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The Effects of Vitamin D_3 During Pregnancy and Lactation on Offspring Physiology and Behavior in Sprague–Dawley Rats

ABSTRACT: *Recent findings show that developmental vitamin D deficiency leads to altered brain morphology and behavioral development in the rat offspring. We examined the effects of different dietary vitamin D levels in rat dams on behavior and biochemistry of the offspring. Females were divided into five conditions and received diets containing 0, 1,5, 3.3, 6.0, or 10.0 IU/g of vitamin D_3 from mating to weaning. Offspring were tested as juveniles and as adults for anxiety, social learning and behavior, and locomotion. Results show that both deficient and excessive levels of vitamin D_3 in juveniles lead to altered physiology and behavior. In juveniles but not adults, variations in vitamin D were related to variations in measures of anxiety and marginally, activity levels. For social behaviors, both juveniles and adults were affected by mothers' diets. In general, offspring of animals receiving abnormal concentrations of vitamin D showed the most deficits. © 2012 Wiley Periodicals, Inc.* Dev Psychobiol 56: 12–22, 2014.

Keywords: *vitamin D; lactation; development; behavior, biochemistry; rats*

INTRODUCTION

There has been growing interest regarding the potential effects of vitamin D_3 deficiency on mental health; including depressive disorders (Humble, 2010), Seasonal Affective Disorder (Gloth, Alam, & Hollis, 1999), multiple sclerosis and other immune conditions (Raghuwanshi, Joshi, & Christakos, 2008; Smolders, Menheere, Kessels, Damoiseaux, & Hupperts, 2008). Furthermore, developmental vitamin D (DVD) deficiency has recently been linked to mental disorders such as schizophrenia (McGrath, 1999; McGrath et al., 2010), Parkinson's Disease (Newmark & Newmark, 2007), and autism (Grant & Soles, 2009).

Diseases with season of birth effects, such as schizophrenia are found to be more prevalent in people born in the winter and early spring months (McGrath, 1999). This is a pattern that is also found in serum vitamin D levels in relation to length of the photoperiod; with lower levels in the winter months (Holick, 1995). The developmental rat model of vitamin D deficiency has recently been employed to examine its potential role in the neurophysiology and behavior in the rat.

Vitamin D is integral to calcium and phosphate metabolism and absorption. Further, vitamin D receptors (VDR) are found in the rat brain throughout embryonic life into adulthood (Prufer, Veenstra, Jirinkowski, & Kumar, 1999; Veenstra et al., 1998), and have been implicated in the development of the brain, mediating various processes; such as neurogenesis, cell proliferation, neurotransmitter synthesis, and cell differentiation

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(Baksi & Hughes, 1982; Cui, McGrath, Burne, Mackay-Sim, & Eyles, 2007; Garcion, Wion-Barbot, Montero-Menei, Berger, & Wion, 2002; Kesby et al., 2010; Wion, MacGrogan, Nevue, Houlgatte, & Brachet, 1991). Additionally, pups exposed to DVD deficiency during the pre-weaning period develop longer brains with larger lateral ventricles and a thinner cortex, as well as reduced glial cell line-derived neurotropic factor and nerve growth factor expression (Eyles, Brown, Mackay-Sim, McGrath, & Féron, 2003). Some of these neural changes persist into adulthood (Féron et al., 2005). Vitamin D deficiency in rats and mice also result in various adverse effects such as reduced learning and memory function (Becker, Eyles, McGrath, & Grecksch, 2005), hyperlocomotion (Burne, Becker, et al., 2004), increased anxiety (Kalueff, Lou, Laaksi, & Tuohimaa, 2004), impaired spatial learning (Altemus, Finger, Wolf, & Birge, 1987), and impaired startle and sensori-motor gating or pre-pulse inhibition (Brune, Féron, et al., 2004). Given the relation between vitamin D_3 and dopamine function it is not surprising that some of the behaviors affected by a deficiency in vitamin D_3 are dopaminergically mediated (Kesby et al., 2010).

