocampus and hypothalamus of VDD mice. We expect that the changes in adult neurogenesis we observed in VDD mice compared to controls might be related to changes in the expression levels of these epigenetic enzymes. As previously shown, VD administration inhibited the expression of KDM5A and KMD6 family of histone demethylases. In addition, loss of Ezh2 caused a decrease in proliferation and neuron numbers in the adult brain as VDD. Therefore, we hypothesise that VDD will lead to a decrease in mRNA expression of KDM6B, KDM6A/UTX, KDM5A, and Ezh2, in VDD mice compared to controls in both hippocampus and hypothalamus.

5.3 Methods and Materials

5.3.1. Animals

See *Chapter 3.3.1* for details about the animals and the treatment used for this study.

5.3.2 Tissue preparation and RNA extraction

For the details of tissue collection and preparation see *Chapter 4.3.* In brief, RNA extraction from the hippocampal and hypothalamic brain tissues were performed with TRIzol (Thermofisher Scientific, UK), following the manufacturers protocol. RNA samples underwent DNase treatment and were reverse transcribed using Superscript™ III from Invitrogen.

5.3.3 qPCR

QPCR reactions were performed using the Rotor-Gene 6000 cycler (Corbett Research) and reactions were performed in triplicates. qPCR was performed as described in *Chapter 4.3.2.5*. One difference to the protocol described in *Chapter 4.3.2.5* was the use of Betaine solution 5M (Sigma, UK) in final concentration of 1µl per PCR sample as an enhancer with *KDM5A* primer. See **Table 5.1** for the details about the genes. Relative expression was calculated by CT^(($Group\ average$) − ($Efficiency$ + 1)) (Pfaffl, 2001). Gene expression was normalized to the geometric average of three control genes (*Hprt1, Pgk1 and CyclA*) according to the GeNorm normalization (Vandesompele *et al.*, 2002).

Finally, the normalised expression of each sample was calculated as $\frac{Relative\ expression}{Normalisation\ facto}$.

	Primer Sequence	Final concentration	Temperature (T_m)
Housekeeping genes			
Hprt1	**F** 5′ cgaggagtcctgttgatgttgc 3′ **R** 5′ ctggcctataggctcatagtgc 3′	500 mM	63°C
Pgk1	**F** 5′ tagtggctgagatgtggcacag3′ **R** 5′ gctcacttcctttctcaggcag 3′	500 mM	63°C
CyclA	**F** 5′ ggcaagttcaatggcacagt 3′ **R** 5′ tggtgaagacgccagtagactc 3′	500 mM	63°C
Genes of interest			
KDM5A	**F** 5′ cacagacccgctgagttttat3′ **R** 5′ cttcacaggcaaatggaggtt3′	500 mM	62°C
KDM6B	**F** 5′ cccccatttcagctgactaa3′ **R** 5′ ctggaccaaggggtgtgtt3′	500 mM	56°C
UTX/KDM6A	**F** 5′ atggaaacgtgccttacctg3′ **R** 5′ ggacctgccaaatgtgaact3′	400 mM	62°C
Ezh2	**F** 5′ acttggattttccagcacaagt3′ **R** 5′ aagggcgaccaagagtacatta3′	500 mM	58°C

Table 5.3 The information of the genes that were used to analyse the mRNA expressionlevel in hippocampus and hypothalamus in this study. Each row shows the gene name, the information of the primer sequence used, the final primer concentration and the annealing temperature used.

5.3.4 Statistics

All statistical analysis were performed using GraphPad Prism version 7.00 for windows, GraphPad Software, La Jolla California USA.

All data was checked for normality using D`Agostino test. Data for all studies were analysed using Student's *t*-test for single comparisons and represented as mean ±S.E.M. The significance threshold was set at $p < 0.05$.

5.4 Results

5.4.1. The impact of VDD at PPS on the gene expression of histone demethylases, KDM5A, KDM6B and UTX in the hippocampus at 3 weeks and8 weeks of age

The quantitative PCR analysis revealed a significant increase in the mRNA expression levels of histone demethylases, KDM5A **(Fig 5.1A.** t (10) =2.333 p=0.0418), KDM6B (**Fig 5.1B.** t (10) =2.511 p=0.0493) and UTX (**Fig 5.1C.** t (10) =2.832 p=0.0178) in the hippocampus of 3 weeks old female VDD mice compared to controls, which is maintained in the hippocampus of 8 weeks old male VDD mice for KDM6B (**Fig 5.1E.** t (18) =2.818 p=0.0114) and UTX (**Fig 5.1F.** t (16) =3.373 p=0.0019) but not for KDM5A (**Fig 5.1D.** t (16) =2.107 p=0.0512).

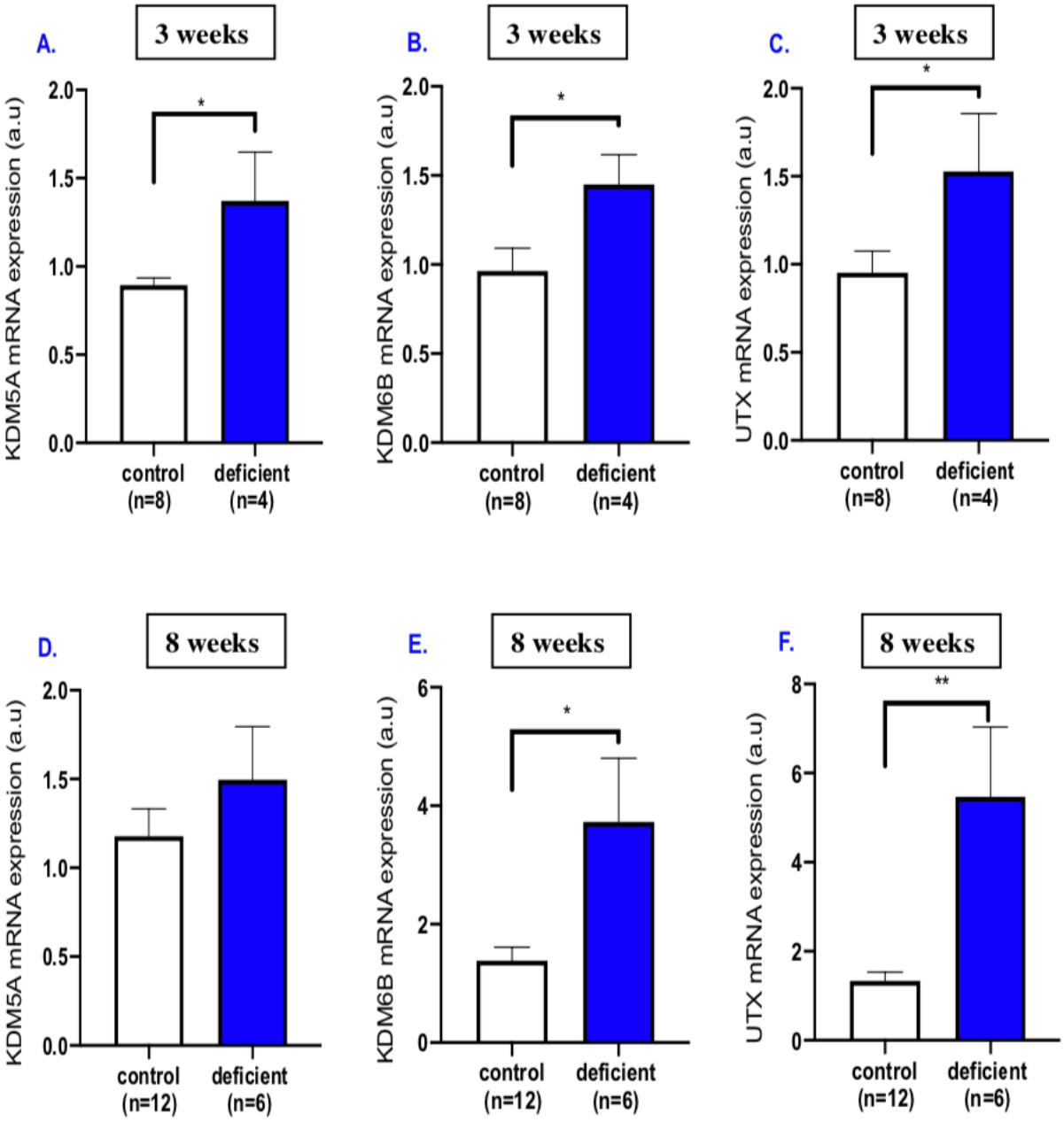


Figure 5.1 mRNA expression levels of **A.** KDM5A **B.** KDM6B **C.** UTX in of 3 weeks old female mice (N=8:4) **D.** KDM5A **E.** KDM6B **F.** UTX of 8 weeks old male mice in hippocampus. (N=12:6) Values represented as mean ± SEM. * $p < 0.05$ ** $p < 0.01$

5.4.2 The impact of VDD at PPS on the gene expression of histone demethylases, KDM5A, KDM6B and UTX in the hypothalamus at 3 weeks and8 weeks of age

The quantitative PCR analysis revealed a significant increase in the mRNA expression levels of several histone demethylases, KDM5A **(Fig 5.2A** t (10) =2.457p= 0.0339), KDM6B (**Fig 5.2B** t (10) =3.225 p= 0.0091) and UTX (**Fig 5.2C** t (10) =2.280 p=0.0195) in hypothalamus of 3 weeks old female VDD mice compared tocontrols. The significant increase of the mRNA expression of the histone demethylases listed above maintained in the hypothalamus of 8 weeks old male VDD mice; KDM6B (**Fig 5.2E** t (16) =3.228 p=0.0053) and UTX (**Fig 5.2F** t (16) =3.569 p=0.0026), except KDM5A (**Fig 5.2D** t (16) p=4.250).

