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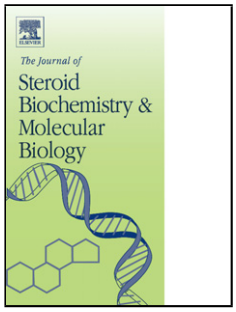

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Review

Developmental Vitamin D deficiency and Autism: Putative pathogenic mechanisms

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Contents

Highlights

- Developmental Vitamin D deficiency during early periods of brain development is linked to Autism
- Developmental Vitamin D deficiency alters response to inflammation
- Vitamin D deficiency alters steroidogenesis
- Developmental Vitamin D deficiency alters foetal brain anatomy
- Developmental Vitamin D deficiency produces behavioural phenotypes relevant to autism

Abstract

Autism is a neurodevelopmental disease that presents in early life. Despite a considerable amount of studies, the neurobiological mechanisms underlying autism remain obscure. Both genetic and environmental factors are involved in the development of autism. Vitamin D deficiency is emerging as a consistently reported risk factor in children. One reason for the prominence now being given to this risk factor is that it would appear to interact with several other epidemiological risk factors for autism. Vitamin D is an active neurosteroid and plays crucial neuroprotective roles in the developing brain. It has important roles in cell proliferation and differentiation, immunomodulation, regulation of neurotransmission and steroidogenesis. Animal studies have suggested that transient prenatal vitamin D deficiency is associated with altered brain development. Here we review the potential neurobiological mechanisms linking prenatal vitamin D deficiency and autism and also discuss what future research targets must now be addressed.

Keywords: Vitamin D, Autism, Brain, neurodevelopment, mechanisms

1. **Introduction**

Autism comprises a heterogeneous group of disorders together referred to as Autism Spectrum Disorders (ASD). ASD includes autistic disorder, Asperger's syndrome, Rett's syndrome, childhood disintegrative disorder and pervasive developmental disorders not otherwise specified. ASD was introduced to Diagnostic and Statistical Manual-III in 1980 (Mayes and Horwitz 2005). It was first described by the American mental health clinician Leo Kanner as a condition of extreme aloneness (Kanner 1968). ASD is characterized by impairments in social interaction, lack of verbal and non-verbal communications, stereotyped repetitive behaviours and hyperactivities to sensory inputs [1]. The lifetime prevalence of ASD is about 1 in a 100. Signs of ASD usually seen before two years of age and persist into adulthood [2]. Some studies claimed diagnosis of autistic features in children younger than 1 year [3, 4]. It has become a substantial socio-economic burden on modern societies [5]. The Global Burden of Disease has ranked ASD within the scope of mental disorder burden in the 1993 World Development Report [6]. It is the leading cause of disability in children under five years of age, and ASD alone is among the 20 leading causes of disabilities from all diseases and injuries in Western Australia [7]. Pronounced increase in its prevalence has been observed during the last 20 years [8]. Some researchers have claimed that this apparent rise in the prevalence is due to a greater level of awareness in parents or due to the diagnostic criteria having been changed [9, 10] while some researchers believe that this increase is a true reflection of the situation, citing an alteration in external environmental risk factors as responsible for this sharp increase [11, 12]. In this review we will concentrate on the emerging evidence that shows how developmental vitamin D (DVD) deficiency may exacerbate numerous events believed to be operating in this disease and suggest future research targets aimed at clarifying this relationship.

2. Maternal vitamin D deficiency as a risk factor for ASD

The association between vitamin D and ASD was first proposed by Dr. John Cannell [13]. Epidemiological studies have suggested a potential role for vitamin D deficiency in the development of ASD [14-18]. A California based study found that children conceived in winter have a 16% elevated risk of ASD diagnosis compared with the children conceived in summer [19]. The effect of season on ASD occurrence has been replicated in other studies based in Boston, Denmark and Sweden [20-22]. These authors concluded that deficiency of vitamin D in pregnant women due to less ultraviolet B radiation may be a plausible etiological factor for the high prevalence of ASD in children conceived in winter. More

importantly, there is direct analytical epidemiological support for this proposal. In a recent systemic review, 9 out of 12 studies clearly show significantly lower levels of vitamin D in ASD children compared to controls [14]. Another meta-analysis of over 800 autistic individuals found that individuals with ASD had lower vitamin D than the healthy controls [23]. Increased prevalence of ASD has also been found in the children of vitamin D deficient Somali mothers [15]. Maternal vitamin D deficiency is more profound in the children of dark-skinned mothers unable to synthesise sufficient vitamin D [24].