In the rat, there is a direct relationship between dietary vitamin D_3 intake and serum vitamin D_3 level (Fleet et al., 2008). Moreover, vitamin D_3 deficiency throughout pregnancy directly results in depletion of fetal vitamin D_3, although serum calcium levels remain unaltered, compensated for by maternal mechanisms (O'Loan et al., 2007). Previous studies examining maternal vitamin D_3 deficiency for different durations during fetal development (Becker et al., 2005; Burne, Becker, et al., 2004; Eyles et al., 2006; O'Loan et al., 2007) show that vitamin D_3 deficiency in the later stages of gestation alone is sufficient to provoke behavioral changes in the offspring (O'Loan et al., 2007). Given that pups vitamin D intake is dependent primarily on the mother via storage obtained in utero as well as the milk until at least 3 weeks of age (Clements & Fraser, 1988), here we explore vitamin D_3 deficiency during both gestation and lactation on basic physiology and behavior of offspring.

In addition to the timing of maternal vitamin D deficiency, recent evidence shows that high levels of DVD may also have adverse effects in humans (McGrath et al., 2010); we therefore undertook a dose–response analysis and investigated the effects of excessive levels of vitamin D_3 in addition to deficient levels. Thus, the purpose of this study was to examine the effects of administering various levels of vitamin D_3 during mating, pregnancy and lactation on behavior and physiology in juvenile and adult offspring. We included behavioral assessments during the juvenile period because previous research found mostly subtle effects of the vitamin D_3 deficiency in adulthood. Hence, we wished to determine how behavior would be affected closer to the time that the deficiency was in place as well as when changes in behavior might first occur. We predicted that prolonged vitamin D_3 deficiency would have similar effects on behavior as it has on physiology. Specifically, we expected to find that vitamin D_3 deficient diets would result in an increase in anxiety and activity level, and impair social learning in juvenile and adult offspring. We had no directional prediction regarding the effect of the highest dosage of vitamin D.

METHODS

Subjects

Female Sprague–Dawley rats ($n = 56$) derived from a stock originally obtained from Charles River Farms (St. Constant, Quebec, Canada) were born and raised at the University of Toronto at Mississauga animal facility. Animals were housed in Plexiglas cages (20 cm × 43 cm × 22 cm) under 12:12 hr light:dark cycle with lights on at 08:00 hr. Temperature and humidity were kept constant between 22–24°C and 60–65%, respectively. Food and water were given ad libitum and females remained on their respective dietary conditions beginning at the time of mating until pups were weaned at postnatal day (PND) 18. All procedures conformed to the Canadian Council on Animal Care guidelines, and were approved by the University of Toronto at Mississauga's Local Animal Care Committee.

Experimental Conditions

Females were randomly assigned to one of five dietary conditions, each with a different concentration of vitamin D_3: 0 IU/g ($n = 10$), 1.5 IU/g ($n = 10$), 3.3 IU/g ($n = 10$), control group; 3.3 IU/g is the standard amount of vitamin D_3 added to compose the regular Rat Chow used for all rats in our vivarium (see http://labdiet.com/pdf/5012.pdf), 6.0 IU/g ($n = 12$) and 10.0 IU/g ($n = 14$). The diet was composed by the TestDiet® division of LabDiet® (Purina Mills, LLC) under the requirements of International Standards Organization. Females were housed under non-UV emitting light and remained on their respective diets (Rat Chow; LabDiet®) from the start of mating until PND 18. Animals were housed under identical UV conditions and given identical diets until the start of our experimental manipulations.

For a subset of the mothers, food intake was monitored daily between PND 2-14 by weighing in and out the powdered diet every day. The 24 hr difference score constituted the measure of daily food intake.

Mating, Parturition, and Offspring

Females were approximately 18 weeks old at the time of mating. Each male was placed with a female in her home cage for 18 hr/day for 8 days. Males were returned to their home

cages for 6 hr each day to receive regular Rat Chow (LabDiet®).

Animals were monitored for parturition 21 days after the first day of mating. If pups were born before 16:00 hr, that day was designated as day of parturition, that is, PND 0. Pups were counted, sexed, and culled to as close to six male and six females as possible on PND 1. Most animals gave birth to litters of >12 pups and there was no significant effect of vitamin D3 dosage on litter size ($p = .507$; data not shown). Pups remained with their mothers until PND 18, at which time they were weaned and placed on a regular diet consisting of 3.3 IU/g vitamin D_3 Rat Chow.