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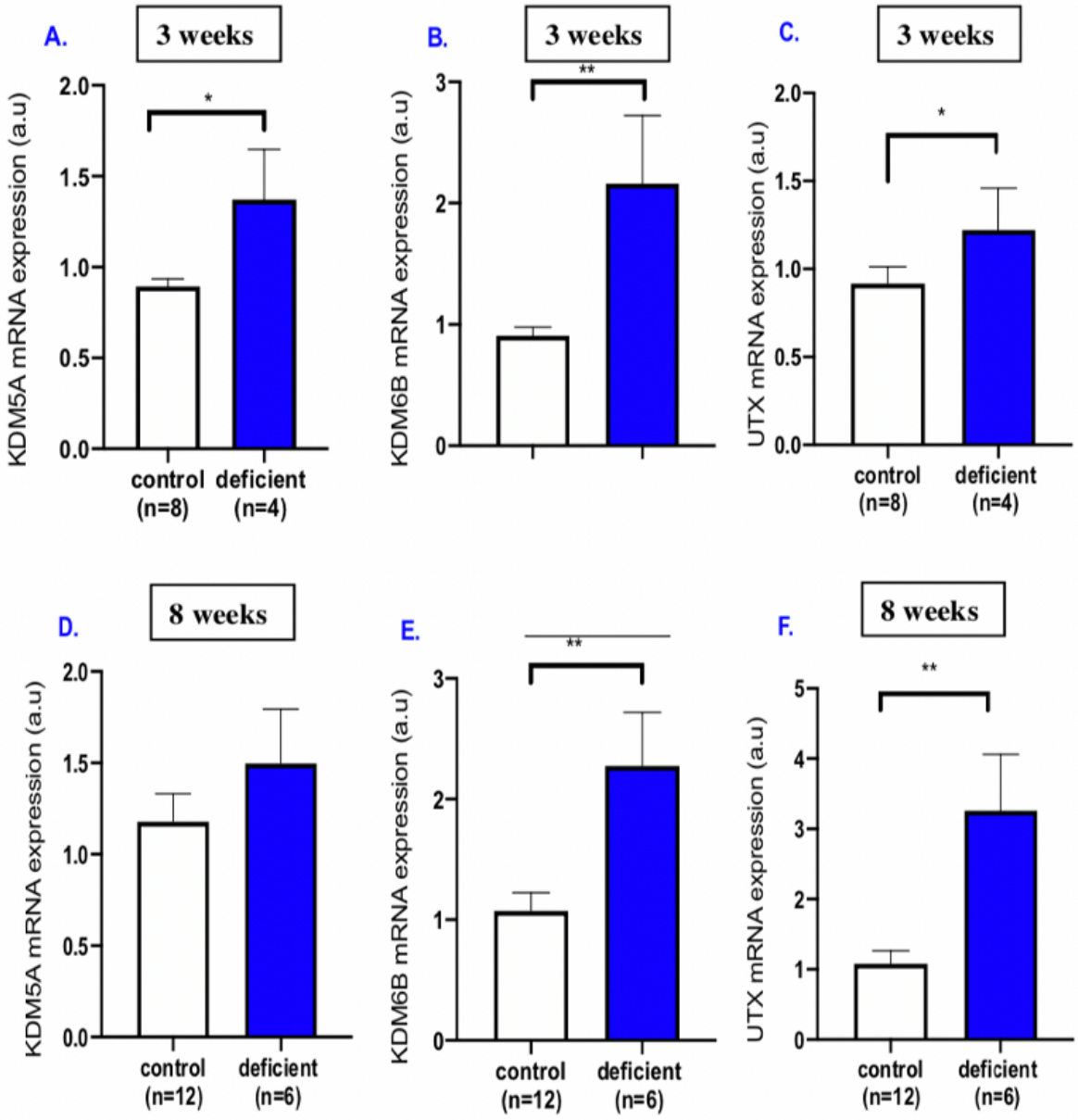

Figure 5.2. The expression levels of **A.** KDM5A **B.** KDM6B **C.** UTX in of 3 weeks old female mice (N=8:4) **D.** KDM5A **E.** KDM6B **F.** UTX of 8 weeks old male mice in hypothalamus (N=12:6). Values represented as mean ± SEM. * $p < 0.05$ ** $p < 0.01$

5.4.3 The impact of VDD at PPS on the gene expression of the histone methyltransferase, Ezh2, in the hippocampus at 3 weeks and 8 weeks of age

Our data showed that the expression of the Ezh2 is not affected (**Fig 5.3A** t (10) =1.411 p=0.1885) in the hippocampus by pre- and post-natal VDD neither at 3 weeks nor at 8 weeks (**Fig 5.3B** t (16) =1.576 p=0.1346) compared to controls.

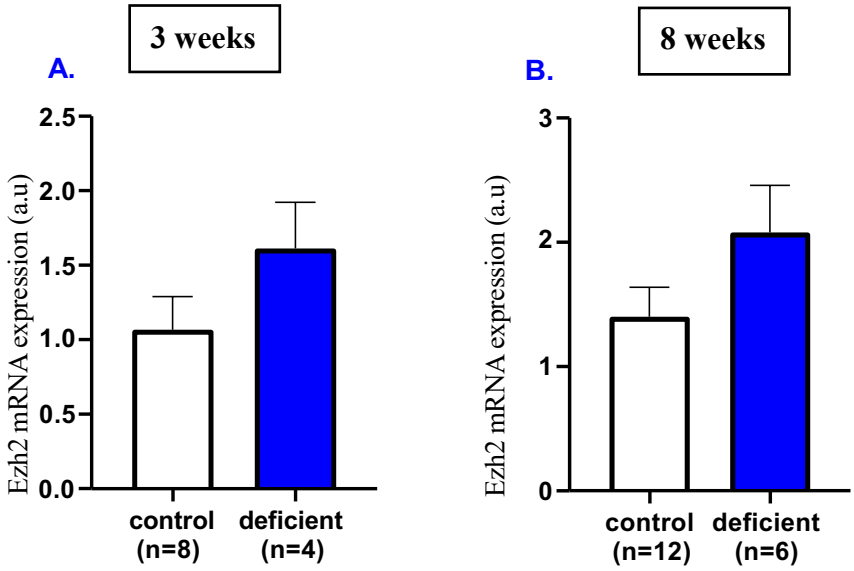

Figure 5.3 Ezh2 mRNA expression in hippocampus **A.** 3 weeks 3 weeks old female mice (N=8:4) **B.** 8weeks old male mice (N=12:6) Values represented as ± SEM.

5.4.5 The impact of pre- and postnatal VDD on the gene expression of the histone methyltransferase, Ezh2, in the hypothalamus at 3 weeks and 8 weeks of age

VDD at pre- and postnatal stages combined had no effect on the mRNA expression of the histone methylase Ezh2 in the hypothalamus of 3 weeks old female mice compared to controls (**Fig 5.4A** t (10) = 1.921), however, it significantly increasedat 8 weeks of age in male VDD mice compared to controls (**Fig 5.4B** t (15) =3.141 p=0.0067).

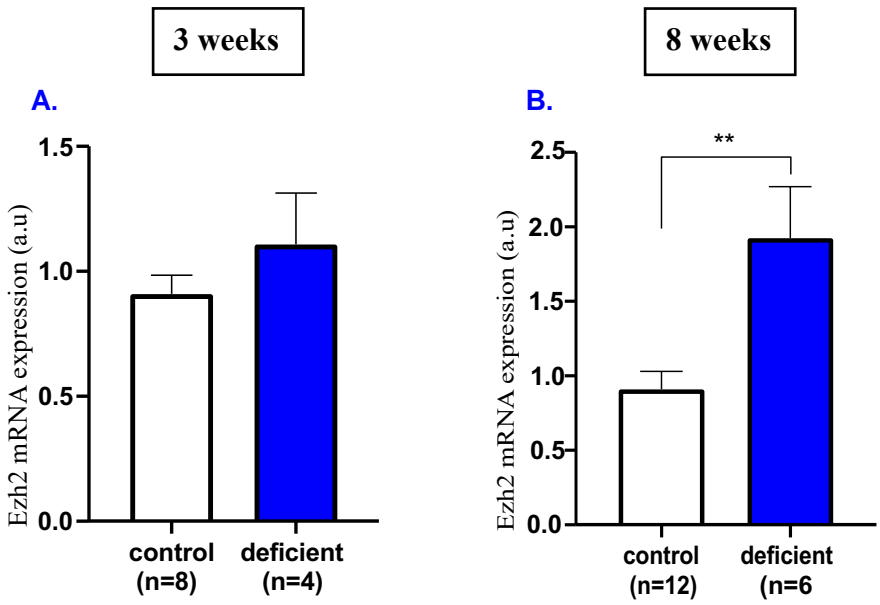

Figure 5.4 A. mRNA expression level of Ezh2 in hypothalamus at 3 weeks old female mice (N=8:4) **B.** at 8 weeks old male mice (12:6) Values represented as mean ± SEM. * $p < 0.05$

5.5 Discussion

In the present study, we investigated the impact of pre-and postnatal VDD on the expression of the histone demethylases KDM6B, *UTX, KDM5A* and the histone methyltransferase Ezh2 in hippocampus and hypothalamus of 3 weeks and 8 weeks old mice. We chose to focus on the expression of these enzymes because these histone (de) methylases are known to be controlled by VD (Pereira F et al., 2011). In addition, these enzymes are among the key regulators that control the proliferation, differentiation, and fate determination of NPCs (Shan Y et al, 2020; Kong SY et al 2018). When genes have methylation at both H3K4me$_3$ and H3K27me$_3$, they are poised to be activated (chapter 1.4.3.1 for details).

5.5.1 Hippocampus

In the hippocampus, VDD led to a significant increase in the mRNA expression of all three histone demethylases KDM5A, KDM6B, KDM6A at 3 weeks of age. As KDM6B and UTX demethylate H3K27me$_3$, which is associated with transcriptional repression, the increased expression of KDM6B and UTX might suggest an increase in H3K27 demethylation that results in increased transcriptional activation of the genes affected. In addition, an increase in KDM5A mRNA expression might suggest an increase in H3K4 demethylation which in contrast, might reverse this poised state. Jepsen and colleagues found that enhanced expression of KDM6B induces neuronal differentiation through demethylating neuronal genes including DCX (Jepsen *et al.,* 2007). Furthermore, deletion of KDM2A, which methylates H3K4, inhibits neuronal differentiation (Greer and Shi, 2012). Following this,

increase expression of KDM5A will lead to an increase in H3K4 demethylation and expected to induce neuronal differentiation. Based on previous studies, we should see that VDD leads to a decreased rate of neuronal differentiation in the adult hippocampus. This disagrees with our hypothesis.

The significant increase in the expression of KDM6B continued at 8 weeks. The increase in KDM5A and with UTX became more significant. The expression of histone methyltransferase Ezh2, on the other hand, was not affected by VDD at 3 weeks or at 8 weeks. The significant increase in KDM6B and UTX and the unaffected Ezh2 gene expression in the hippocampus leads an unbalanced demethylase and methyltransferase activity on H3K27. Due to H3K27 methylation being associated with gene silencing, our results (increase in H327-specific histone demethylases) indicate a shift towards demethylation thus, favour transcriptional activation. The unaffected KDM5A and increased UTX might suggest a decrease H3K27 methylation and increase in H3K4 trimethylation, which is associated with transcriptional activation. These results are also not in agreement with our hypothesis.

Our results showed that VDD at PPS lead to increased expression of KDM6B, KDM5A and UTX. KDM6B and UTX are the only known enzymes that demethylate $H3K27me_3$. expecting as previous studies the upregulation of KDM5A by VD treatment might lead to gene repression (Pereira *et al.,* 2011). The reason for this discrepancy could be that KDM5A is only one of the enzymes that demethylates H3K4. There are many other epigenetic enzymes that are known to be regulated by VD and might contribute to transcriptional modulation in the hippocampus. So,the other enzymes that demethylate H3K4 such as KDM5B, 5C, 5D and NO66 (Hyun

et al., 2017) could compensate for this outcome. Future studies may investigate the expression of these enzymes upon VDD. The trend we have seen towards transcriptional activation via increased mRNA expression of the KDMs might also be explained by the body compensating for VDD over time. Future studies might look into the impact of VDD at different times of development: embryonic, at birth and postnatal stages separately.

5.5.2 Hypothalamus

Like the hippocampus, VDD also led to an increase in the expression of KDM6B, UTX and KDM5A in the hypothalamus. However, a clear difference was the significant increase of the Ezh2 expression at 8 weeks of age. The significant increase of Ezh2 expression might contribute towards repression of the genes affected through increased H3K27 methylation. However, this impact might be counteracted by the increased expression of KDM6A and UTX. As hypothalamus is another neurogenic area, our findings were not expected.

