Chapter 5

The Developmental Vitamin D (DVD) Model of Schizophrenia

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Abstract

It is now widely acknowledged that exposure to adverse environmental factors in utero may not only affect how the brain develops but have long-lasting consequences for later brain function in the adult off-spring. This idea has gained particular prominence amongst researchers interested in the etiology of neurodevelopmental disorders such as schizophrenia and autism. Approximately 10 years ago we proposed that developmental vitamin D (DVD) deficiency may explain several epidemiological features of this disease, most noticeably the winter/spring season of birth effect. In 2003 we published results from our first study indicating there were structural changes in how the brain develops in these offspring. Since then we have firmly established that DVD deficiency not only affects brain cell differentiation and gross anatomy but also produces alterations in behavior in these offspring as adults. In this chapter we describe how we came to construct the model we use today. Over the past 7 years the model has proved informative producing both structural brain changes (ventriculomegaly) and behavioral alterations (hyperlocomotion in response to NMDA antagonists) that are thought to be relevant to schizophrenia.

Key words: Vitamin D, brain, animal model, development, schizophrenia, behavior.

1. Introduction

Despite many decades of concerted, multi-disciplinary research, the etiology of schizophrenia remains poorly understood. In keeping with its clinical heterogeneity, schizophrenia is almost certainly a syndrome with many different etiological factors. The symptoms that contribute to making a clinical diagnosis include hallucinations, delusions, disordered thoughts, and alterations in affect and cognitive impairments – all higher cognitive features. Additionally although the heritability of the disorder is claimed to be as high as 80% (1), the identification

of genetic candidates in all populations has remained elusive (2). For these reasons, it is not surprising that schizophrenia has proved a difficult, some would even suggest impossible, disorder to model in animals. Nevertheless, there remains a stubbornly persistent developmental epidemiology that strongly supports the neurodevelopmental hypothesis of schizophrenia (3, 4). For instance, prenatal exposure to infection, pregnancy and birth complications, and winter/spring birth are all associated with increased risk of developing schizophrenia in later life (5). In general, it is thought that these adverse exposures act against a backdrop of a "vulnerable" genome, which in turn adversely impacts brain development. The neurodevelopmental hypothesis suggests that there are critical early periods of brain development when certain adverse genetic and/or environmental factors contribute to disease susceptibility (6).

Despite the inherent difficulties in trying to develop an animal model of a disorder that affects higher cognitive ability (7), results from animal models based on clues from epidemiology have provided important new clues (8). For example, rodent models based on prenatal exposure to virus-like agents (e.g., the synthetic double-stranded RNA and poly I:C), or bacterial membranes, have been used to explore the neurobiological correlates of maternal infection (9). Similarly, pre- or perinatal hypoxia has been used to study obstetric complications (10). Our group has developed an animal model related to prenatal vitamin D deficiency as a plausible explanation for several important epidemiological observations in schizophrenia risk factor biology.

Many studies have shown that those born in winter and spring have a significantly increased risk of developing schizophrenia (11). The size of the winter/spring excess increases at higher latitudes (12) and the incidence and prevalence of schizophrenia is also greater in sites at high latitudes (13). Curiously, the incidence of schizophrenia is also significantly higher in dark-skinned migrants to cold countries compared to the native born (14). Given that hypovitaminosis D is more common (a) during winter and spring, (b) at high latitudes, and (c) in dark-skinned individuals (15), low prenatal vitamin D "fits" these key environmental features.

It is now 10 years since we first proposed that low prenatal vitamin D may be a risk factor for schizophrenia (16). Initial support for this idea came from studies that established that vitamin D supplementation in the first year of life significantly reduced the risk of schizophrenia in males from a large Finnish birth cohort (17). In addition, a pilot study found that 25-hydroxyvitamin D serum levels in 26 mothers whose children developed schizophrenia were nonsignificantly lower than those of 51 control mothers whose children did not develop the disease, but this group difference was more prominent in mothers with dark skin (18). Larger

studies investigating a direct link between neonatal vitamin D levels and risk for schizophrenia in later life are ongoing.