Blood Chemistry and Body Measurements

On PND 9, body weights of males and females from each litter were measured in groups and their average weight was calculated. On PND 18, two males and two females from each litter were sacrificed via live decapitation for brain and blood collection. Their body weight, brain weight, body length, and head length were measured. Body lengths were measured from nose-to-rump, head widths from ear-to-ear and head lengths from nose to the end of skull.

Twenty-five (OH) vitamin D_3 in serum was assessed by a competitive enzyme-linked immunoassay (ELISA), VitaKit D™ (SciMed Technologies Inc., Alta, Edmonton, Alberta, Canada). This kit was based on fluid milk samples and therefore an alternative extraction was needed from serum. Ten microliter of 50% DMSO in phosphate buffered saline was added to 10 μl of serum, mixed, and left at room temperature for 15 min. Next, 50 μl of hexane was added and mixed, followed by 20 min in a 4°C centrifuge at 4,500 rpm. The hexane layer (50 μl) was carefully extracted from the surface and 10 μl of this hexane extract was used per well in the ELISA assay. Absorbance was measured at 450 nm on a spectrophotometer and the amount of vitamin D_3 in each sample was determined from a standard curve. Measurements were expressed by the ELISA kit in units of IU/ml.

Serum calcium was measured by a commercial calcium kit (Pointe Scientific, Inc., Canton, MI). Calcium in the serum reacts with Arsenazo III to form a purple complex that can be detected by a spectrophotometer at 650 nm. Concentrations were calculated from a standard sample and presented in units of mg/dl.

Serum phosphate was measured using a commercial inorganic phosphorus kit (Pointe Scientific, Inc.). Inorganic phosphorus in serum reacts in acid solution with ammonium molybdate to produce a complex that absorbs light at 340 nm. Samples were measured in a spectrophotometer and concentrations were calculated from a standard sample and presented in units of mg/dl.

Serum urea was measured using a commercial urea nitrogen kit (Pointe Scientific, Inc.). Serum urea reacts with the enzyme urease to produce ammonia. This then reacts with hypochlorite and phenol in an alkaline medium to produce a complex that can absorb light at 630 nm. A spectrophotometer was used to measure the reacted samples. Concentrations were calculated from a standard sample and presented in units of mg/dl.

Behavioral Tests

Subjects were tested as juveniles between PND 35–40, and as adults between PND 100–105. Testing was done in both age groups to assess the permanence of any effects of vitamin D_3. One male and one female from each litter were assigned to the anxiety, social behavior and learning, and locomotor activity test conditions. The assignment of animals' behavioral tests remained consistent between the two phases of testing. However, separate statistical analyses were undertaken on the effects of dosage and sex at the two timepoints.

Elevated Plus Maze

Anxiety was tested using an elevated plus maze (EPM) modified from a previous design (Pellow, Chopin, File, & Briley, 1985). It consisted of two open arms (10 cm × 50 cm × 45 cm) and two closed arms (10 cm × 50 cm × 45 cm) that extended from a central platform (10 cm × 10 cm). The maze was constructed from wood, painted over in black and was elevated 50 cm from the base. Subjects were transported from their colony room and tested in a separate room between 10:00 and 13:00 hr. Each rat was placed in the centre facing the experimenter at the beginning of the test, and then allowed to explore the maze for 10 min. The apparatus was cleaned with 70% ethanol and warm water after each test. The amount of time spent in each arm and the centre, along with grooming and rearing behaviors were recorded using Behavioral Evaluation Strategy and Taxonomy (BEST) software (Educational Consulting, Inc., Hobe Sound, FL).

Social Behavior and Social Learning

Subjects were tested in their home cage in their home colony room. Cage mates were removed from home cages and each subject was allowed to interact with a same sex conspecific of similar age and weight for two consecutive days: 30 min on the first day and 10 min on the second day. On the second day, subjects were randomly selected to receive either the same conspecific as Day 1 (familiar) or a new conspecific (unfamiliar) fitting the same criteria. The interactions were video recorded and then coded using BEST. The following measures were analyzed for both frequency and duration: anogenital sniffing, body sniffing, head sniffing, play fighting, and grooming (Macbeth, Edds, & Young, 2009).