There is also indirect support for maternal or DVD-deficiency increasing ASD risk. In a study in Swedish born autistic and healthy siblings who shared the same environment and genetic background, siblings born in spring were more likely to develop ASD than the sibling born during other seasons. Interestingly, in a later study autistic siblings had significantly lower blood level of vitamin D at birth compared with non-affected siblings [25].

3. Other risk factors for ASD

Like all complex psychiatric conditions, ASD involves both genetic and environmental factors which interact to presumably adversely affect brain development. Abnormalities have been found in multiple genes functionally linked with ASD each with small individual effects [26]. ASD is a familial and highly (50-80%) heritable disorder [27, 28]. Monozygotic twins have high concordance rate (50%) in comparison with dizygotic twins (15-20%) [29, 30]. Incomplete concordance rates for monozygotic twins and phenotypical variabilities indicate the likely influence of environmental risk factors in the development of ASD. A growing body of evidence has revealed the association of several prenatal environmental risk factors for ASD such as advanced paternal age, being male, obstetric complications, maternal infections and stress during pregnancy [31] [32] [33].

Evidence indicates that the offspring of aged parents and grandparents are at increased risk of ASD [34-36]. *De novo* mutations in the male germ cell line due to advanced age is a possible explanation of high occurrence of ASD in such children [37-39]. ASD is four to five times higher in males than in females [40]. The reasons behind gender biased ASD prevalence are still not clear. Sex steroids particularly testosterone and estradiol may have a role here as they have been shown to differentially regulate novel ASD related genes, for example retinoic acid-related orphan receptor-alpha [41].

Obstetric complications may increase the risk of ASD. Obstetric complications linked with ASD include maternal bleeding, gestational diabetes, preeclampsia and maternal hormonal

imbalances [42-46]. Infection during pregnancy is also a well-recognized environmental risk factor for ASD [47]. Maternal viral infection in the first trimester and bacterial infections in the second trimester are highly associated with ASD [48, 49]. Maternal stress during mid to late gestation is associated with increased risk of ASD. Studies showed that fetuses exposed to stress during last two trimesters had 3.8 times higher risk of developing ASD [33, 50].

An under-recognised feature of this diverse risk-factor epidemiology is that many of these risk-relationships may be regulated by DVD-deficiency (Fig.1). Vitamin D deficiency is directly linked with several maternal factors such as gestational diabetes, preeclampsia, dysregulated steroidogenesis, maternal depression and infection [51, 52]. Additionally, vitamin D supplementation during pregnancy has been shown to reduce the risk of complicated pregnancies and obstetric complications [53]. Vitamin D also plays an important role in modulation of immune responses against infection and gestational hypovitaminosis D is associated with bacterial vaginosis [54]. Collectively, these observations suggest that DVD-deficiency may modulate or even amplify the effects of other developmental ASD risk-factors.

4. Vitamin D and brain

Vitamin D is potent neurosteroid, which mediates numerous actions in several body tissues including brain. Immunohistochemical presence of vitamin D synthesizing enzyme CYP27B1 and catabolic enzyme, CYP24A1, in neural cells of cerebral cortex and cerebral Purkinje cells suggest vitamin D can be formed locally in the brain [55-57]. Localization of CYP27B1 in human fetal brain suggests this steroid may also exert influence in the developing brain [58]. Like other steroid hormones, vitamin D exerts its actions via both genomic and non-genomic pathways [59]. Genomic actions are mediated by binding of vitamin D to the vitamin D receptor (VDR) which is a member of nuclear receptor super family. Vitamin D-VDR complex heterodimerizes with retinoid X receptor and binds to vitamin D response elements (VDREs) within the target gene to regulate its expression. The presence of VDR has been confirmed in adult and embryonic (E) rodent brain [56, 60-62].

Vitamin D mediates its rapid non-genomic actions through several pathways. One of these non-genomic pathways involves membrane bound VDR. Non-genomic actions involve several signalling pathways mediated by kinases and phosphatases [59]. A novel vitamin D binding receptor protein 1,25D3-MARRS has been identified involved in the rapid non-

genomic actions of vitamin D [63]. The activities of 1,25D3-MARRS have been detected in newborn and adult rat brains [64].

5. Animal models of vitamin D deficiency and the developing brain

The impacts of maternal vitamin D deficiency on fetal brain development have been widely studied using a Sprague-Dawley rat model of DVD deficiency in our laboratory. This model was developed by feeding vitamin D depleted diet (Specialty Feeds, WA) to the females for the period of six weeks before mating. Dams remain on this diet until birth. The diet has normal levels of calcium and phosphorus. The developing fetus is totally dependent on the availability of vitamin D from the dam. Lack of vitamin D in maternal circulation induces vitamin D deficiency in developing the fetus. Several clinical studies have shown a positive correlation among the levels of 25-hydroxyvitamin D (25OHD) in maternal and fetal blood [65-67].