In order to establish the biological plausibility of this hypothesis, we have developed an animal model to study the impact of developmental vitamin D (DVD) deficiency on a range of brain outcomes (structure, function, neurochemistry, genomics, proteomics, and behavior). We have shown that the brains from DVD-deficient neonates have larger lateral ventricles (19). Brain differentiation also appears delayed with a generalized increased cellular proliferation, increased neurogenesis, and reduced apoptosis (19–21). Ventricular enlargement persists in these animals as adults (22). Behaviorally, adult DVD-deficient rats are more active than controls in novel environments (23, 24). DVD-deficient rats also have enhanced locomotion in response to psychomimetic agents (agents that induce psychosis) such as the NMDA antagonist MK-801(24, 25). The DVD-deficient adult rat is also more sensitive to the widely used antipsychotic haloperidol, a dopamine 2 (D2) receptor antagonist (24).

Several features of the DVD-deficient phenotype are therefore informative for schizophrenia research: (a) increased lateral ventricular volume is one of the most consistent neurobiological correlates of schizophrenia (26); (b) behavioral sensitivity to NMDA antagonists is also displayed by patients and is thought to reflect an underlying abnormality in neurotransmission consistent with the hallucinatory or positive symptoms of schizophrenia (27). Intriguingly, our most recent experiments have established that dopamine ontogeny appears to be affected in the developing brains of DVD-deficient animals and that there are also several learning deficits in animals from this model (unpublished observations). In this chapter, we outline how we create a DVD-deficient rat and pitfalls in the process of developing this model.

2. Materials

Three diets are used in this model:
- The control diet used during gestation and rearing is AIN93G rodent diet custom formula #110700 (Dyets, Inc., Bethlehem, PA, USA) – with #210025 salt mix and #310025 vitamin premix. The amount of pre-vitamin D, cholecalciferol (*see* **Note 1**), is 1000 IU/kg, calcium is 5 g/kg, and phosphorous is 1.5 g/kg.
- The deplete diet used during gestation and rearing is AIN93G rodent diet custom formula #119266 – (Dyets) with the same salt and vitamin premix – minus cholecalciferol (*see* **Note 2**). Calcium and phosphorous contents are

the same. A casein rather than a cereal-based diet is used as it can be stripped of vitamins and then added back in. This is not possible in non-purified diets such as the grain-based diets.

- Weanling diet (all experimental animals) – Rat & Mouse Cubes (Specialty Feeds, Glen Forrest, WA, Australia). Cholecalciferol content is 2000 IU/kg, calcium 8 g/kg, and phosphorous 7 g/kg.

The control and deplete diets need to be stored in a dry, cool, dark location, in airtight containers. Shelf life is ~3 months at 4°C (refrigerated) but up to 6 months if stored at less than –20°C (*see* **Note 3**). The micronutrient contents of these diets are vulnerable to various sterilization procedures such as irradiation and autoclaving (procedures required in certain animal houses) (*see* **Note 4**). Finally we recommend a close inspection of diet constituents if prepared locally (*see* **Note 5**).

A measure of serum 25-hydroxyvitamin D3 (25OHD3) is the best indication of overall vitamin D3 status (28). We have developed an assay using LC/MS/MS technology that requires a small volume of blood (3 μl) to routinely assess maternal vitamin D status (29). A single drop of blood is drawn routinely from the saphenous vein (30). A commercial radio-immunoassay (Dia Sorin, Inc., Stillwater, MN, USA) can also be used; however, this requires substantially more whole blood to obtain the 50 μl sera required. PTH was measured using a commercial ELISA kit (Immutopics, San Clemente, CA, USA) and calcium and phosphate were analyzed independently with an AutoAnalyser (Hitachi Instruments, Tokyo, Japan).