Locomotor Activity

Subjects were tested for 1 hr each for 3 consecutive days in a locomotor activity box (47 cm × 26 cm × 20 cm) between 10:00 and 13:00 hr. Each test box was connected to a computer, which recorded the animal's activity using infrared lights nodes. On the bottom and top of each test box, there are rows of corresponding infrared light nodes, each pair of corresponding nodes connected to make a beam. Each time a beam was interrupted, the program recorded a "Cut," and each time the animal traversed the entire length of one row of nodes, the program recorded a "Length." Data were collected

into 5-min bins. Each test box was cleaned with 70% ethanol between trials.

Statistical Analysis

For most outcome measures two-way ANOVAs were performed, comparing the five vitamin D_3 groups as well as between males and females, followed by Tukey's post hoc tests comparing pairs of groups. For behavioral measures, figures are included for juvenile performance where group differences were significant or marginally significant, and, for comparison purposes, on adults on the same measures where group differences were no longer found.

RESULTS

Biochemistry

There was a significant effect of maternal vitamin D_3 diet on serum vitamin D_3 concentrations in the offspring at PND 18 (see Fig. 1A). Two-way ANOVA revealed a significant effect of vitamin D3 ($F_{(4,45)} = 171.67$, $p < .001$) but no effect of sex. As expected, decreased vitamin D_3 dose in the maternal diet resulted in partial depletion of serum vitamin D_3 in the offspring. Tukey's post hoc analysis showed that differences among all groups were significant except between 0 and 1.5 IU/g groups ($p < .05$). Two-way, ANOVA also showed a significant effect of dietary maternal vitamin D_3 level on serum calcium levels in the offspring ($F_{(4,55)} = 18.812$, $p < .001$; see Fig. 1B); again, there was no main effect of sex or interaction. Post hoc analysis revealed that the 10.0 IU/g group had significantly higher serum calcium levels than the other groups, while the 6.0 IU/g group had significantly higher serum calcium levels compared to those in the 1.5 IU/g group ($p < .05$).

No effects were found in serum phosphate levels for vitamin D_3 dosage or sex ($F_{(4,55)} = 2.429$, $p > .05$; $F_{(1,55)} = .115$, $p > .05$). Two-way ANOVA showed both a main effect of vitamin D dosage ($F_{(4,55)} = 4.025$, $p < .05$) and of sex ($F_{(1,55)} = 32.059$, $p < .001$) on serum urea levels. Further investigation reveals that vitamin D_3 dosage has a significant effect in males ($F_{(4,27)} = 4.04$, $p < .05$; data not shown) but not in females.

Body Length, Body Weight, and Brain Weight

Two-way ANOVA showed no significant effect of vitamin D_3 dosage or sex on pups' body weight on PND 9. However, by PND 18, there were significant effects of maternal vitamin D_3 levels on pups' body lengths ($F_{(4,109)} = 4.617$, $p < .05$; Fig. 2A), body weight ($F_{(4,109)} = 6.19$, $p < .001$; Fig. 2B), and brain weight ($F_{(4,108)} = 5.87$, $p < .001$; Fig. 2C). When pups' brain weights were corrected for pups' body weights, there is still a significant effect of vitamin D3 dosage ($F_{(9,107)} = 4.906$, $p = .001$; data not shown). There was no effect of sex on pup's body weight, body length, or brain weight, and no interactions on PND 18. Tukey's post hoc analysis revealed that rats in 10.0 IU/g groups weighed significantly less than those in 3.3 and 6.0 IU/g groups ($p < .05$), while those in 10.0 IU/g group were shorter in body length compared to 0, 3.3, and 6.0 IU/g groups ($p < .05$). Post hoc analysis also showed that the brains of animals in 10.0 IU/g group weighed less than animals the other groups ($p < .05$).