Most of the studies mentioned below have been conducted using the same DVD-deficient model unless otherwise described. DVD-deficient animals exhibit a number of neurodevelopmental alterations of potential relevance to ASD. For example, brain structural abnormalities include an enlarged brain at birth, increased lateral ventricle volumes, increased overall cell proliferation and altered neurotransmission [61, 68]. In behavioural domains the most replicated finding is hyperlocomotion, increased sensitivity to N-methyl-D-aspartate (NMDA) antagonists and reduced cognitive functions [61, 69, 70].

6. Pathophysiology of ASD and role of vitamin D

The exact pathophysiology and mechanisms underlying ASD are still poorly understood. However DVD-deficiency in animal models has been shown to reproduce phenotypes of relevance to ASD in the domains of neuroanatomy, expression of certain molecular factors and steroidogenesis.

6.1. Neuroanatomical changes in ASD and DVD-deficiency

Autistic children exhibit initial brain overgrowth followed by abnormally slowed growth later in life [71, 72]. Total brain volume is reported to stay higher even after correcting for body height and length [73-75]. Cross sectional and longitudinal magnetic resonance imaging (MRI) studies have shown that ASD children from birth to 12 months of age had abnormally large brain volumes compared with age matched control children [76-78]. Early brain enlargement is associated with the increased cerebral gray matter, enlarged lateral ventricles and striatum [79, 80]. At the cellular level, enlargement of ASD brains involves an excess

number of neurons in the prefrontal and dorsolateral prefrontal cortex [81, 82]. These findings suggest altered cell proliferation and differentiation in autism. Consistent with this proposal cell cycle length in ASD derived induced pluripotent stem cell is reduced. This may contribute an overall increase in cell proliferation in ASD [83].

The effects of DVD-deficiency on neuroanatomy and neural cell proliferation have been studied in newborn rat pups. These pups had larger and longer brains along with enlarged lateral ventricles and thinner neocortex in comparison with control pups. These changes in the brain structure were associated with generalized increased cellular proliferation [61]. This finding was accompanied by reduced expression of apoptotic genes in vitamin D depleted brains compared with controls [84]. Moreover neurospheres cultured from the subventricular zone of DVD-deficient brains showed increased numbers of neurospheres [85]. Neurospheres are floating clusters of neural stem cells that can be obtained by exposing progenitor cells to different growth factors [86]. Adult CYP27B1 null mice lacking ability to produce the active vitamin D hormone also showed increased cellular proliferation in dentate gyrus [87]. Taken together, these finding suggest that low gestational vitamin D leads to increased cell proliferation, reduced apoptosis and de-differentiated fetal brain.

Vitamin D has pleiotropic effects in several tissues including brain. Vitamin D supplementation promotes anti-mitotic activities and reduced cell division in hippocampal cells along with increased neurite growth [88]. Vitamin D has been shown to prevent cell proliferation by inducing cyclin-dependent kinase inhibitors p21 and p27 in different cells [89, 90]. Vitamin D prevents expression of other proteins required for cell cycle such as proliferating cell nuclear antigen and cyclin D1 [91]. Anti-apoptotic protein encoded by Bcl2 gene is also enhanced by vitamin D, which further increase cell proliferation in hippocampal neurons.

6.2. DVD-deficiency induces calcium signalling abnormalities in the brain with relevance to ASD

Calcium signalling is critical for normal dendritic growth and synaptic transmission [92]. Calcium homeostasis is vital for neuronal survival and elevated calcium level is a well-established cause of brain excitoxicity [93]. Not surprisingly cells in the brain are equipped with complex mechanisms for regulation of calcium involving voltage gated and ligand gated calcium channels. Dysregulation of these channels leads to abnormal intracellular calcium homeostasis.

ASD associated mutations have been seen in genes encoding calcium channels and calcium regulated ion channels [94-96]. Both the L-type voltage-sensitive calcium channel (L-VSCC) and T- type calcium channels are abnormally regulated in ASD [97].

The DVD-deficient adult rat exhibits dysregulated calcium regulating genes such as voltage-dependent anion channel 1and calnexin [64]. Furthermore, CYP27B1 KO mice lacking active form of vitamin D displayed calcium toxicity by upregulation of L-VGCC expression in subgranular zone and granular cell layer within the hippocampal region [87]. An increase in L-VSCC density is associated with hippocampal cell death [98].Vitamin D protects brain from calcium induced toxicity by downregulating L-VSCC expression in hippocampal and cortical neurones. This occurs through reduction of alpha(1C) and alpha(1D) subunits of L-VSCC [99, 100]. The addition of vitamin D has been shown to decrease (37%) membrane density of functional L-VSCC in hippocampal neurons [100]. These findings suggest that vitamin D is important for neural haemostasis and DVD-deficiency may disrupt calcium signalling. This is one possible mechanism linking vitamin D deficiency and ASD.