In the hypothalamus, tanycytes are diet-responsive adult NPCs and are known to can differentiate into neurons and astrocytes in the adult brain. They act as metabolic modulators (Lee *et al.,* 2012). Thyroid hormone, RA metabolism (Venkataraman *et al.,* 1999) and VD are all regulated by epigenetic mechanisms. So, we think a similar mechanism will happen. As coordination between metabolic cues and gene expression is essential to maintain energy balance, proper gene expression in tanycytes is necessary for their function. Deiodinase enzyme 2, Dio2, converts T_4 into T_3 to be released into the hypothalamus for the control of orexigenic enzymes, and it is photo-periodically sensitive. Ross and colleagues found that

several genes encoding retinoic acid receptor, RXR/RAR and histamineH3 receptor were downregulated in short-day photoperiods in hypothalamic tanycytes (Ross *et al.,* 2005). RA, a heterodimeric partner of RXR, regulates Dio2promoter. Therefore, we can hypothesize that VDR which also heterodimerizes with RXR upon binding of the $1.25OH_2D_3$, can influence the expression of these genes including Dio2. In Chapter 4, we saw a significant increase in the VGF expression in VDD mice compared to controls. VGF expression and function is influenced by both T_3 and VD (Lewis JE *et al.,* 2016). As VD is linked to epigenetics by histone methylation, this could point out that the impact of VD on VGF is via the change in these epigenetic enzymes. Thus, it might be possible that VD and underlying epigenetic mechanisms might contribute to energy homeostasis and appetite regulation. Chapter 4 results showed a significant increase in VGF expression in VDD mice, and this might be compensatory as our VDD mice were pre-and postnatally VDD throughout the study. In addition, VGF have different responses in different regions of the brain (Eagleson *et al.,* 2001). So, the results might be region-specific. Changes in histone methylation by VD could influence energy homeostasis by methylation at the promoter of the Vitamin D binding protein (DBP) gene. Indeed, studies have shown that di-tri methylation of H3K4 and H3K9 dynamically occur at the promoter region of DBP gene. In addition, immunostaining for DBP showed that it is present in rat hypothalamus (Jirikowski *et al.,* 2008) and display circadian oscillations (Ripperger *et al.,* 2000). Energy homeostasis (regulation of fast/feeding states) is affected by circadian rhythms and hypothalamus is an area of the brain where mammalian circadian rhythms are controlled (Cedernaes *et al.,*2019). They are dependent on transcriptional feedback loops involving CLOCK, BMAL1, Period

(per1) and period 2 (per2) genes. BMAL1/CLOCK is shown to bind to the promoter of DBP gene and drives its transcription. Given that DBP influences the availability and bioactivity of active metabolite of VD, $1.25OH_2D_3$, VD might affect circadian function in the hypothalamus.Furthermore, *Ezh2* has been shown to alter circadian rhythm in SCN of hypothalamus. The CHIP study showed that Ezh2 is involved in the circadian function through H3K27 methylation on promoter of the Per1 gene (Etchegaray *et al,* 2006). This might suggest an independent role of Ezh2 in hypothalamus, hence,why we saw a significant change in the mRNA expression of Ezh2 in the 8 weeks of age in this brain region.

These results collectively may suggest that VDD at PPS might impair both sleep/wake cycles and fast/feeding states, eventually resulting in metabolic disorders, possibly through epigenetic changes. Further metabolic experiments needed.

CHAPTER 6 The effect of Vitamin D on cell proliferation-differentiation *in vitro*

6.1 Background

6.1.1 *In-vitro* models of VDD

VDR is present widely in almost all the cells in the body. According to the Chip-seq studies, VDR- binding sites (VDREs) are identified anywhere from 1000 to 13000 in a variety of cells that were examined, involving 3-4% of the whole genome (Ramagopalan *et al,* 2010) the majority being cell-specific, present at almost anywhere, at multiple locations on each gene (Carlberg, 2014). Cell culture studies have been greatly used to explore the effects of VDD. One of the earliest studies was conducted using bone tibia from pups delivered from VDD mothers in mice. They showed changes in circulating concentrations of Ca, Mg, and Pi ions from tibia of VDD pups, and further alterations upon 1.25OH2D3 treatment (Ramp et al, 1986). Moreover, 1.25OH2D3 play an important role in regulating cell growth and differentiation, while *in vitro* studies have shown, in variety of cells, that proliferation is either inhibited or stimulated (Walters, 1992). VDR, as well as CYP27B1 and CYP24A1, are known to be expressed in most tumours so variety of was downregulated while CYP24A1 was induced by $1.25OH_2D_3$ (Lechner *et al.,* 2007). In his previous study, Bikle D and colleagues showed that VD can act as a tumour suppressor (Bikle *et al,* 2015). $1.25OH_2D_3$ binding blocks β- catenin regulated proliferation through activating VDR transcriptional activity and in a calcium-

dependent manner. This promotes differentiation in keratinocytes via binding to VDR through $1.25OH_2D_3$ - dependent and independent manner (Shah S et al., 2006; Cianferotti *et al*., 2007). In addition, VD can induce differentiation of certain cancer cells into granulocytes (Miyaura *et al.*, 1985). It has been found that it can inhibit the proliferation of *M. tuberculosis* in human monocytes *in vitro* (Rook G et al, 1986). In coloncarcinoma, CRC, cell lines, VD exerts antiproliferative actions. In SCC25 cell lines, $1.25OH_2D_3$ has shown to promote the interaction between VDR and forkhead box O (FoxO) protein and its regulators including sirtuin1 (Sirt1), which are the transcription factors known to suppress proliferation and increase cell survival (Lin R et al, 2002). Conversely, Shirazi and colleagues found that *in vitro* administration of $1.25OH_2D_3$ to NSCs generated from the C57BL/6 mice significantly enhanced NSCs proliferation and differentiation into neurons and oligodendrocytes, but not astrocytes (Shirazi *et al.,* 2015). Molecular control of the endogenous NSC activation and progenitor mobilization is important for understanding neurogenesis in detail thus, give various chances for therapeutic approach in future. Previous study observed that Sox2, Oct4, Klf4 and c-Myc can convert fibroblast into pluriropotent stem cells (Takashi and Yamanaka, 2006). Enhanced cell proliferation is the earliest response observed upon expression of these reprogramming factors (Smith *et al,* 2010). Several transcription factors including sex-determining region Y-box 2 and 9 (Sox2&Sox9), homeodomain transcription factor Jag1 neuronal differentiation (NeuroD) regulates adult neurogenesis. Sox2 regulates different stages of adult neurogenesis including precursor cell proliferation, neuronal maturation, and migration whereas Sox9 mediates neuron to glial fate determination (Vong *et al.,*

2015). Jag1 controls postnatal and adult neurogenesis in DG: it promotes proliferation and survival of NPCs via interaction with Notch signalling pathway (Lavado and Oliver, 2014). Transcription factors NeuroD1 and Synaptophysin are two of these reprogrammingfactors which are required for the pluripotency, self-renewal of the embryonic stem cells and for synaptic density. Synaptophysin (Syp) is a presynaptic protein that is expressed in synaptic vesicles in the brain. An *in vivo* study where neurogenesis and synaptic plasticity were observed by injecting *Oct4*, *Sox2*, c-*Myc*, and *Klf4* coupled with a fluorescent protein into the lateral ventricles, they observed an increase in Syp expression, which shows an increase in synaptic density, compared to the control group. The enhanced long-term memory and decreased anxiety they observed in the treated animals might be due to enhanced neurogenesis and synaptic plasticity in the brain (Wi *et al.,* 2016).

Neuronal differentiation 1(NeuroD1) belongs to the basic helix-loop-helix proteins, whichplays a crucial role in determining cell fate during development. Neuro D is expressed in post-mitotic cells has a role in survival and differentiation of the adult-born neurons in theSGZ and SVZ (Gao *et al.,* 2009). NeuroD1 is an important factor for neurogenic differentiation, shown by both in vivo (Pan *et al*., 2009) and in vitro (Kamath *et al.,* 2005) studies.

Neurogenesis involves concerted action of many transcription factors reprogramming epigenetic landscape. VD's direct relationship to NeuroD1 and Syp is currently not known.However, given that they are known markers of neurogenic

cues, we found it appropriate to use these markers to observe neuronal proliferation/differentiation in our study.

6.1.3 N2a cell line

The N2a cell line (also known as Neuro2a) is a Mus Musculus neuroblastoma brain cancer cell line derived from the spontaneous mouse C1300 neuroblastoma tumour whenit is first established by R.J Klebe RJ and F.H Ruddle in 1969. According to ATCC (Neuro-2a ATCC® CCL-131), N2a cell line is neuronal amoeboid stem cell which when differentiated, has neuronal cell type morphology and physiology such as having neurofilaments. N2a cell line is also known to be fast-growing. In 1972, Prasad and colleagues induced morphological differentiation in culture by prostaglandin (Prasad *et al.,* 1972). N2 cell line was then used in several other studies related to differentiation andgrowth (Mao *et al.,* 2000; Namsi *et al.,* 2018). Previous studies have indicated that reduced serum concentration in the presence of retinoic acid (RA) can be used to inducea change in differentiation of the N2a cells. Significant growth in neurite length and branching pattern was observed when the N2a cells are subjected to culture conditions with RA present (Kumar and Katyal, 2018). Retinoic acid is derived from the liposoluble vitamin A (retinol). VDR belongs to the steroid and hormone nuclear receptor family whichincludes all-trans retinoic acid receptors (RARs), 9-cis retinoic acid as well as thyroid hormone receptors (TRs) and PPARs and many others (Mangelsdorf *et al.,* 1995). To exert its function, upon $1.25OH_2D_3$ binding, VDR forms a heterodimer with retinoid X receptor (RXR). The structure of the Vitamin D response elements (VDREs),

identified over a decade ago, is composed of two direct repeats separated by three nucleotide pairs:G G G/T T G/C A and G G G/T T G/T/A A (Ozono *et al.,* 1990). More than one VDRE binding sites has been identified as binding sites for VDR/RXR heterodimer. Direct interactions between RA and VD signalling pathways have been identified previously (Schrader *et al,* 1993). In fact, Bland and colleagues showed a formation of a complex between VDRE, RA, $1.25OH_2D_3$ and a thyroid hormone, T3 (Bland *et al.,* 1997). Therefore, N2a neuroblastoma cell line provides an ideal in vitro model for our study to observe VD's effects on cell differentiation and proliferation.

6.2 Aims and Objectives

The purpose of the study is to investigate the effects of VD on neuronal proliferation anddifferentiation *in vitro* using N2a cell line firstly by measuring cell proliferation by MTT assay, measuring differentiation by axonal outgrowth and then by analysing the gene expression levels of NeuroD1 and Syp, neurogenic transcription factors.

The objective is to discover the effect of VD administration on cell proliferation and differentiation and to confirm our previous findings from *the in vivo* studies.

6.3 METHODS

6.2.1. Culturing the cells

The N2a cells are purchased from Sigma, UK. Cells were maintained in a culture medium containing Dulbecco's modified Eagle's medium with 2 mM L-glutamine (Sigma, UK), supplemented with 10% foetal bovine serum (Sigma, UK) and 2% Penicillin/G Streptomycin antibiotic mixture (Sigma, UK). The cells were seeded at 1 x 10^4 cells/mL in T25 flasks.

6.3.2 Maintaining the cells

The cells were routinely split 1:2 and passaged at 3-day intervals. They were left undisturbed to attach. Once confluent, the cells were detached from the monolayer

bygentle jets of growth media. When all the cells are in cell suspension, cells were pipetted out into a sterile universal tube. Light microscope was used to ensure all thecells at monolayer were harvested. Cell suspension was centrifuged at for 10 minutes at 1000rpm. Then, supernatant was discarded, and the cells were divided up into T25 flasks in sufficient fresh growth media. All the work was carried out under a sterile cell culture hood.