3. Methods

3.1. Preparation of the Dams

All procedures were performed with the approval of the University of Queensland Animal Ethics Committee, under the guidelines of the National Health and Medical Research Council of Australia. Every 2 weeks, eight-four-week-old female Sprague–Dawley rats (*see* **Note 6**) from a specific pathogen-free source are selected. These females come from two litters and four females from each litter are used. Two females from each of the two litters are assigned to a control diet (#110700) and a corresponding two females from the same two litters are assigned to the vitamin D deplete diet (#119266). All breeding animals are housed in groups of four in incandescent light devoid of ultraviolet B radiation on a 12-h light/dark cycle (lights on 0600 h), at a constant temperature of $21 \pm 2°C$ and 60% relative humidity, with food
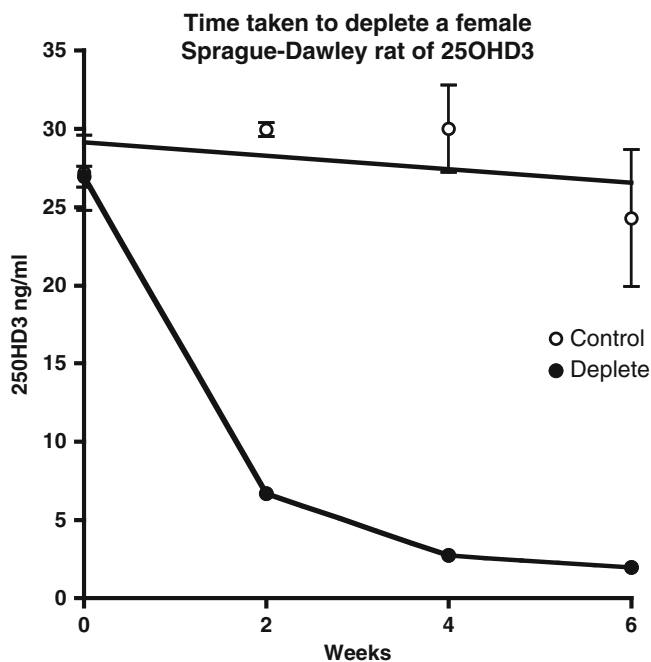
Fig. 5.1. Time taken to deplete a female Sprague–Dawley rat of 25OHD3 (●) compared with a control dam (○) over the same pre-breeding period (mean ± SD). After 6 weeks on a diet deficient in cholecalciferol (119266; Dyets, PA, USA), 25OHD3 is virtually absent.

and water provided ad libitum. These conditions are maintained for 6 weeks prior to mating. Six weeks is sufficient to ensure depletion of 25OHD3 (**Fig. 5.1**) (*see* **Note** 7).

3.2. Preparation of the DVD-Deficient and Control Offspring

At this 10-week period, both vitamin D-deplete females and control females are mated with vitamin D normal males. Males are placed in with the females for 7 days. For experiments in embryos or fetuses where gestational time is required, dams are plug checked and vaginal smears are taken to ensure sperm presence once a day for the first 5 days (or until sufficient dams are confirmed). It is plausible that vitamin D-deficient females could absorb some vitamin D through grooming the vitamin D-replete male during breeding. We cannot rule this possibility out; however, to date we have not observed any increase in vitamin D levels in DVD-deficient females post-mating. This breeding protocol results in approximately 67% pregnancy rates independent of diet. Prospective pregnant dams remain on their respective diets throughout gestation and are housed in their same groups of four, until embryonic day 20 (E20), when they were housed individually and provided with nesting materials. When neonates are required, expectant dams are checked twice a day, beginning with the morning of E21, until litters are born. The first appearance of pups was recorded within 12 h of birth, and this day is referred to

as postnatal day 0 (P0). On the day dams litter, both control and deplete dams are placed on the casein vitamin D-containing diet (#110700) and they remain under the same lighting conditions until the pups are weaned, at which time they are transferred to a separate animal holding room with standard fluorescent lighting and the dam is culled.