6.3. Mitochondrial dysfunctions and oxidative stress

A growing body of evidence suggests that mitochondrial dysfunction (MD) and oxidative stress (OS) could play some role in the onset of ASD [101, 102]. About one in twenty individuals with ASD have been diagnosed with MD [101]. A population based study in Portugal and Azores Islands screened 120 autistic children. This study concluded that 7.2% of autistic children met the criteria for MD [103]. Higher blood levels of pyruvate, lactate, pyruvate-lactate ratio and increased metabolism of pyruvate to lactate and alanine has been found in ASD children [103-105]. A postmortem study has found deficiencies in electron transport complexes and pyruvate dehydrogenase in frontal cortices of autistic brain compared with controls [106]. Magnetic resonance spectroscoy (MRS) studies showed decreased levels of N-acetylaspartate in amygdala, orbito-frontal cortex and cerebellum of autistic brains [107, 108].

Mitochondria localized in brain are involved in calcium homeostasis and energy production. These processes produce a high amount of reactive oxygen species [109]. The depletion of glutathione exposes the brain to OS [110]. ASD children have significant high concentrations of plasma nitric oxide and hydrogen peroxide [111, 112]. A recent meta-analysis from 29 studies found 27% reduction in reduced glutathione, 18% reduction in glutathione peroxidase

and 45% elevation in the level of oxidized glutathione in the blood of autistic children than control [113].

Vitamin D deficiency induces MD and OS by dysregulating expression of several mitochondrial enzymes localized in the frontal cortex and hippocampus of DVD-deficient rats when measured as adults. These enzymes include ATP synthase β-chain, cytochrome c oxidase, cytochrome b5, ATPase H+ transporting V1 B2 and electron transfer flavoprotein complex [64, 114]. Oxidative phosphorylation and redox balance are two processes disrupted in the DVD-deficient brains. DVD-deficiency also downregulates several mitochondrial proteins in the nucleus accumbens such as NADH dehydrogenase 1 α subunit 10, pyruvate dehydrogenase E1 component subunit β and isocitrate dehydrogenase 3 α [115]. DVD-deficiency may increase OS in rat brains by decreasing expression of anti-oxidative enzymes such as catalase and Mn-containing superoxide dismutase [64].

Vitamin D has been shown to inhibit production of nitric oxide synthase in neural cells [116]. Glutathione, an important antioxidant and mostly involved in the removal of nitric oxide is reported to be enhanced by vitamin D in newborn rat astrocytes [117]. Vitamin D promotes other molecules involve in glutathione biosynthesis including glutamate cysteine ligase and glutathione reductase [118]. Collectively, these studies show that vitamin D may confer a protective role at mitochondrial level and DVD-deficiency may contribute to MD and OS in the brains of ASD individuals.

6.4. Neurotransmission

6.4.1. Glutamatergic and gamma-aminobutyric acid abnormalities

Glutamate and gamma-aminobutyric acid (GABA) are two important neurotransmitters that are connected to extensive synaptic communication in brain. Glutamate is metabolized by glutamic acid decarboxylase 65 (GAD65) and glutamic acid decarboxylase 67 (GAD67) to GABA. Many studies have reported dysregulation in glutamatergic and GABAergic neurotransmission in autistic brains. Abnormal levels of glutamate and reduced expression of glutamate metabolizing enzymes have been reported in the brain of ASD individuals. One study showed significantly higher levels of glutamate and glutamine in amygdala and hippocampal regions of adult ASD brain compared with controls [119]. In contrast to this, another study has shown reduced subcortical glutamate and glutamine in adult ASD brains [120]. The cerebellum is one brain region repeatedly shown to be implicated in ASD. The cerebellum mediates motor coordination, cognitive and sensory functions. A comprehensive

post-mortem study have shown reduced (48–60%) protein levels of GAD65 and GAD67 in cerebellar and parietal cortices of autistic brains compared with age matched control brains [121]. A significantly reduced level of GAD65 and GAD67 mRNA in cerebellar Purkinje cells and cerebellar dentate nuclei of ASD brains has also been reported [122, 123]. It has even been suggested that the stereotyped repetitive behaviours found in ASD is in part due to reduced inhibitory control in autistic brains.