Differentiation

To induce differentiation, cells were kept in a serum free media: DMEM — 10% fetal bovine serum (FBS). To induce differentiation, cells were treated with 10μM all-trans-RA (Sigma, UK). A 3mg/ml solution was prepared in DMSO and stored at -20^{o}C (light- protected). Initial concentration was 100mg, 300.44 g/mol MW. In 95%ethanol at 2.7mg/ml; dilutions prepared in DMEM with ethanol (0.1%) 1 μl RA was diluted in 500 ml DMEM for use (20 μM).

6.3.3 Experimental preparation of the cells

When cells are confluent the protocol for ‘maintenance of the cells’ were followed to produce a cell suspension. They were centrifuged for 10 minutes at 1000 rpm. Supernatant was discarded and the pellet was resuspended in 1 ml of growth media. From the 1 ml cell suspension, 1:10 dilution of the cell suspension (5 μl in 45 μl PBS). 10 μl of the cell suspension above was mixed with 10μl Trypan blue. The cell suspension was resuspended and transferred onto a reusable chamber slide. Total number of cells counted by an automated reader (Bio-Rad). Then, they were plated out onto a well-plate.

6.3.4 (4,5-Dimethylthiazol-2- yl)-2,5-diphenyl tetrazolium bromide (MTT) reduction assay

To prepare the MTT solution, 5 mg MTT was dissolved in 1ml of PBS. 50 µl of MTT solution were added to each well. After the end of the incubation period, medium was removed and discarded from the wells. 1ml of DMSO was added to the wells and plate was gently agitated. Then, absorbance was read using a spectrophotometer (Thermo Scientific, UK) at a wavelength of 570nm.

6.3.5 Fixing and Staining

Procedure:

Each well was rinsed with TBS 5 mM (Tris (6.06g) and 20nM NaCl (11.67g)) several times to remove the serum the medium, if present. Then, 90%methanol/TBS solution (10 ml of TBS in 90 ml methanol) was added to the plate and was stored overnight at -20C. The next day, the 90%methanol /TBS solution was removed from the wells and replaced by Coomassie brilliant blue stain (1.25 g of R-250 Coomassie blue, 200 ml, 40% (v/v) methanol) for several minutes until absorbed by the cells. Then, the excess stain was removed from the wells and thewells were washed with ddH_2O twice. The plate was tap dried and left overnight to air dry. At the end of the experimental procedure, the stored plates were taken to the light microscope for the image analysis.

6.3.6 Image analysis with Fiji

Total of three images were taken from each well during the study. A total of 10 axon lengths were measured from each of these with ImageJ/Fiji and mean averages were used.

Experiment 1. Dose-Response

We made a serial dilution from a 100 μM VD stock continued by a series 1:1 dilution for the doses of $1.25OH_2D_3$.

SFM (control) 20μM RA 100nM 50nM 25nM 40nM

Procedure:

6 x 24-well plates were used for the experiment in total (2 plates for each day) over the treatment time of 3 days. On day 1, 2 plates were removed from the incubator. Undifferentiated cells were plated in 2 24-well plates at 25,000 cells / 0.5 ml respectively for MTT assay and fixing/staining (**Figure 6.1**).

48 hours post VD treatment. For 3x 24-well plates.

2.5x10^4/0.5ml cells were used. For 3x 6-well plates1x10^5/1ml cells were used to calculate the cell density per plate.

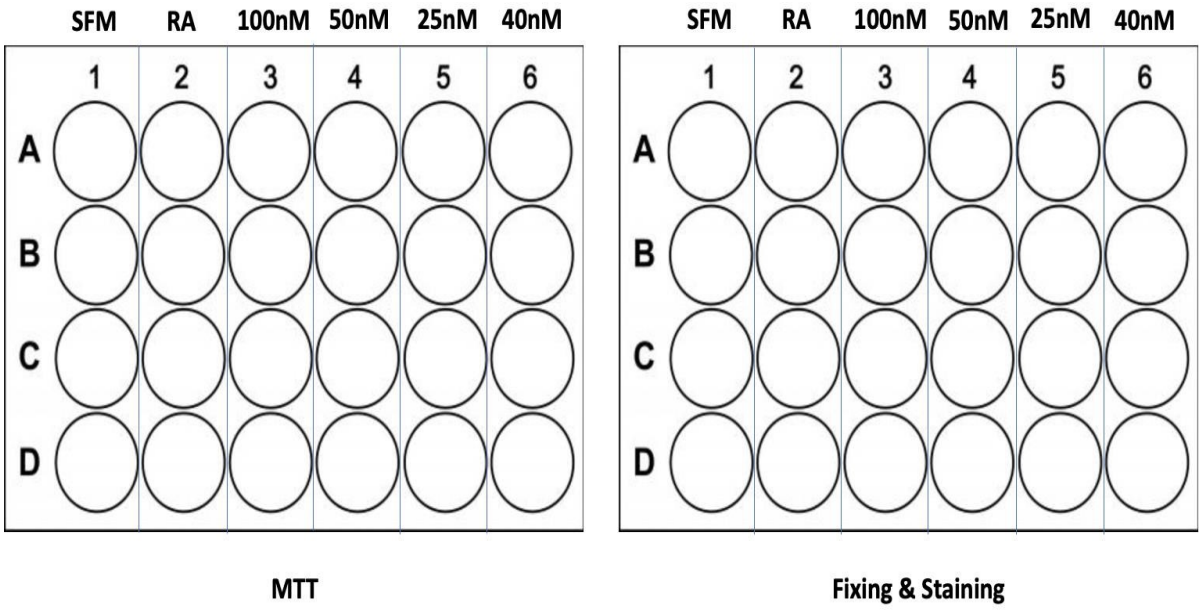

Figure 6.1 Representative figure of the distribution of the individual groups on the 24-wellplates that were used for each day over the treatment time.

Experiment 2. Proliferation and Differentiation

Cells were maintained at the logarithmic phase of growth, routinely split.

When the cells are confluent, monolayer was detached by using gentle jets of growth media then pipetted out to a universal sterile tube. They were seeded on uncoated plastic plates (**Figure 6.2**). When all cells are harvested, cell suspension were

centrifuged for 10 min at 1,000rpm and the cells are seeded on uncoated plastic plates.

A 24-well plate (A-D/1-3) was divided into two so that one half of the plate was treated with MTT to determine proliferation and the second half of the same 24-well plate (A-D/4-6) was used for fixing and staining procedure to measure the neurite outgrowth using image analysis. Initial concentration was 416.64 g/mol MW. 40nM dose of VD used for the following experiments. It was diluted in 95%ethanol at 2.7mg/ml; dilutions prepared in DMEM with ethanol (0.1%). 1µl of the (100 µM) stock diluted in 2499µl DMEM (*40*nM).

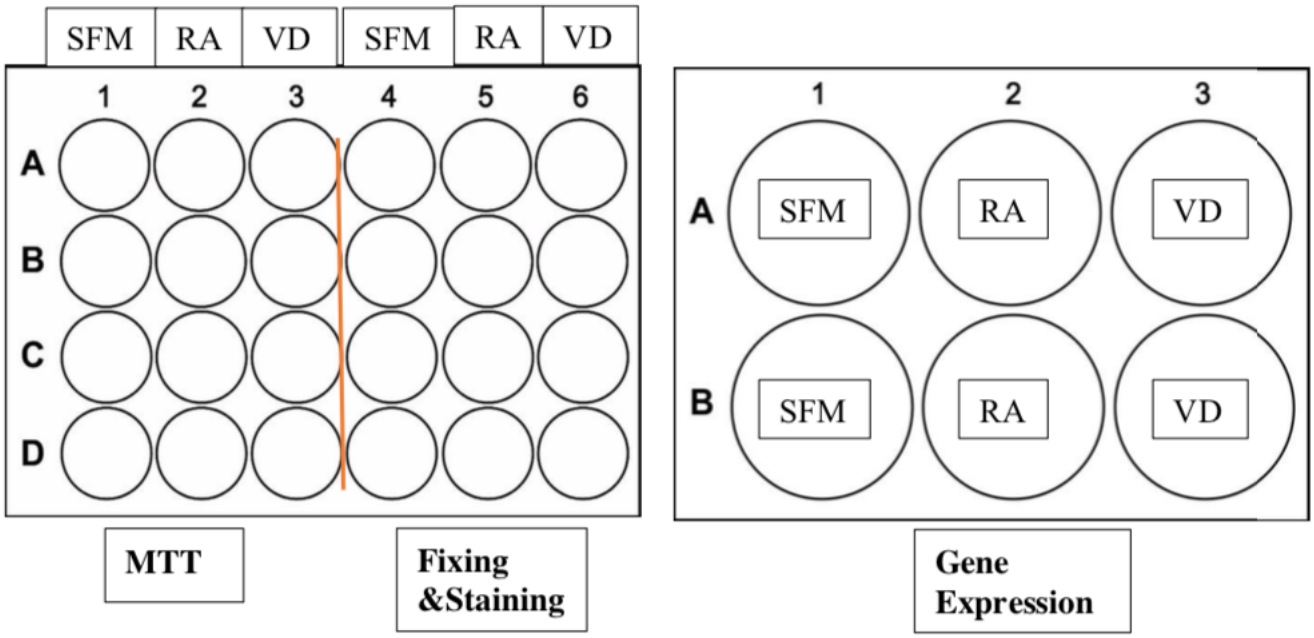

Figure 6.2 Representative figure of the distribution of the individual groups on the 24-well and 6-well plates that were used each day over the treatment time.

Experiment 3. Gene expression

For gene expression, cells were grown and harvested on a 6-well plate at 100,000 cells / 2 ml, shown in **Figure 6.2**. The cells were collected from the wells by adding TRIzol reagent (Invitrogen) for detachment. The cell count was calculated using an automated cell counter. Then, they were transferred to cryovials and stored in -80 freezer. Total RNA was extracted using TRIzol reagent and equal amounts of (1 µg) of RNA were reverse transcribed (see chapter2). Mouse gene primers for Syp and NeuroD1 was obtained from Sigma-Aldrich. Real-time PCR and QPCR were performed according to the manufacturer's protocol (for details of the gene expression protocols see Chapter 2).

Primers:

Synaptophysin

Forward (5'-3')	AGGTGCTGCAGTGGGTCTTT
Reverse (3'-5')	CGAAGCTCTCCGGTGTAGCT

NeuroD1	
Forward (5'-3')	CAAAGCCACGGATCAATCTTC
Reverse (3'-5')	GCGAATGGCTATCGAAAGACA

6.3 Statistics

Statistical analysis was performed using GraphPad Prism version 8 software (USA).

Image analysis was performed using ImageJ software (Fiji Image J 2.0, USA). Data was analysed by two-way ANOVA for multiple group comparisons, Tukey's multiple comparisons when appropriate. Normality was confirmed by D'Agostino test. Statistics were reported to show the mean standard error (SEM).

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6.4 RESULTS

6.5.1 EXPERIMENT 1. DOSE REPONSE RELATIONSHIP

MTT assay was used to measure the cell density at 24, 48 and 72 hours respectively afterVD treatment. While MTT assay shows that the 40nM dose had the least cell density, it had the most neuronal outgrowth over the course of 2 days.