Litter sizes of between 8 and 18 are considered normal for Sprague–Dawley rats (31). At birth, litters of size less than 8 or greater than 18 are rejected (*see* **Note 8**). Both control and vitamin D-deficient dams and their respective offspring remain on the control diet (diet #110700) till weaning. In pups, 25OHD3 levels return to control levels by weaning (**Fig. 5.2**). All pups are weaned at 21 days into same sex groups of no specific number and all offspring are placed on a standard cereal-based rat chow that contains vitamin D (diet #119266). At 4 weeks of age, animals are split into groups of 2, 3, or 4, and housed in open-top wire cages, with no additional environmental enrichment. All animals remain under these conditions until behavioral testing (5, 10, or 20 weeks) after which the brain is analyzed for gross or cellular anatomy or protein or mRNA expression.
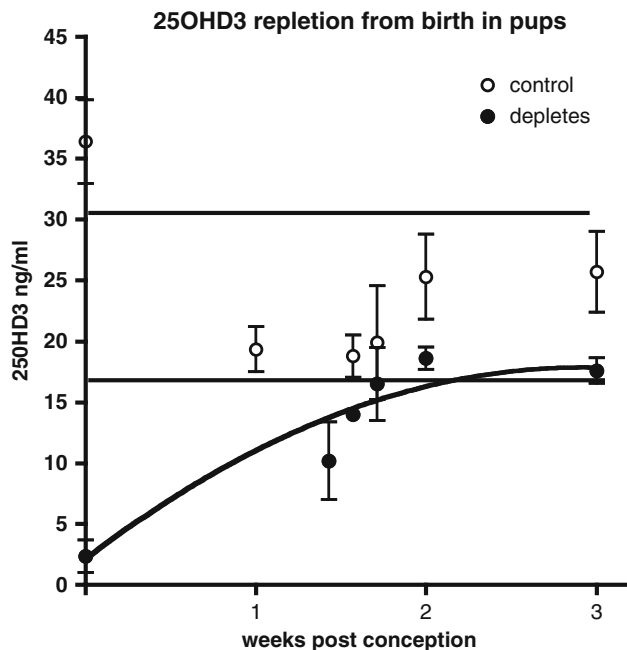

Fig. 5.2. Time taken to replete a vitamin D-deficient dam (•) compared with a control dam (○) over the same postnatal period (mean ± SD). 25OHD3 levels in vitamin D-deficient dams return to control levels 2 weeks after vitamin D is reintroduced in the diet. *Horizontal lines* indicate 25OHD3 concentration range (mean ± 1 SD) in control dams over this same postnatal period.

Vitamin D deficiency had no effect on dam or offspring weight gain either during pregnancy or after conception (**Table 5.1**). Serum calcium and phosphate measures were also normal in vitamin D-deficient dams despite a slight elevation in PTH across the period of pregnancy (**Table 5.2**). Rates of pregnancy and fecundity were also unaltered by maternal vitamin D depletion (**Table 5.3**). These findings conflict with other more drastic models of maternal vitamin D deficiency (see below).

Table 5.1
Vitamin D deficiency had no effect on dam or offspring weight gain either during pregnancy or after conception

Time	Control dam weight (g)	Vitamin D–deplete dam weight (g)	Control offspring weight, male (g)	DVD-deficient offspring weight, male (g)	Control offspring weight, female (g)	DVD-deficient offspring weight, female (g)
Conception	269±12	258±9				
E7	281±6	294±16				
E14	308±4	328±16				
P0	298±5	321±14	6.7±0.8	6.6±0.9	6.4±0.8	6.5±0.7
P7	338±7	337±10	21.5±1.9	21.9±1.4	20.9±1.1	20.8±1.3
P14	335±6	339±11	42.7±2.2	43.7±5.6	42.3±2.9	42.8±4.1
P21	328±6	325±6	61.4±3.0	64.7±8.8	60.4±4.6	61.6±6.0
P35			184.9±12.6	178.4±11.6	147.6±7.6	139.7±8.8
P70			461.1±38.6	475.8±43.2	265±20.9	264.3±30.5

Table 5.2
Vitamin D deficiency did not affect serum calcium or phosphate at any stage of pregnancy. PTH levels were however elevated

Time	Ca^{2+} (mM)		PO_4 (mM)		PTH (pg/ml)	
	Control dam	Vitamin D-deficient dam	Control dam	Vitamin D-deficient dam	Control dam	Vitamin D-deficient dam
Pre-conception	2.92±0.02	2.88±0.02	2.68±0.18	2.51±0.21	177.4±21.3	350.6±59.5*
E7	2.86±0.04	3.01±0.03	2.20±0.14	2.47±0.12	98.5±20.8	280.5±55.8*
E14	2.94±0.06	2.95±0.03	2.52±0.13	2.57±0.15	75.3±22.8	189.5±73.1‡
P0	3.38±0.07	3.25±0.08	2.21±0.29	1.82±0.27	233.7±104.0	725.2±191.2‡

*$P<0.01$; ‡$P<0.1 >0.05$ relative to control PTH.