DVD-deficiency decreases gene expression of GABA-Aα4 receptor in newborn [124] and NMDA receptor density in the adult rat brain [125]. The impacts of vitamin D deficiency were also examined in adult mice which were vitamin D depleted for 10 weeks before testing. Adult vitamin D deficient mice had significantly lower protein levels of both GAD65 and GAD67 [126].

Vitamin D protects brain against glutamate induced neurotoxicity by upregulating VDR expression in rat cortical neurons [127]. Chronic treatment of rodents with vitamin D further increases GABA synthesis in several brain tissues including prefrontal cortex, anterior cingulate cortex and hippocampus [128, 129]. Interestingly, the same study has found upregulation of GAD65 and GAD67 in limbic regions and hippocampus respectively [129]. Therefore diminished regulation of inhibitory control induced by vitamin D deficiency may in part explain the repetitive behaviours found in ASD.

6.4.2. Dopamine

Dopamine (DA) is a neurotransmitter in the brain that plays important role in several behavioural domains such as motor control, reward-motivation, regulation of emotions and social interaction [130, 131]. There is a small amount of evidence that DA signalling may be altered in patients with ASD [132, 133]. *De novo* mutations have been found in DA transporter (DAT) gene (SLC6A3) in ASD individuals [133]. DAT is a membrane protein localized on nerve terminal and regulates transmission of synaptically released DA across the membrane and plays important role in maintaining DA homeostasis in the brain [134]. Polymorphism of a major DA catabolic enzyme (catechol-O-methyltransferase (COMT) have also been found in several ASD populations [135-137].

Altered DA signalling is a persistent finding in DVD-deficient rat model. DVD-deficiency may alter DA turnover by decreasing the expression of COMT in neonatal rat brains [68]. Interestingly, vitamin D treatment increases COMT gene expression in SH-SY5Y cells [138]. DVD-deficient embryonic brains exhibit decreased expression of genes required for DA

specification such as Nurr1 and p57(Kip2) [139]. Both Nurr1 and p57(Kip2) are critical for DA neuron development and maturation [140]. DVD-deficient adults have elevated levels of DAT and increased binding affinity for DAT ligands in caudate putamen and nucleus accumbens [141]. Increased uptake of DA due to elevated levels of DAT results in OS and neuronal damage [142]. Vitamin D has also been shown to increase production of tyrosine hydroxylase (DA synthesising enzyme) and DA metabolites such as homovanillic acid and 3 methoxytyramine in VDR expressing neuroblastoma SH-SY5Y cells [138, 143].

6.4.3. Serotonin

Serotonin is an important neurotransmitter and plays significant role in neurogenesis and neuronal differentiation during brain development [144]. Few studies have shown abnormal serotonin system in ASD brain. A positron emission tomography study of autistic and non-autistic adults found low serotonin transporter binding throughout the autistic brain particularly in anterior and posterior cingulate cortices [145]. ASD post-mortem brains show low densities of serotonin receptors including 5-HT1A and 5-HT2A in fusiform gyrus compared with controls [146]. Serotonin synthesis capacity is also compromised in frontal cortex, thalamus and cerebellum of ASD individuals [147]. In contrast to a relative decrease in serotonin signalling in the ASD brain, autistic patients have a significantly high level of serotonin in blood compared to healthy individuals. Hyperserotonemia is found in 30% ASD patients and their first degree relatives [148, 149].

Vitamin D treatment upregulates the serotonin synthesizing gene tryptophan hydroxylase 2 (THP2) in human and rat glioblastoma cell lines [150]. Moreover, sub-chronic treatment of rats with high dose of vitamin D induced expression of TPH2 and monoamine oxidase A, which increased concentration of 5-hydroxyindoleacetic acid (major serotonin metabolite) without changing serotonin status in prefrontal cortex [129]. It has been suggested that Vitamin D may induce the THP2 in brain and repress TPH1 in gut as central mechanisms in ASD [151]. This has yet to be demonstrated in patients or any ASD model.

The impacts of vitamin D deficiency on serotonin system have been observed in adult vitamin D deficient mice. The level of 5-hydroxyindoleacetic acid was significantly increased in the brains of these mice compared to the controls. The ratio of serotonin to 5-hydroxyindoleacetic acid was also altered which may reflect an increase in serotonin turnover in vitamin D deficient brains [126].