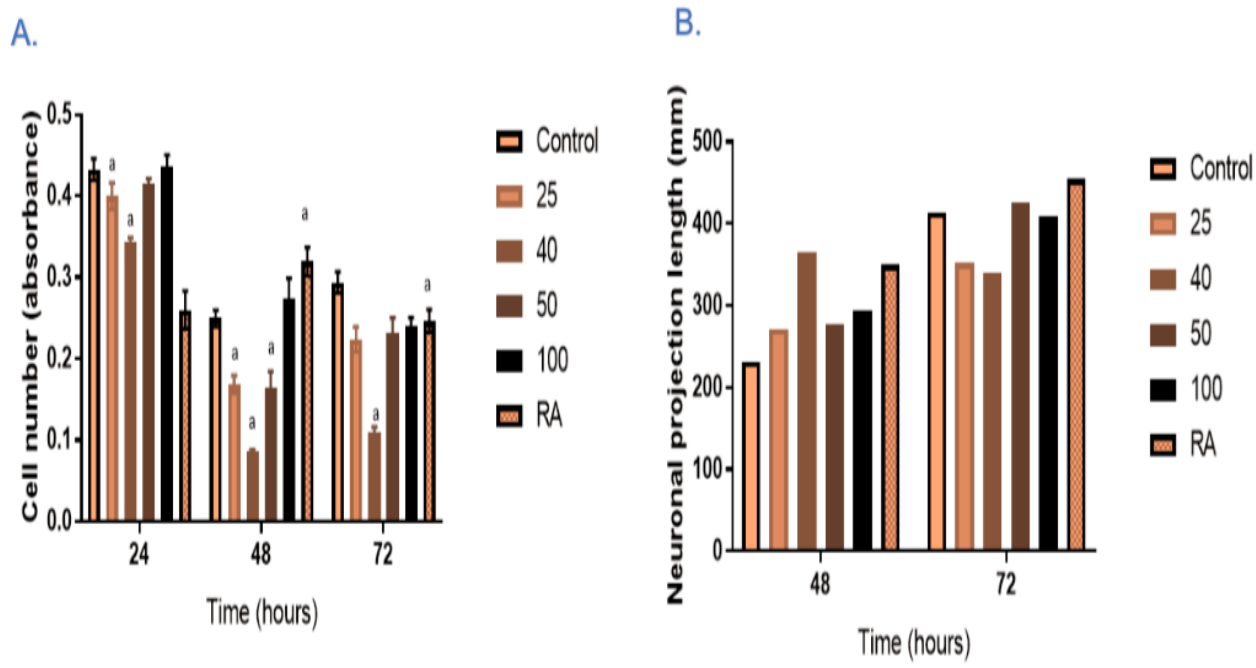

Figure 6.3 A. Data showing the cell viability of each group from the MTT assay at 570nm wavelength over the treatment time. **B.** Data showing the distribution of the neuronal projection outgrowth for each group at 48hr and 72 hr.

6.5.2 EXPERIMENT 2. PROLIFERATION: DIFFERENTIATION

MTT assay was used to measure the cell viability/metabolic activity over the time course of3 days (group x interaction $F=11.51$ $p<0.0001$). On day 2, the cell viability of RA (20μM)($p=0.0007$) and VD (40nM) ($p<0.0001$) both significantly decreased compared to control. Conversely, on day 3, the cell viability of RA ($p=0.0066$) and VD ($p=0.0008$) both significantly increased compared to control (**Fig 6.4A**). After the fixing and staining protocol, images were captured with the light microscopy and axon outgrowth was measured with ImageJ as a cell differentiation marker. On the courseof all 3 days, cells treated with RA (day1 $p= 0.0039$; day2 $p=0.0463$; day3 $p<0.0001$) and VD (day1 $p= 0.0252$; day2 $p=0.0048$; day3 p=0117) showed significantly increased axon outgrowth. Cells treated cells treated with RA (day1 p= 0.0039; day2 p=0.0463; day3 $p<0.0001$) andVD (day1 $p= 0.0252$; day2 $p=0.0048$; day3 p=0117) showed significantly increased axon outgrowth. Cells treated with RA had more axon outgrowth compared to VD on days 1 and 3 (**Fig 6.4B**).

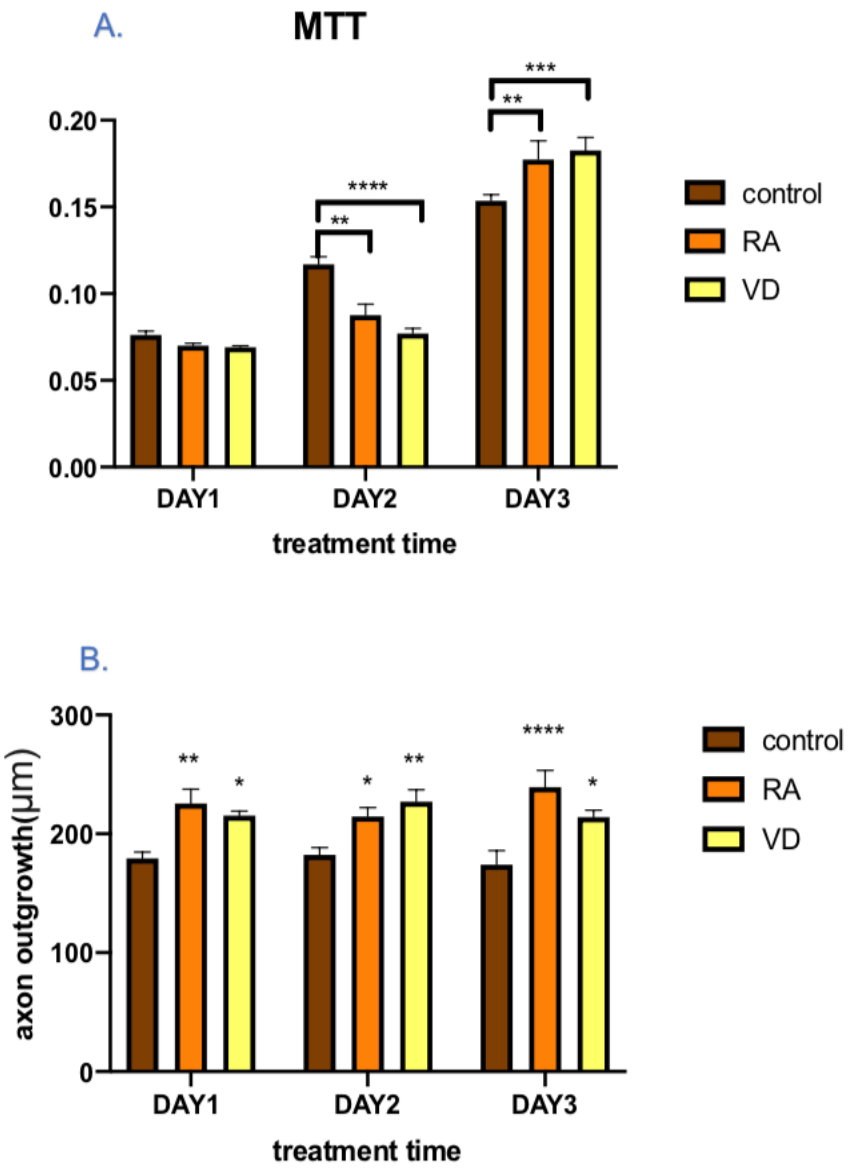

Figure 6.4 A. Data showing the MTT assay comparison of each group through the treatment time, measured at 570nm wavelength **B.** Data showing the distribution of the neuronal projection outgrowth for each group over the treatment time. Values represented as mean ± SEM. * $p < 0.05$ ** $p < 0.01$ *** $p < 0.001$ **** $p < 0.0001$

6.5.2 Experiment 3. GENE EXPRESSION

The effect of VD on specific markers of cell differentiation, Synaptophysin and NeuroD1,were evaluated. Changes in the mRNA expression levels were observed. There was no significant effect of VD on NeuroD1 mRNA expression (**Fig 6.5A**). The expression of Sypsignificantly increased in day 1 between RA and VD groups (p=0.0238) (**Fig 6.5B**).

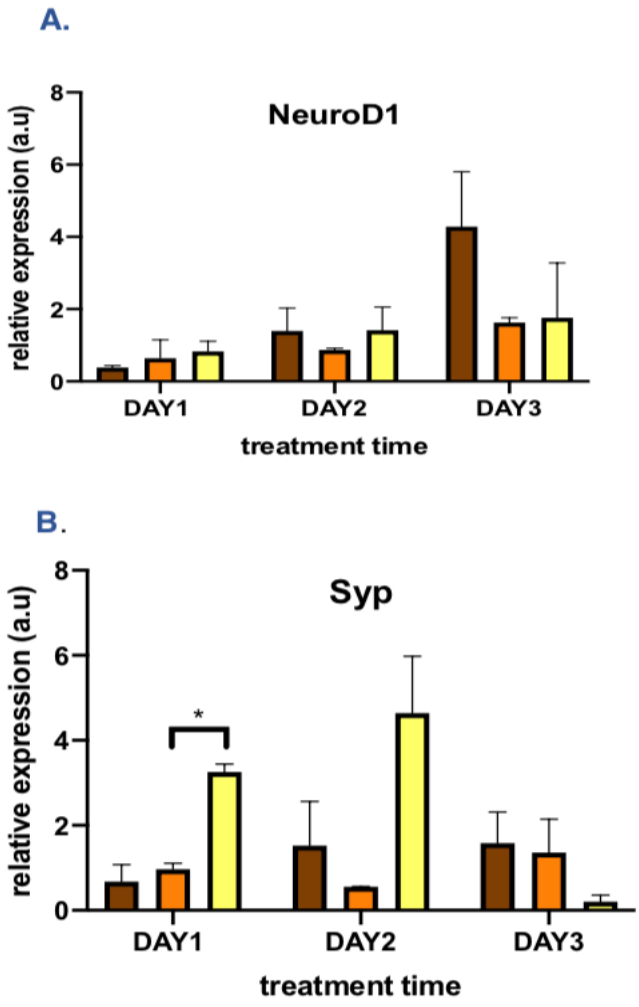

Figure 6.5 mRNA expression of **A.** NeuroD1 **B.** Syp over the course of 3 days.

Values represented as mean ± SEM. * $p < 0.05$

6.5 DISCUSSION

The aim of this study was to evaluate the cell proliferation and cell differentiation rateof N2a cells when treated with RA and VD. For this purpose, the first experiment wasconducted to observe the dose-response relationship between $1.25OH_2D_3$ and possible proliferation and differentiation rates. This allows to find the optimal dose to be used in the next experiments. After we establish the optimal dose of VD to be used,the second experiment was designed to measure the rate of cell proliferation and differentiation rate. We selected Syp and NeuroD1 as selective markers of cell differentiation and we observed mRNA expression levels for confirmation of cell differentiation.