Table 5.3
Rates of pregnancy and fecundity are unaltered by maternal vitamin D depletion

Measure	Control	DVD deficient
%Pregnancies	67.8	67
Average litter size	11.4	11.8
Male/female ratio	1.1	1.0
Litters <8 pups	7.2%	6.4%
Litters >16 pups	3.0%	0.8%

The levels of 25OHD3 and 1,25OH2D3 in pups at birth reflect those seen in dams during pregnancy (25). DVD-deficient newborns are also normocalcemic (i.e., neither the dams nor their offspring have the rickets-like phenotype that would result in more chronic vitamin D depletion) (*see* **Note 9**). Observation of the offspring from birth to weaning indicated that maternal vitamin D depletion did not affect the progression of normal development or physical maturity. For instance, there were no significant differences between dietary groups on physical maturity scores (eye and ear opening, ear unfolding, fur development and teeth protrusion, self-righting reflex, and posture or stepping activity) (25). All the above physical and endocrine measures were also normal at the time of behavioral testing at either 10 or 20 weeks.

4. Other Models of Maternal Vitamin D Deficiency and General Measures of Pup Health

We have considered the effects of varying the duration of maternal vitamin D deficiency in the development of this model. We have examined both a shorter period of vitamin D depletion (e.g., gestation only) and two longer periods extending DVD deficiency (e.g., until weaning and throughout adulthood). Although gestational deficiency was also sufficient to produce important behavioral deficits in the offspring (e.g., NMDA antagonist-induced hyperlocomotion) (25), the level of the active form of the hormone 1,25OH2D3 in maternal animals was still well within control levels despite profoundly deficient levels of 25OHD3 (25). Extending maternal vitamin D deficiency until weaning produces changes in gross brain architecture consistent with schizophrenia (22); however, deficiency extended into the period of rearing increases possible associated physiological abnormalities such

as hypocalcaemia, maternal weight loss, and reduced fertility (32). Lifelong vitamin D depletion apart from being completely non-physiological produced hypocalcaemia and associated cardiovascular and kidney abnormalities (33) and was therefore rejected.

We have consistently shown that this model produces no gross abnormalities in birth outcomes, growth rates, or calcium status in either the vitamin D-deficient dams or DVD-deficient pups. However, other models of maternal vitamin D deficiency have not been so benign. Studies in the early 1980s reported that if female rats were kept vitamin D deficient for ≥90 days prior to mating, and during the period of gestation and rearing, then maternal growth and fecundity was reduced (34–36). Hypocalcemia in dams was also prevalent, but not universal (37). Curiously, where assessed, it appeared that in these earlier studies, pups appeared to be calcium normal. It has been suggested that fertility issues in the long-term vitamin D-depleted dams were secondary to low serum calcium and phosphorus rather than vitamin D deficiency per se (38).

The duration of maternal vitamin D depletion prior to mating used in our DVD model (42 days) is insufficient to affect maternal calcium and has no adverse effects on fertility, fecundity, or various measures of pup growth. However, this issue would appear to be not completely resolved with a recent study by a Japanese group showing that in female rats depleted in a similar fashion to that used here, both the gravid Sprague–Dawley dams and their offspring were hypocalcemic and fetuses were growth restricted (39). We advise those establishing the DVD-deficient model to first determine that their conditions do not have any adverse effects on either serum calcium levels or the aforementioned indices of maternal or fetal growth.

5. Conclusions

Most of our published data have been generated from offspring who experience a transient gestational period of vitamin D deficiency. Since our first studies were published (19), we have refined certain experimental factors such as lighting, litter size, and maternal calcium supplementation: We remain interested in what impact variations in the duration of developmental vitamin D depletion would have for brain development and function. We are also interested in the application of this model in other experimental animals such as wild-type (40) and transgenic mice. Other models could also be employed that, although having less face validity for the environmental nutrient deficiency being studied, could also reveal much about how vitamin D

affects brain development. For example, studies that allow prolonged but less severe hypovitaminosis D also warrant inspection. Additionally, after birth, maternal vitamin D deficiency could be more rapidly reversed than the simple dietary intervention used here either with injections of the active hormone 1,25(OH)2D3 or by cross-fostering DVD-deficient offspring to control vitamin D normal dams. However, these models would require a substantial amount of preliminary studies to establish the correct drug/dosage/dosing interval prior to any consideration of their suitability for investigation.