6.5. DVD-deficiency alters immune function; relevance to ASD

There is a well-established association between maternal infection and increased incidence of ASD in children. Maternal cytokines can cross the fetal blood brain barrier through placenta and lead to neuro-inflammation in developing brain [152]. A number of post-mortem studies have suggested that ASD brains have on going neuro-inflammation regardless of age [153-156]. In general neuro-inflammation is characterized by activation of the inflammatory cells in the brain astrocytes and microglia. These cells, particularly microglia which are the major phagocyte-like cell in the brain, when stimulated increase expression of cytokines and chemokines. Activated microglia and increased microglial density have been found in dorsolateral prefrontal cortex of autistic brain [157]. Persistent ongoing inflammation leads to loss of connectivity and neural cell death [158].

A growing number of studies suggest that vitamin D induces an anti-inflammatory response in several tissue types including brain [159, 160]. The proof of concept that vitamin D modulates immune systems arises from the fact that VDR is present in almost all immune cells including neutrophils, macrophages, T cells, B cells and dendritic cells (DC) [161, 162]. Vitamin D inhibits synthesis of pro-inflammatory cytokines by targeting mitogen-activated protein kinase phosphatases which are essential in the regulation of immune responses [163]. Vitamin D via its genomic actions inhibits propagation of uncommitted T helper cells and promotes production and accumulation of immunosuppressive T regulatory cells at the site of inflammation [164]. It also suppresses proliferation of activated B lymphocytes, suggesting an anti-inflammatory role of vitamin D in autoimmune diseases [165]. The absence of dietary vitamin D may therefore have implications for normal immune function.

DVD-deficiency in rats induced persistent changes in the morphology of immune organs such as enlarging spleen and thymus [166]. Cultured lymphocytes from DVD-deficient offspring exhibited increased levels of IL-2 and IL-10 after a ionomycin challenge [166]. The effects of vitamin D deficiency were also examined on microglial phagocytic rates that were stimulated with toll like receptor (TLR) agonists lipopolysaccharide (LPS) and polyinosinic:polycytidylic acid (poly I:C). After such treatment DVD-deficient microglial cells engulfed fewer E.coli than cells exposed to normal levels of vitamin D [167]. Vitamin D deficiency also decreased the immune response of microglial cells by reducing production of TNF-α and IL-6 [167]. Additionally, VDR and CYP27B1 knockout mice are more vulnerable to exogenously induced inflammatory bowel disease [168-170]. VDR null mice are sensitive to dextran sodium phosphate and show significant high expression several cytokines

including of IL-1β, TNF-α, IL-10 and INF-γ compared with wild type mice [171]. Taken together then, DVD-deficiency may lead to increased inflammatory outcomes associated with ASD.

6.6. Steroid dysregulation in ASD and vitamin D

Studies have consistently shown that males are over-represented four-fold in ASD [40, 172]. Sex steroids particularly testosterone and estradiol may be crucial here. The brain develops differentially in both sexes because of exposure to distinct steroid hormones. It is hypothesized that hypermasculinization due to exposure of high levels of prenatal testosterone leads to extreme manifestation of cognitive and emotional behaviour observed in ASD individuals [173, 174]. Consistent with this, significantly higher levels of androgens and testosterone have been reported in the saliva and urine of ASD individuals [175, 176]. Another study found higher levels of testosterone in the amniotic fluid of the children who were later diagnosed of having autistic traits [177]. The mRNA levels of estrogen synthesizing enzyme aromatase and estrogen receptor β are decreased by 38% and 35% respectively in middle frontal gyrus of ASD brains compared with healthy brains [178]. Genetic studies revealed associations of steroidogenic enzymes with ASD including CYP17A1, CYP11B1 and aromatase [179]. These enzymes are involved in the conversion of cholesterol into different steroids such as progesterone, 17α hydroxy-progesterone and estrogen. Interestingly, a recent study using the Danish Historic Birth Cohort has found elevated levels of several steroids including progesterone, 17α hydroxy-progesterone, androstenedione, testosterone and cortisol in the amniotic fluid of ASD children [180]. Elevated corticosterone levels and abnormal activation of hypothalamic–pituitary–adrenal (HPA) axis has also been cited in ASD individuals [181-183]. Together, these studies suggest that ASD is associated with a dysregulation of steroidogenic activities mediated by cytochrome P450 and other enzymes involved in steroid synthesis.

Vitamin D has been shown to regulate the expression and synthesis of many steroids. Clinical studies in 1959 found a negative correlation between the concentrations of vitamin D and urinary androstenedione and dehydroepiandrosterone (DHEA) [184].