VD is anti-proliferative, and it controls cell cycle progression. First, we used doses of25,40,50 and 100nM of VD, RA, and control, to find the optimal dose and to investigateits effects. Our study showed that 40nM dose showed the most profound effect, an increased differentiation rate compared to other doses compared to RA and the control. At 40nM, they took the morphological form of neuronal differentiation with extended axon projections. So, we decided that 40nM dose of VD is the optimum doseto be used to explore the effects of VD with N2a cells. A previous *in vitro* study observed dose and time-dependent changes of VD (doses up to 1000nM) on MCF-7. A significant decrease in cell numbers were apparent in the cells treated with 10nM $1.25OH_2D_3$ in the dose-dependent manner. The cell numbersat 48hr significantly reduced when the cells were treated with 100nM, consistent with our study. However, they found no effect on MCF-7 cell proliferation

in their previous study under the same conditions (Zheng W *et al.,* 2017). Another study using MCF-7 cell line treated with Vitamin D concluded that the decrease in cell numbers is due to either decrease in cell growth or increase of apoptosis, in a dose and time dependent manner (Saracligil *et al.,* 2017). Another study used an endothelial cell culture. They also found a dose-dependent effect of VD when they observed that it inhibited endothelial cell sprouting and morphogenesis, also had a significant effect on cell proliferation (Mantell *et al.,* 2000). There are conflicting results regarding the dose- dependent effects of VD *in vitro* (Walters, 1992). The conflicting results might be due to the different strains of cells used as in vitro models. We used doses of 25,40,50 and 100nM of VD, RA, and control. We used MTT reduction assay to measure the metabolic cell activity as a marker for cell proliferation rate (day 2 between RA-VD ** $p < 0.01$, day 3 between RA-VD *** $p< 0.001$). There is a reduction in the metabolic activity and cell viability when either cells undergo apoptosis or when they are differentiating. The linear relationship between the number of cells and the signals produced provides an accurate quantification of the changes in cell proliferation (ATCC: ScienceDirect/topics/MTTassay). In our current study, the MTT assay showed that RA and VD significantly alters cell proliferation. Interestingly, the time response showed a significant decrease in cell viability on day 2 and a significant increase on day 3. N2a cells, as mentioned before, is known to be of neuronal origin and they take a neuronal morphology as they differentiate. We measured axon outgrowth as an indication of cell differentiation. Our results showed that, the N2a cells treated with VD had significantly increased axonal projection. They take similar morphology of a differentiated neuron at each day of the treatment time.

This result was expected and is consistent with the previous studies. In our study, we did not see any significant effect on NeuroD1 mRNA expression.Each group has a tenancy to increase over the treatment time. NeuroD1 is expressedin the central and peripheral nervous systems at the time of terminal differentiation into mature neurons (Lee *et al.,* 1995). An increase in NeuroD1 expression was expected in RA and VD groups compared to control as the differentiation rate increases. There can be number of limitations regarding this. In a human embryonalcell line, NTERA-2, expression of NeuroD1 was induced when the cells becomepostmitotic (Przyborski *et al.,* 2000) and plays a major role in terminal differentiation of the postmitotic neurons (D'Amico *et al.,* 2013). Syp mRNA expression showed a significant increase on day1 compared to RA and control groups ($p < 0.05$). Our results showed that its expression decreased gradually in VD group over treatment time of 3 days. Syp is a marker of mature neural cells. Syp expression increased in phase 3 of neuronal differentiation and NeuroD1 expression in phase 2, both after the first week of retinoic acid-induced NTERA-2 cell line differentiation (Przyborski *et al.,* 2000) which seem to contradict our results.

6.7 Conclusion

We showed that the expression of VD in N2a cell line may have a dose-dependent manner.

There were couple of limitations with our study. Firstly, N2a cell line might not be the best suitable cell line for this study as they as very fast-growing and, they could not be maintained for longer times. For this reason, it may be best to use a cell line such as SHSY-5Y cell line. The SHSY-5Y is a cell line derived from the human

neuroblastoma cells. It is a cell line which can be maintained for longer treatment times.

Secondly, we planned to observe the gene expression of Sox1,2 and 3, Oct4 and Pou5f1, which are other differentiation markers that are expressed in different times of neurogenesis. Due to time limitations, it was not possible for us. However, it should be considered for future studies to understand neuronal differentiation rates upon VD administration in detail.

CHAPTER 7 GENERAL DISCUSSION

Optimal VD levels is essential during pregnancy to ensure the infant`s metabolic health via epigenetic programming. We started addressing this hypothesis by investigating the relationship of VDD and MetSyn incidence. Our meta-analysis showed a statistically significant negative association between serum concentrations of VD and the presence of MetSyn. Despite the increasing evidence on the importance of VD in health, it is difficult to know the mechanism and causality. Problems can be due to variations on data between trials such as ethnicity, obesity, age, baseline $25D_3$, measuring VD levels or dose size given. Also, incomplete data collected on physical activity, smoking, drugs can affect the outcome. Further studies needed to clarify the causality. The optimal supplementation during pregnancy is still contradictory (De-Regil *et al.,* 2012; Umaretiya *et al.,* 2017) even though VD is important especially during this period. If causality can be answered, then the adequate intake of VD can be introduced by medical authorities.

VDD is common worldwide. In adults, it is hypothesized that VDD is emerging as a risk factor for components of the MetSyn including obesity and diabetes (Foss, 2009) in addition to complications in pregnancy (Christesen *et al.,* 2012). There is a high prevalence of maternal VDD among pregnant and lactating women due to various ethnicities, living in different latitudes and limited access to sunlight, change of lifestyle and minimal intake of VD via diet. It is well known that maternal environment can affect later life health, particularly the development of MetSyn. Therefore, VDD during prenatal stage may predispose individuals. As well as affecting the offspring metabolic health, thedeficiency, having *less than 20 nmol*/L VD levels during pregnancy is associated with high risk of pre-eclampsia, low birth weight, caesarean section, preterm delivery, and gestational diabetes (Urrutia *et al.,* 2012).

It is currently recognized that *in utero* environment plays a key role in the pathogenesis and predisposition to diseases in later life, which seem to propagate to subsequent generations (Kong *et al.,* 2020). The primary aim of this thesis was to investigate the long- term effects of VDD on offspring's metabolic health and behaviour and determine the potential underlying mechanism. We hypothesized that VDD at PPS effects the offspringhealth and behaviour via epigenetic mechanisms.

Exposure to sufficient VD during early development is thought to be critical for neurodevelopment (Pet *et al.,* 2016) and yet no public health intervention has been designed considering the number of women of childbearing age deemed to have VDD isincreasing in both developing and developed countries. Results from an epidemiological study of 346 multi-ethnic, pregnant women living in Northwest

London has shown that 18% of them had ‘adequate’, 36% ‘deficient’ and 45% had ‘insufficient’ VD levels (McAree*et al.,* 2013). Evidence has shown fetuses exposed to insufficient maternal VD levels have low birth weights, are small for gestational age, have reduced bone growth, poor skeletal mineralization, and lower bone mineral content (Mahon *et al.,* 2010). Thus, maintaining optimal levels maybe crucial.

VDR is present in a wide range of tissue including the brain, and human observational studies suggested that VD may play a role in various neuropsychiatric and neurological disorders including depression (Penckofer *et al.,* 2010), schizophrenia (Sheikhmoonesi *et al.,* 2016), attention deficit disorder (Naeini *et al.,* 2019), Alzheimer’s disease (Sato *et al*, 1998), dementia (Oudshoorn *et al.,* 2008) and Parkinson's disease (Knekt, 2010). To further support this, foetuses exposed to VDD have enlarged brains possibly due to decreased levels of apoptosis, increase meiosis and decrease in the levels of theneurotrophic factors (Hawes *et al.,* 2015). The complications that arise due to VDD are discussed in the introduction section in detail.

Metabolic conditions such as diabetes is associated with increased risk of anxiety-related disorders (Smith *et al.,* 2012). VDD in early life has broad effects throughout the body. So, we have looked at the effects of maternal VDD on offspring metabolic health. Our meta-analysis showed a statistically significant negative association between serum concentrations of VD and the presence of MetSyn. VDD is associated with MetSyn, having at least 3 conditions of abdominal obesity, high blood pressure, high blood sugar,high serum triglycerides, and low high-density lipoprotein levels. Lu and colleagues and Ford and colleagues found a significant inverse

association between serum $25D_3$ concentrations and developing MetSyn (Lu *et al.,* 2009; Ford *et al.,* 2005). Our results are consistent with these previous findings. In a predictive study of a cohort of 524 non - diabetic adults, the higher baseline serum $25D_3$ concentration, the higher the risk of developing MetSyn 10 years later (Cheng *et al.,* 2010). So, this correlation between MetSyn and VD is highly important for offspring's future metabolic health later in life.

Maternal VDD exposes the placenta to higher levels of glucose and lower insulin (Hay Jr*et al.,* 2006). Indeed, children aged 5-19-years exposed to an environment of abnormal glucose intolerance *in utero* had a significantly higher weight and glucose concentration following challenge, than those who didn't (Pettitt *et al.,* 1983). Thus, to study the relationship between metabolic state and VDD, we developed a rodent model. We observed offspring born to VDD dams weighed less at birth and by postnatal day 11 were heavier than the control mice. Interestingly, further analysis confirmed that this change isdue to reduction in energy metabolism, not due to the food intake. Furthermore, we observed a significant increase in anxiety and a tendency towards decreased memory and learning. *Bax* gene is required for most of the adult-born neuronal cells to undergo apoptosis before reaching maturity (Kim *et al.,* 2009). A study used a *Bax* null mouse demonstrated that increased neurogenesis leads to reduced anxiety in mice, promoting antidepressant like behaviour (Hill AS et al., 2015). Moreover, several studies on both mice and rats showed that VDD during adulthood affects range of brain functions from behavioural to neurochemical (Groves *et al,* 2013: Byrne JH et al, 2013). Consumption of a VDD diet for 10 weeks impaired behavioural phenotypes in both C57BL/6J and BALB/cmice. Furthermore, Yates and colleagues showed that VDD during pregnancy and lactation impaired

learning and memory of the adult male offspring rats (Yates *et al,* 2018).Although the behavioural findings have been inconsistent and dependent on the background strain, our results in the current study are in line with the research mentioned above.

However, there is a limitation to our study. The VD levels of dams and off-spring were not confirmed. An enzyme linked immunosorbent assay, ELISA, would confirm that the female mice were serum VDD for the accuracy of the study. The concentrations would confit the level of deficiency, as it well known that VD can be stored in tissue in the body, meaning that despite being on a VDD diet the mice many have not been VD. VD is stored in many tissues throughout the body, mainly the adipose tissue. A double-blinded, prospective study found that when a weekly supplementation of 20.000IU of VD for 3-5 years stopped, VD group has significantly higher concentrations of serum 25D3compared to placebo, even after 12-month of follow-up period (Martinaityte I et al.,2017). Although the results of animal models cannot be directly correlated to humans, obtaining the levels of serum VDD levels would be essential for validating that the results of the experiments presented in this thesis. The changes in metabolic state of the offspring can demonstrate that the prenatal VDD can increase the susceptibility for obesity and DMT2 which are disorders strongly dependent on bodyweight (i.e., BMI, waist circumference). Figure below is a schematic of the core components of MetSyn. The World Health Organization (WHO) first developed its definition in 1998 (Alberti and Zimmet, 1998). They believed strongly that insulin resistance is at the core of MetSyn i.e., fasting glucose levels, HOMA-IR. In addition to the insulin resistance, two of the following had to be met: obesity, dyslipidaemia, and hypertension. So, the essential core components of the MetSyn came together by the definition of WHO. European Group for the study

of Insulin Resistance (EGIR) agreed with this current definition to require the two additional criteria from listed above. EGIR simplified insulin resistance to only fasting insulin, while WHO used waist-to hip ratio or BMI. In 2001, the National Cholesterol Education Program (NCEP) Adult Treatment PanelIII (ATP III) devised a definition for the metabolic syndrome (National Cholesterol Education Program, 2002), which was updated by the American Heart Association and the National Heart Lung and Blood Institute in 2005 (Grundy et al., 2005 : Huang PL, 2009).