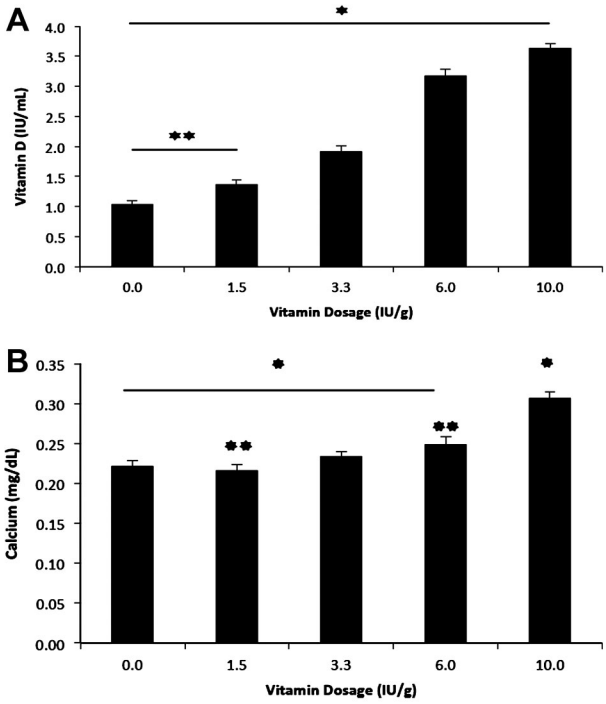


FIGURE 1 (A) Serum vitamin D concentration in the offspring at the time of weaning on PND 18 (mean ± *SE*). Decreased vitamin D dose in the maternal diet resulted in partial depletion of serum vitamin D in the offspring, $F_{(4,44)} = 178.\ 36$, $p = .000$. Post hoc analysis shows that animals in the 0 and 1.5 IU/g group had significantly lower serum levels of vitamin D3 than those in the 3.3, 6.0, and 10.0 IU/g groups ($^{*}p < .05$, $^{**}p < .01$). (B) Serum calcium levels in the offspring on PND 18 (mean ± *SE*). Higher levels of dietary maternal vitamin D resulted in increased serum calcium levels, $F_{(4,54)} = 19.864$, $p = .000$. Tukey's post hoc analysis showed that animals in the 6.0 IU/g and 10.0 IU/g groups had significantly higher serum calcium levels compared to those in the 0 IU/g and 1.5 IU/g groups. As well, animals in the control (3.3 IU/g) group had significantly lower serum levels of calcium than animals in the 10.0 IU/g group ($^{*}p < .05$, $^{**}p < .01$).