Vitamin D deficiency has been shown to increase circulatory glucocorticoid concentrations in pregnant mice [185, 186]. Glucocorticoids are lipophilic compounds that can diffuse from placenta to the fetal circulation. The placental expression of HSD11β2 protects the fetus from glucocorticoid exposure by inactivating corticosterone to 11-dehydrocorticosterone. DVD-

deficiency further exposes the developing fetus to higher levels of maternal glucocorticoids by reducing the expression of HSD11β2 in placenta [185]. However by adulthood it would appear that HPA function was normal in DVD-deficient offspring [186]. Ablating the VDR induces abnormalities in both male and female steroidogenesis. VDR mutant male mice showed transient increase in testicular weight but testosterone level was not measured. However female VDR null mouse from this same study had dysfunctional ovaries and these animals were unable to produce sufficient estrogen. Aromatase activity was also reduced in ovaries and testicles of VDR knockout mice along with decreased levels of estrogen in the blood [187].

In vitro, vitamin D has been shown to regulate expression of steroidogenic enzymes in different non-neural cell lines. It induced activation of CYP11A1 and CYP17A1 and decreased synthesis of androstenedione, DHEA, DHEA-sulphate and corticosterone in human adrenocortical carcinoma cells [188]. The testosterone catabolic enzyme HSD17β2, was found to be upregulated by vitamin D [189]. Vitamin D exhibits tissue specific effects on the expression of the major catabolic enzyme for testosterone, aromatase [190]. Vitamin D increases estrogen and progesterone synthesis in cultured human trophoblast cells [191], whereas in breast cancer cells, aromatase expression was reduced following vitamin D treatment [192]. The impact of vitamin D deficiency has not been studied on steroidogenic activities of these enzymes in DVD-deficient model. Future studies need to address if this is a contributing mechanism to ASD.

7. Vitamin D and placental functions

Placenta becomes an active site of extra-renal synthesis of vitamin D and contributes 20-50% of the circulating vitamin D in pregnancy [193, 194]. Locally produced vitamin D facilitates maternal immune tolerance by inducing expression of VDR and CYP27B1 in placental immune cells [195, 196]. In vitro studies showed that vitamin D prevents maturation and differentiation of placental DC by reducing cell surface expression of major histocompatibility complexes [197]. It is worth noting that during the first trimester, placental DC become tolerogenic becoming less capable of presenting antigens compared with maternal DCs [198, 199]. The role of vitamin D also been studied in placenta in the presence of an exogenous immune challenge. Both in vivo and in vitro LPS exposure induces CYP27B1 and VDR expression which again is consistent with vitamin D signalling being anti-inflammatory [200]. Genetic ablation of CYP27B1 or VDR leads to increased placental inflammatory cytokines in response to an LPS challenge [200].

8. ASD-related behavioral phenotypes in DVD-deficient animals

8.1. Ultrasonic vocalizations

Impairments in verbal communications suggest language delays and poor pragmatics in ASD individuals. Interestingly, some studies found unusual patterns of vocalizations in ASD infants [201, 202]. Obviously rodents do not use language for communication but rather they emit ultrasonic Vocalizations (USV) in different situations. For example, neonates emit USV when isolated from mother and littermates, juveniles during social play and adults during mating and aggression [203]. Reduced USV have been reported in some genetic and environmental animal models of neurodevelopmental diseases including ASD [204-206]. The patterns of USV emitted from a pup isolated from its nest initiate exploratory and retrieval behaviour in mothers [203]. Evidence shows that dams spend more time with pups vocalizing at high amplitude than the pups with low amplitude [207]. USV by newborn pups and maternal retrieval behaviours have been studied in DVD-deficient animals. There was no significant effect of diet on USV and calling pattern by pups, however the DVD-deficient dams retrieved their pups earlier and spent more time with them than the control dams [208]. Because pup USV have been shown to alter maternal retrieval behaviour, it was speculated that altered maternal retrieval behaviour in DVD-deficient dams was due to alteration in the calling amplitude of their pups.

8.2. Stereotyped repetitive behaviour

Stereotyped repetitive behaviour has been considered central to ASD. Autistic children show stereotyped movements such as hand or finger flapping, spinning and jumping [1]. Spontaneous motor stereotypies can be modelled in rodents by assessing the time spent on self-grooming, circling, jumping or excessive digging [209]. Interestingly, significant effect of maternal diet has been found on the grooming frequency in juvenile rats. DVD-deficient juvenile rats showed significantly more grooming behaviour than the vitamin D sufficient rats [210].

8.3. Anxiety-related behaviour

Children with anxiety disorders score high for ASD traits compared to healthy children [211, 212]. There is an overlap in behavioural characteristics between children with ASD and anxiety disorders with respect to Diagnostic and Statistical Manual of Mental Disorders-IV-TR diagnostic criteria [213]. In fact anxiety related concerns are common in toddlers who develop ASD [214].