Figure also shows the potential roles of VD, written in purple. Hyperglycaemia triggers the pancreas to produce insulin. The skeletal muscle, liver and adipose tissue impacts serum glucose. In insulin resistance, these tissues do not respond appropriatelyto insulin. In the skeletal muscle, VD regulated normal calcium influx and regulates these processes. VD, increases insulin secretion in pancreas, increases adipocyte apoptosis inadipose tissue. It also regulates FFA metabolism in

these tissues. In response to hypertriglyceridemia, there is an increase in VLD production and decrease in its renal clearance. VD contributes to its breakdown

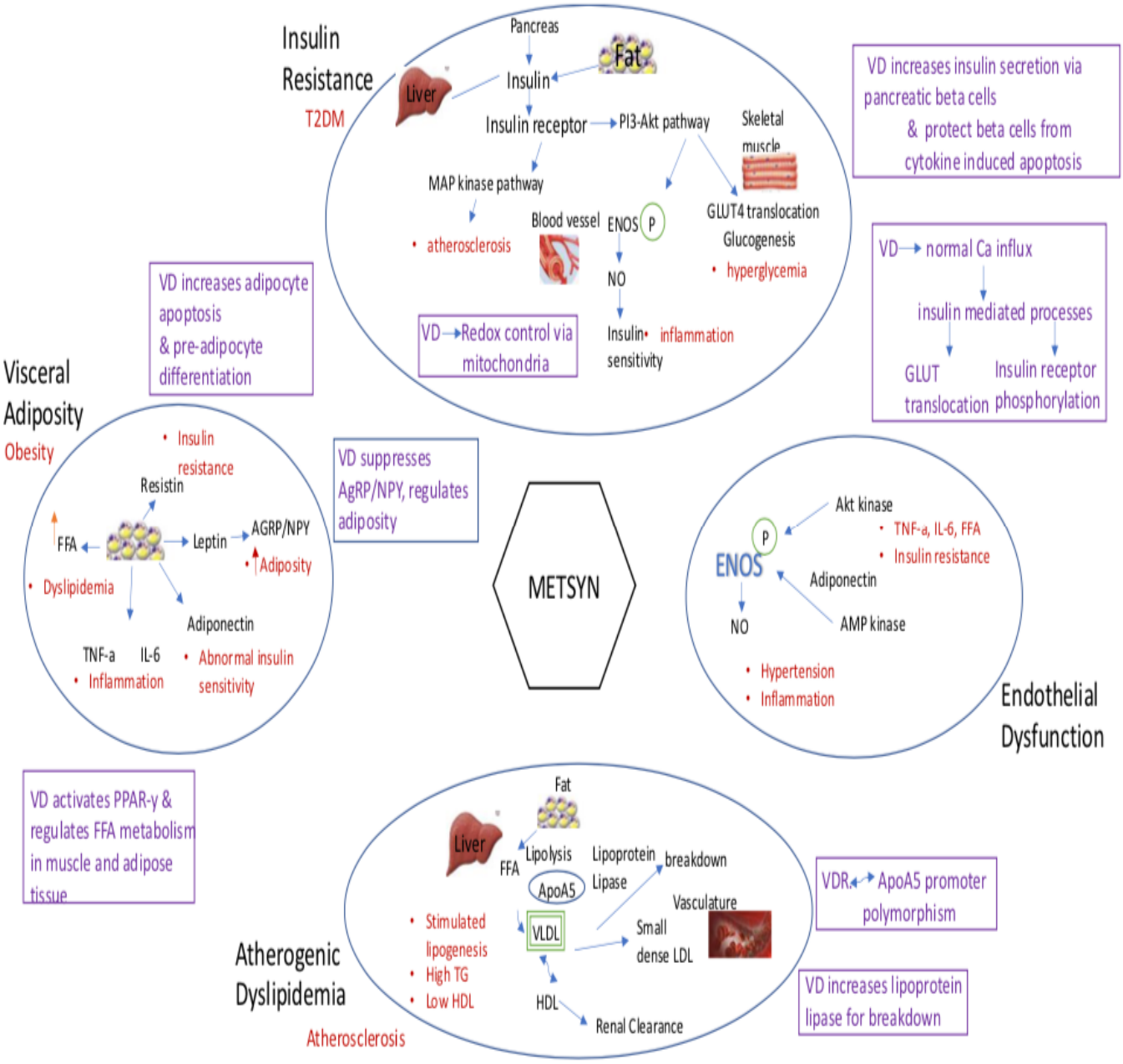

Figure 7.1 *Adapted from Disease Models and Mechanisms, 2009.* A figure summarizing the pathophysiology of Metabolic Syndrome and the relevant functions of VD. The NCEP ATP III has the most comprehensive and widely used definition of MetSyn for diagnosis. The key features are visceral/abdominal adiposity, endothelial dysfunction, atherogenic dyslipidaemia and insulin resistance. The text written in purple represents the relevant roles of the VD. The text written in red are the pathology, the consequence of the abnormality in the tissues. The blue arrows represent the cascade of mechanisms. P = phosphorylated

In the current study, we could not look at the molecular mechanisms underlying the changes in bodyweight and adiposity, but we speculate that that they may be due to the altered expression of the Agouti-related protein (AgRP) in the brain. Hypothalamic AgRP neurons are known to orchestrate complex behaviours in mice such as food intake and energy balance. Accumulation of systemically administrated AgRP in liver, adipose tissue and the adrenal gland shows that it has a role in regulating energy balance (Stutz AM et al, 2005). Indeed, hypothalamic AgRP has been found to be elevated in obese and diabetic mice (Shutter JR et al, 1997). AgRP is associated with negative state of energy balance. Recent studies showed that AgRP influences systems beyond feeding. AgRP can regulate HPA axis: i.c.v administered AgRP enhanced the ability of the interleukin IL-1β to increase ACTH, possibly by affecting hypothalamic α-MSH receptors (Xiao *et al.,* 2003). VD normally suppresses AgRP/NPY (Farhangi *et al.,* 2017) (**Fig 7.1**). VDD favours visceral adiposity through stimulation of AgRP by suppressing POMC. Interestingly, increased visceral adiposity has been linked to abnormal cognitive function but AgRP

hasbeen shown to effect dopamine cell function independent of adiposity (Dietrich *et al.,* 2012).

Inflammation is another reoccurring MetSyn phenomena. Increased adiposity and impaired energy metabolism in VDD mice are likely to cause hyperglycaemia, dyslipidaemia, and insulin resistance. The increased ROS in the body, through the NF- κB pathway, leads to impaired mitochondrial respiratory chain reaction, thus, increased peripheral oxidative stress (Wang Y et al, 2014). Studies on cancer cell lines on mitochondrial function of VDR have demonstrated that VD suppresses mitochondrial (Consiglio et al*.,* 2015: Ricciardi *et al,* 2015). Abnormal amounts of ROS causes number of chronic inflammatory diseases including atherosclerosis (Hulsmans *etal.,* 2012), hypertension (Montezano *et al.,* 2015), diabetes (Ahmad *et al.,* 2017), obesity(Ghosh *et al.,* 2011) and NAFLD (Spahis *et al.,* 2017). Several studies confirmed that diabetes and obesity are associated with increased pro-inflammatory markers such as TNF-alpha, CRP, and IL-6 so they can be considered as inflammatory conditions (Dandona *et al.,* 2003).

The recent studies correlating VD to mitochondrial metabolism may suggest that the negative metabolic effects of VD could be the result of mitochondrial modulation. Pregnancy is a state of increased oxidative stress. The systemic inflammatory response results in high amounts of ROS (Burton and Jauniaux, 2011). Oxidative stress in the brain causes nervous system impairment and plays a significant role in neurological diseases (Mosley *et al.,* 2006). Supported by both *in vitro* (Holmes *et al.,* 2013) and *in vivo* (da SilvaMachado *et al.,* 2016) studies, VD is thought to have an antioxidant function and remove the ROS. Therefore, lack of sufficient VD can contribute to neuronal damage. Farhangi and colleagues showed

that VD administration reduced food intake and weight gain whilealso reducing the inflammatory markers IL-1, IL-1β, NF-Kβ and acetylcholine concentration and BDNF concentration in hippocampus and hypothalamus in the high-fat diet induced obese rats (Farhangi *et al.,* 2017). While Dursun and colleagues reported that VD significantly lowered iNOS mRNA expression in primal hippocampal neurons after 48hr treatment (Dursun *et al.,* 2014). Obesity can induce brain inflammation and is associated with cognitive impairment including spatial learning (Boitard *et al.,* 2014) andanxiety (Bouayed *et al.,* 2009). Therefore, it is possible that impaired energy metabolism and increased susceptibility to weight gain and obesity might cause increased anxiety and impaired memory in maternally VDD mice.

Altered cell proliferation and differentiation in the hippocampus is the potential mechanismbehind behavioral changes. Following this, we wanted to determine the impact of long- term VDD on adult neurogenesis by assessing the number of Ki-67^{+} and DCX^{+} expression in the hippocampus of VDD mice. Our study showed that VDD at PPS did not have any effect on proliferation in DG (Ki-67+expression) while differentiation (DCX+ expression) significantly decreased at 3 weeks of age in females. However, the effect was not present at 8 weeks of age in males. Our findings have demonstrated that exposure to VDD at PPS had noeffect on cell proliferation in the adult DG. As the effect of VDD on Ki-67^{-}positive cell number wasn't significant in pups, the developing hippocampus may be capable of compensating for a lack of circulating VD postnatally, as supported by current literature, where VDD augmented proliferation prenatally but not postnatally (Ko *et al.,* 2004). In the SVZ, VDD at PPS significantly decreased proliferation (Ki-67+ expression) at 3 weeks ofage in females. Interestingly, it significantly increased at 8

weeks of age in males. Differentiation (DCX+ expression) significantly decreased at 3 weeks in females, however, did not have any effect at 8 weeks of age in males. So, treatment with DCX and Ki-67 markers showed that cell proliferation and differentiation is altered in both DG and SVZ, therefore, VDD at PPS altered adult neurogenesis. In corroboration, Zhu and colleagues showed that VD3 1α-hydroxylase KOmice had decreased neurogenesis at 8 weeks of age (Zhu *et al.,* 2012). They developed VD3 1α-hydroxylase KO mice, which cannot synthesize calcitriol, had a 50% increase in the rate of proliferation whereas neurogenesis was 50% lower when compared to WT controls. While Cui and colleagues showed an increase in the number of neutrospheres and clusters of NSCs in the SVZ at birth in mice born to dams provided VDD diet during pregnancy only, indicating an increased rate of neural proliferation (Cui *et al.,* 2007). While some of these studies agree with our results, it's important to note that virgin female mice were placed on a VDD diet prior to mating and offspring remained on this diet over following birth and throughout the study.