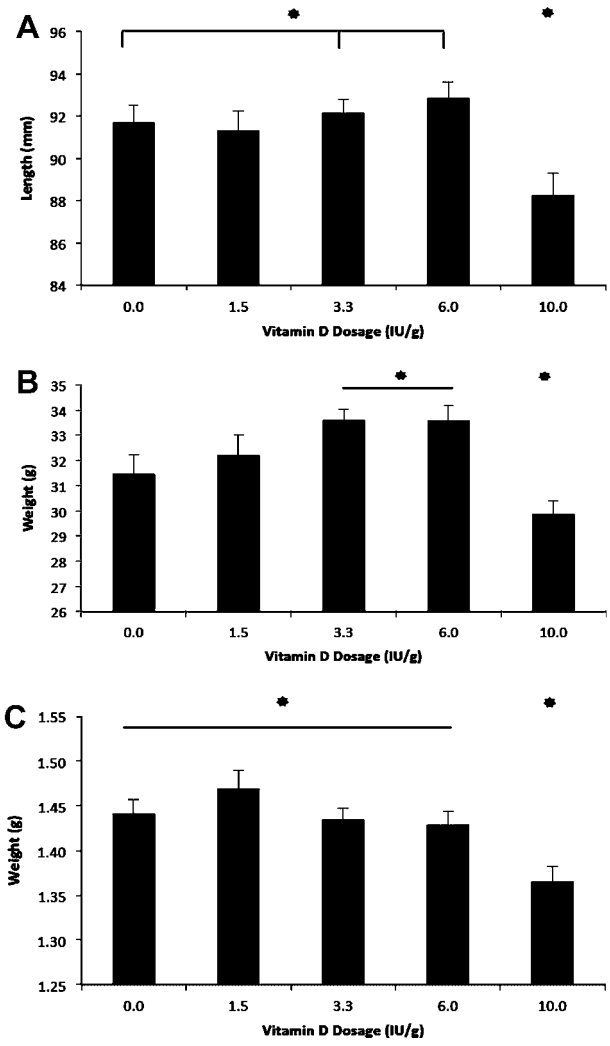


FIGURE 2 (A) Pup body length (nose to rump) measured on PND 18 (mean ± *SE*). Pups that received the highest level of dietary maternal vitamin D had reduced body length, $F_{(4,108)} = 4.571$, $p = .002$. Tukey's post hoc analysis showed that offspring in the 10.0 IU/g group had significantly shorter body lengths compared to those in the 0, 3.3, and 6.0 IU/g groups ($^{*}p < .05$). (B) Maternal dietary vitamin D levels influenced pup body weight at the time of weaning on PND 18 (mean ± *SE*). Pups showed increased body weight with higher levels of maternal vitamin D but the effect was reversed in 10.0 IU/g group. $F_{(4,108)} = 6.361$, $p = .000$. Tukey's post hoc analysis showed that offspring in 3.3 and 6.0 IU/g groups weighed significantly more compared to those in the 10.0 IU/g group (* $p < .05$). (C) Brain weight of the pups on PND 18 was affected by the mother's dietary vitamin D level (mean ± *SE*), $F_{(4,107)} = 5.714$, $p = .000$. Mothers that received the highest level of Vitamin D3 in diet produced pups with the lowest brain weight. Post hoc analysis indicated that the offspring in the 10.0 IU/g group had significantly lower brain weight compared to all other groups ($^{*}p < .05$).

Mothers' Food Intake

One-way ANOVA showed no significant effect of vitamin D_3 levels in mothers' diets on mothers' food intake over the first 10 days after parturition. Whether it does over the next week prior to weaning, we do not know.