6. Notes

1. Cholecalciferol is pre-vitamin D3. This compound is oxidized largely in the liver to form 25OHD3. 25OHD3 undergoes further oxidation in a variety of organs but primarily in the kidney to form the active hormone 1,25(OH)2 vitamin D3. This form of the vitamin is far more labile.

2. When the vitamin D-deficient diet is prepared, making the control and deficient diet of different colors can help reduce mistakes in the animal house.

3. Storage conditions for the control and deplete diets are also particularly critical due to the lack of antioxidants and preservative agents. We have observed that if the diet becomes compromised via storage artifact (i.e., temperatures >4°C, high humidity, and continuous exposure to air and light) or if used beyond extended shelf life (more than 6 months at less than –20°C or 3 months at less than 4°C), pregnancy is severely compromised. If possible, we recommend diets should be vacuum sealed and stored at –20°C in the dark to reduce the amount of oxidation.

4. Some countries require strict sterilization procedures prior to importing food. Similarly most animal houses require food to be sterilized. We have found that treatments such as irradiation and autoclaving also interfere with viable animal breeding presumably due to reduced vitamin and nutrient content. The weanling diet is cereal based and can be stored at room temperature prior to autoclaving, for up to 6 months. Once autoclaved, food should be used within 4–6 weeks. The control and vitamin D-deficient diets are casein based and do not withstand autoclaving. We have observed that when sterilized with gamma ionizing irradiation from cobalt-60 at a minimum dose of 2.5 Mrad (25 kGy), successful breeding is also dramatically reduced. If possible we

recommend another sterilization procedure. To avoid the use of radiation in Australia, we have had to separate our animals during breeding and gestation into separate quarantine-approved premises.

5. We have also had the experience of local manufacturers modifying the original formula to suit locally available raw materials but resulting in compromised pregnancy. Close inspection of each recipe is recommended. The American Institute of Nutrition's prescribed formula for the AIN93G rodent diet states the requirement of two fatty acids linoleic [18:2(n-6)] and linolenic [18:3(n-3)] as dietary essentials and defines soybean oil as the ideal source of fat to provide these in the right ratio. Substitution of soybean oil for locally available oils, such as canola oil, not only alters the ratio of these essential fatty acids but also often has different biological interactions with vitamin E, hence exacerbating the volatility of vitamin E and possibly increasing the risk of impaired pregnancy. Additionally we have experienced alterations in the prescribed carbohydrates (cornstarch and dextrinized cornstarch) in preparations. This will alter pellet formation and appearance (look/feel/touch), therefore influencing palatability as well as vitamin and mineral dispersal during manufacturing.

6. Almost all published studies on DVD deficiency and behavioral or brain outcomes have been published in rats rather than mice. We have one study outlining DVD-deficient behavioral outcomes in mice (40). An outbred strain such as a Sprague–Dawley rat is possibly subject to greater variation in experimental outcomes compared to say an inbred mouse strain. This strain however is available internationally and has been widely used in behavioral studies modeling schizophrenia.

7. The level of maternal vitamin D depletion in the model is marked; however, these levels have been reported in pregnant women during winter and spring months (41, 42).

8. This has not always been the case. For instance, in the first version of the DVD-deficient model, litters were culled to only two male pups (19). In later versions of the model, only males were tested from litters which were culled to six males and two females at birth (24, 25). A further variation was to restrict litter size to three males and three females (22). In recent years we have abandoned the "cult of culling" in favor of using offspring that reflect the natural variance in litter size from Sprague–Dawley rats.

9. This model has been adopted by collaborators who amongst other minor modifications have included 2 mM Ca^{2+} in

the drinking water of both controls and vitamin D-deficient females. We see this as an unnecessary step as serum calcium status is unaffected by this degree of vitamin D depletion.

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