In addition, we looked at the mRNA expression two of important neurotrophic factors, BDNF and VGF in both hippocampus and hypothalamus. VDD at PPS had no effect on VGF gene expression in hippocampus of 3-week-old female mice but significantly increased at 8 weeks of old male mice. In the hypothalamus, VGF mRNA expression had significantly decreased then significantly increased at 8 weeks old male mice in male mice. BDNF mRNA expression had no effect at 3 weeks of age in the hippocampus of female mice, however it significantly increased at 8 weeks of age in male mice. In the hypothalamus, VDD at PPS had no effect on the expression of BDNF. Here we showed that interestingly BDNF expression significantly changes in

hippocampus however, not in the hypothalamus at 8 weeks of age in males. The overall aim of this experiment was to assess the proliferation and differentiation rates in the hippocampus and hypothalamus, regions of high plasticity, to see if VDD at PPS influenced these rates that might affect the offspring's future cognitive health. We did cell counting in DG and SVZ, two of the main regions that adult neurogenesis occurs, to determine the change in the proliferation and differentiation of neurons in the brain. Then, we measured the gene expression of neurotrophic factors BDNF and VGF. We were particularly interested in these two neurotrophic factors because of their roles in energy metabolism and cognition(Noble, 2011; Lewis *et al.,* 2015), with regards to our findings from Chapter 3. Neurotrophic factors are a family of secreted proteins which were extensively studied for their roles during development, supporting proliferation, survival, and differentiation of the neurons.However, now it is known that some also have important roles in adult nervous system regarding neuronal physiology. BDNF is a unique member of neurotrophic factors which impacts proliferation, differentiation, survival, and death of both neuronal and other cell types (Lee *et al.,* 2001). Both BDNF and VGF have robust expression and immunoreactivity in the CNS, particularly hippocampus and hypothalamus, in the developing and mature nervous system (Katoh-Semba *et al.,* 1997; Kawamoto et al*.,* 1996). Neurotrophic factors, especially BDNF is involved in plasticity processes during adulthood. So, with regards to our findings, it is expected to see a change in the expression of BDNF and VGF in the hippocampus. The significant increase of VGF mRNA expression we have observed in both tissues might also be due to the neuroprotective effects of VGF (Noda *et al.,* 2019). Furthermore, VGF is known to be induced by overexpressing BDNF. So, this may also contribute to the

significant findings we have in the hippocampus. It is important to note that hypothalamic knockdown of VGF in the adult mice was found to increase adiposity, decrease core body temperature, reduce energy expenditure, and disturb metabolic processes without effects on food intake (Foglesong *et al.,* 2016). This highly important role of VGF in the hypothalamus might explain the significant changes we observed in VGF mRNA expression in the hypothalamus independent of BDNF. The changes that we have seen between offspring that are 3 weeks, which are juvenile, and 8 weeks of age, which are adults, might suggest a compensatory mechanism. However, due to the difference in sex between these two time points we cannot rule out an effect of sex.

One of the molecular mechanisms that regulate neurogenesis is epigenetic programming.Appropriate epigenetic regulation of the genome during this period via epigenetic markers such as DNA methylation and histone modifications and noncoding RNA is crucial for expression of the genes essential for development.

At a cellular level, epigenetic reprogramming is essential for the functions such as proliferation, differentiation, cell lineage commitment and survival (Hemberger *et al.,* 2009). Although some epigenetic states are reversible, some of these dysfunctions of the epigenome are permanent and can persist into adulthood, thus lead to neurological and metabolic disorders. VD can interact of the epigenome as discussed in detail in chapter 4. Human studies report conflicting findings: Cohort studies from Norway (Suderman *et al.,* 2016) and UK (Curtis *et al.,* 2019) showed no significant correlation between maternal VDD and DNA methylation status at birth. Although the studies had a large sample size the existing hypothesis that maternal VDD effects offspring's epigenome should not be excluded. The methodology used to

measure sensitive epigenetic changes is challenging. Indeed, it is possible that the changes were too sensitive/weak to detect in the cord blood or that theywere more pronounced in another tissue. Also, the measurements were either taken or normalised to 18 weeks and 28 weeks gestational periods. A more throughout measurement during pregnancy and a follow-up during the postnatal life of the offspring might support a different conclusion. Lastly, serum VD levels fluctuates during each trimester (highest in third trimester) and the maternal diet. So, the weak associations found might be due to the higher socio-economic status of these countries which might supplement with VD during pregnancy. On the other hand, a Midwestern US cohort case/control study showed significant changes in DNA methylation in infant leukocytes upon maternal supplementation of 3800IU vs 400IU VD during pre- and post-natal development, starting from the second trimester (Anderson *et al.,* 2018). Junge and colleagues also reported a significant association between low serum VD levels and DNAmethylation kin children in the first 3 years of life (Junge *et al.,* 2016). Considering the ethical limitations of the human studies (seasonal, cultural, diet, VD levels during pregnancy etc.) and the previous findings from *in vivo* and *in vitro* studies, the evidence supports the correlation between VDD and the epigenetic changes. Further research to fully understand the underlying mechanisms will contribute to informed clinical advice onprenatal care and support the mother and the offspring's health. Several rodent studies resulted in significant correlation between maternal VDD and DNA methylation (Wen *et al.,* 2018; Zhang *et al.,* 2014). Another way which VDR can interact with epigenome is through recruitment of coactivators and corepressors which lead to genome-wide changes in chromatin patterns (Meyer MB et al, 2014). A study has shown that VD levels are

inversely correlated with methylation levels at the p21 gene promotor in human mammary epithelial metastatic carcinoma cell cultures. The transcriptional regulation a the p21 was dependent on histone deacetylation and recruitment of the histone demethylases HDAC4 and LSD1 (Saramaki *et al.,* 2009). In our study, we found that VDDat PPS significantly increased the expression of several histone demethylases*, KDM5A, KDM6B, UTX* in the hippocampus and hypothalamus of 3 weeks and 8 weeks old mice. VDD at PPS did not influence the expression of Ezh2, the histone methyltransferase, in hippocampus at 3 weeks and 8 weeks of age. Maternal VDD did not influence the expression of Ezh2 in hypothalamus at 3 weeks however, the expression of Ezh2 significantly increased at 8 weeks of age. Our results are consistent with the previous research on VD treatment (Pereira *et al.,* 2012; Fu *et al.,* 2013) and maternal VDD modifications (Xue *et al.,* 2016) regulating epigenome through histone modifications. However, it is important to note that our study is the first to use VDD dams during pregnancy through lactation and gestation and the offspring stayed on a VDD diet throughout the experimental duration. Methylation of histones are important for the regulation of gene activity. KDM6B, UTX, KDM5A and Ezh2 (de)methylate H3K27me andH3K4me which keeps the promoters in repressive and active state respectively. The balance between H3K27 and H3K4 keeps the developmental genes in a poised state. Upon activation of the genes, cellular differentiation occurs. KDM6B promotes demethylation of neuronal genes including neuronal migration protein Doublecortin,which induces neuronal differentiation.

Our findings show that the gene expression of tyrosine hydroxylase, TH, is increased in hypothalamus of the 8 weeks old maternally VDD mice. This result has not been included; however, it is important to note. TH catalyzes the first reaction in the

catecholamine synthesis pathway and TH gene expression is regulated by regulatory mechanisms including epigenetic, transcriptional, and posttranscriptional levels (Lenartowski and Goc,2011). TH gene, together with genes for insulin and insulin growth factors, are distinguished in human pancreatic **β** cells by increased levels of histone acetylation and H3K4 di-methylation. *TH* has a positive role in energy homeostasis. Optogenetic stimulation of the arcuate TH neurons in hypothalamus has increased food intake and caused weight gain in rodent model (Zhang and van den Pol, 2016). *TH* is also associatedwith the release of dopamine hormone. VDR has been shown to be expressed in TH+ neurons in the brain (Cui *et al.,* 2013); also $1.25OH_2D_3$ has been shown to increase the expression of TH *in vitro (*Puchacz et al*.,* 1996) *and in vivo* (Trinko *et al.,* 2016). Gene expression of TH in the brain has significantly reduced at E17.5 in maternal VDD mice (Hawes EJ et al., 2015). The gradual increase of TH expression in hypothalamus over time suggests that VD can influence the expression of TH, therefore can affect energy and glucose homeostasis in the body, which is consistent with our previous results (see chapter 3).

In vitro studies are well suited for observing biological processes and are being used extensively in studies of neurotoxicity and chemical responses. They can provide direct information on mechanistic processes eliminating the complex experimental questions involved in designing *in vivo* experiments (Macleod *et al.,* 2015; Mignini *et al.,* 2006). To confirm the effects of VD on proliferation and differentiation of neurons using in vitro tissueculture using N2a cells. We measured cell proliferation by using MTT assay and cell differentiation by measuring axon outgrowth. We also analyzed the expression of neurogenic transcription factors NeuroD1 and Synaptophysin. To start with, we conducted a dose-response relationship study between VD and the

proliferation and differentiation rates in order to find the optimal dose of VD to be used in further experiments. Among different doses used (25,40,50 and 100nM of VD, RA and control), 40nM gave us the most profound effect. The MTT assay showed that RA and VD significantly alters cell proliferation (group x interaction $F=11.51$ $p<0.0001$). We measured axon outgrowth as an indication of cell differentiation. Our results showed that, the N2a cells treated with VD (day 3; p=0117) had significantly increased axonal projection. Furthermore, we measured at the gene expression levels Synaptophysin and NeuroD1 as selective markers of cell differentiation and we observed mRNA expression levels for confirmation of cell differentiation. Syp mRNA expression showed a significant increase on day 1 compared to RA and control groups. Our results showed that its expression decreased gradually in VD group over treatment time of 3 days and there were no changes in expression of NeuroD1. Limitations are discussed in detail chapter 6. Other markers of differentiation such as Sox1,2 and 3, Oct4 and Pou5f1 can be measured for confirmation of cellular differentiation rates upon VD administration. However, I believe this data paved a way towards measuring the *in vitro* effects of VD on cellular proliferationand differentiation using N2a cell line.

LIMITATIONS AND FUTURE WORK

- The most pronounced limitation of our study is the sample numbers. We might have relatively small numbers of mice; however, we found several significant data thus making our findings robust. For metabolic changes, we need power analysis. For example, we do not have significant results for the SAY test. Sample size calculations were not carried out for this study however, these data can form the bases of sample size calculations for future studies to assess the molecular effects of the VDD in the brain.
- Due to breeding issues, the 3-week-old mice were females, and the 8 weeks old mice were males. In C57BL/6J mice, Roughton *et al* (2012) found 4-week $BrdU^{+ve}$ and DCX^{+ve} cell numbers in the DG were significantly higher in females versus males. However, not all studies have reported a significant gender effect (Lagace *et al.,* 2007).
- ELISA assay has not been conducted to confirm the VD serum levels of both dams and the offspring. It is recommended this to be measured for further studies.

i) It is hard to differentiate between the acute, short-term effects or long-term effects of the VDD at PPS on the offspring during pregnancy and in adulthood. So, future studies should consider exploring the impact of VDD with an *in vivo* study with larger sample sizes and experimental groups with: :

ii) Measurements at different stages of development i.e., embryonic, at birth, juvenile and adulthood.

iii) Analysis within both genders and different ages.

Based on our study, future research also can:

- look at subsections of hypothalamus to understand the mechanisms of how VDD impacts the central network of energy metabolism.
- measure the gene expression levels of other neurotrophic factors, i.e., NGF.
- measure the gene expression levels of the histone (de)methylases *in vitro* and the gene expression of other proliferation and differentiation markers mentionedin Chapter 6.
- investigate the impact of VDD on inflammation by measuring blood levels of cytokines in the offspring.

CONCLUSION

Accumulating evidence suggests that maternal VDD affects the development of the vital organs and tissues of the foetus during development. To date, there are only few studiesthat have observed the effects of maternal VDD on offspring's metabolic health and cognition and it resulted in controversial findings. The data reported in this thesis enhances our understanding of the effects of VDD at PPS on offspring health and gives an insight to potential underlying mechanisms. Given the significant correlation between metabolic health, cognition and maternal VDD, further research is needed, potentially with larger sample numbers, in hope that it will direct the authoritative bodies to set definite cut-off points for VDD. Hence making diagnosis and treatment more efficient and enhancing public health.

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