Open field exploration and behaviours in the elevated plus maze are well established and validated tests to assess anxiety-like behaviours in rodents [215]. Usually, rodents spent more time in the periphery compared to the central area of an open field. Time spent in the central area is considered a measure of non-anxious behaviour [215]. In an open field test, adult DVD-deficient rats spend more time in the sides and less time in the corners compared with controls suggesting some form of anxious behaviour in DVD-deficient rats [216]. However, in an EPM which is a reliable measure of anxiety in rodents DVD-deficient rat behaviour was normal (Burne 2004).

8.4. Hyperlocomotion

Children with ASD show hyperactivity to sensory inputs [1]. Hyperlocomotion is one of the most consistent findings in DVD-deficient rats, which has been observed across different behavioural tests including open field, elevated plus maze and hole-board [69, 217]. Both DVD-deficient adult rats and mice show spontaneous hyperlocomotion [186, 218]. DVD-deficiency induced hyperlocomotion is independent of HPA-axis as these animals had normal corticosterone release in response to restraint stress [186]. Juvenile DVD-deficient rats travelled further when vitamin D deficiency was extended until weaning. Similar to these observations, DVD-deficiency increased sensitivity to psychostimulant induced locomotion in adult rats during open field testing [69, 125, 141]. Systemic injections of the NMDA antagonist MK-801 or amphetamine increased hyperlocomotion in DVD-deficient rats compared with controls. These findings suggest DVD-deficiency is associated with impaired NMDA receptor and DA signalling. Interestingly, administration of DA and NMDA antagonist haloperidol eliminates both spontaneous and MK-801 induced locomotion in DVD-deficient animals [69].

9. Conclusion and future directions

In this review we have summarized fundamental pathways that may be key targets in understanding the pathophysiology of ASD. We have outlined the impact that vitamin D deficiency may have on the regulation of these pathways both in vivo and in vitro. Both human and animal studies suggest that an optimal level of vitamin D during development and early life is important to avoid ASD-like behavioural phenotypes. The DVD-deficiency model we have developed is an ideal experimental platform to explore molecular mechanisms linking DVD-deficiency and development of ASD. We think it highly likely that the molecular mediating mechanisms between DVD-deficiency and ASD are likely to involve

both direct regulation of the fetal/placental immune response and alterations in steroidogenesis. However our model requires 2 major alterations. First, to date we have explored behaviours primarily in adults. We now wish to assess ASD relevant behaviour in DVD-deficient young and juvenile animals mimicking the period of ASD onset. Second, to date we have restricted the period of vitamin D deficiency to gestation in the rat. This is inadequate when assessing the effects of vitamin D deficiency across an equivalent period of primate brain development. Therefore we wish to now extend the period of DVD-deficiency to weaning. DVD-deficient model may prove relevant for the investigation of possible therapeutic strategies to reverse both these putative mechanisms and optimally ASD-like phenotypes. DVD-deficiency may only represent one environmental factor contributing to this disease. However the ability to intervene with such a simple, safe and affordable factor during pregnancy makes this an important public health concern.

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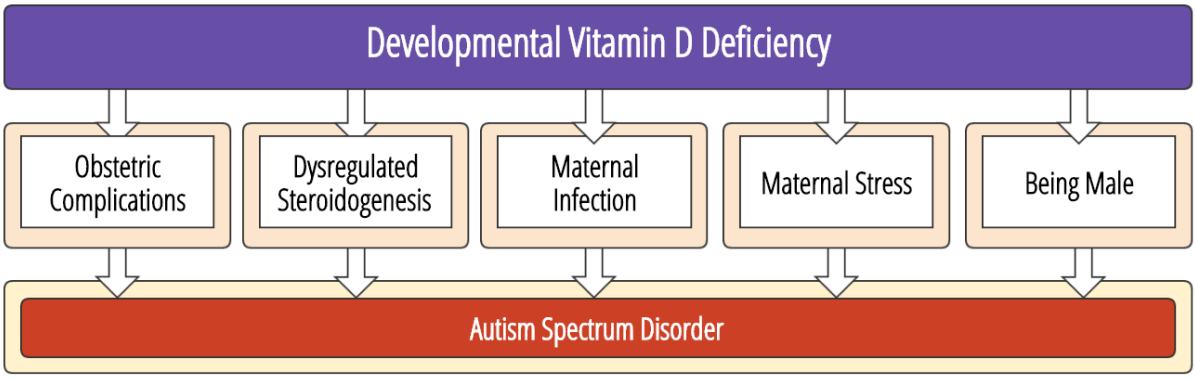

Fig. 1: DVD-deficiency interacts with wide range of other environmental risk factors to modulate multiple pathophysiological processes relevant to ASD