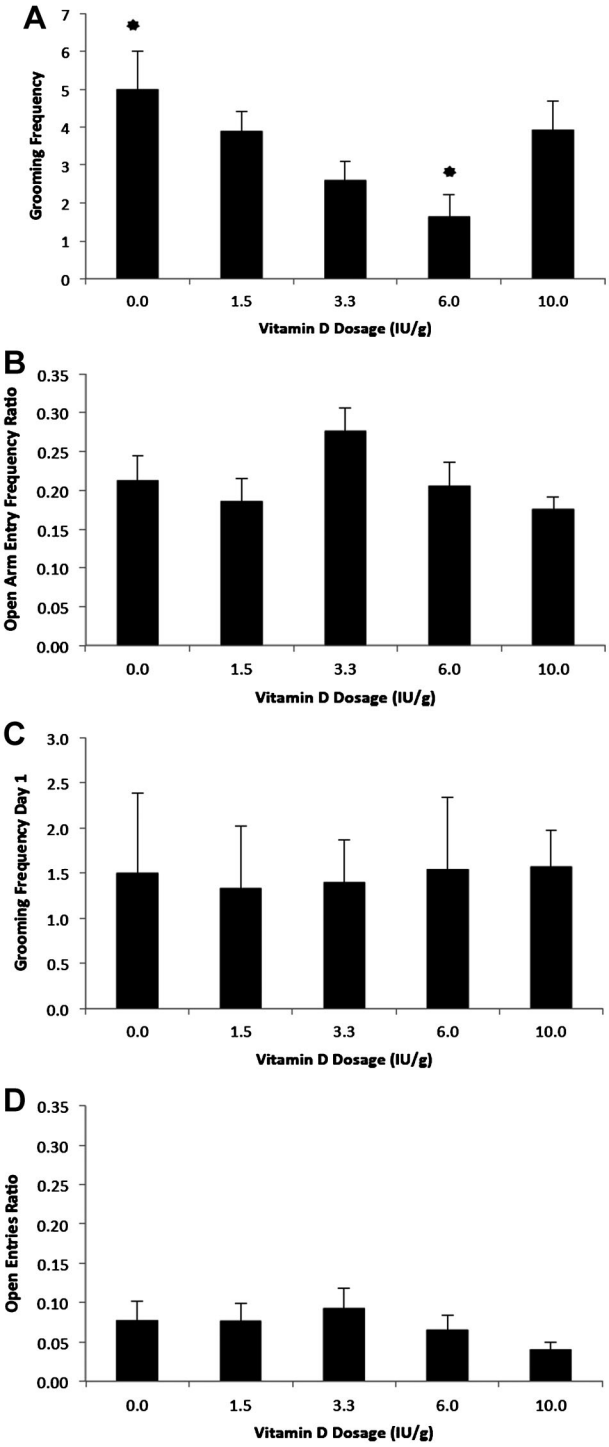
Behavioral Tests

Elevated Plus Maze. Two-way ANOVA revealed a significant effect of vitamin D_3 levels in mothers' diets on grooming frequency in the EPM test in offspring between PND 35-40 ($F_{(4,54)} = 2.916$, $p < .05$; see Fig. 3A), but no main effect of sex and no interaction. Tukey's post hoc analysis showed a significant difference between the 0 and 6.0 IU/g groups ($p < .05$); animals in 0 IU/g group showed significantly more grooming behaviors than those in the 6.0 IU/g group. While there were no significant group effects at $p < .05$ (2T) of vitamin D_3 on time or proportional time spent in open arms, or number of arm changes, for both the ratio of open arm entries ($F_{(4,54)} = 2.135$ $p = .09$ (.045, 1T, Fig. 3B) and total number of arm changes in the maze ($F_{(4,54)} = 2.319$, $p = .07$ (.035, 1T, not shown), there were marginal overall group differences. For the ratio score the 3.3 IU/g group showed higher levels than both the lowest concentration group (0 IU/g) and the highest concentration group (10.0 IU/g). There was no effect of sex on any of the above measures in the EPM. For comparison purposes, as can be seen in Figure 3C,D, there were no significant or marginal effects of vitamin D on any measure of anxiety in adult offspring between PND 100-105.

Social Behavior and Learning. In the juveniles, there was a group effect on social sniffing only in males on the first day of testing ($F_{(4,28)} = 3.116$, $p < .05$, Fig. 4A), but no effect on social learning (reflected in the differential responses to the familiar vs. the unfamiliar conspecific on Day 2 and the proportional change in responses from Day 1 to 2). The 10.0 IU/g male animals showed the highest levels of sniffing in comparison to all other groups. In addition, there was a significant group effect on frequency and duration of self-grooming, with the 3.3 IU/g animals showing the lowest levels and the extreme groups showing higher levels ($F_{(4,49)} = 3.15$, 3.45; $p < .015$–.022, Fig. 4B). There were no other significant effects in juveniles. However, among adults a somewhat different pattern was shown. There was no group effect on sniffing or grooming (Fig. 4C), but there was a significant group effect on "playfighting" duration ($F_{(4,55)} = 2.65$, $p < .05$; see Fig. 4D). Post hoc analysis showed that

rats in the sufficient groups (3.3, 6.0 IU/g) spent more time playfighting than those in the 1.5 IU/g group.

Locomotor Activity. There was a significant effect of vitamin D_3 levels in mothers' diets on locomotor activity levels in the offspring between PND 35–40 ($F_{(4,56)} = 3.217$, $p = .021$; see Fig. 5A); but no effect of sex. Tukey's post hoc reveals a marginally significant difference between the 0 and 3.3 IU/g groups, $p = .091$, but no significant differences between any other pairs of groups. There was a significant effect of testing days on the mean distance travelled ($p < .001$) in both males and females with a gradual decrease of activity level over the three testing days. There was also a significant days effect on the number of droppings animals deposited in the test box ($p < .05$). Lastly, there were also no significant effects of maternal vitamin D levels on locomotion in the adult offspring (Fig. 5B).



DISCUSSION

The current study found that the administration of different concentrations of vitamin D_3 at mating and during pregnancy and lactation are transmitted to the offspring through the mothers' milk during nursing, producing levels of vitamin D_3 and of calcium in the offspring circulation that are related to the dose present in the mothers' diet. Furthermore, this shows that variations in vitamin D_3 are related to juvenile behavior, such that very low levels administered to mothers produce in offspring (a) enhanced anxiety, as indicated by increased grooming frequency and lowered probability to enter the open arms; (b) enhanced anxiety in social contexts as shown by increased self-grooming during the social interaction task; and (c) reductions in

FIGURE 3 (A) Grooming frequency of pups in the EPM in juveniles as a function of vitamin D dosage administered to mothers. Deficient maternal vitamin D diet resulted in increased anxiety level as measured by grooming frequency in the EPM in the juvenile offspring, $F_{(4,54)} = 3.256$, $p = .019$. The effect was reversed when the mother received the highest level of dietary vitamin D (10.0 IU/g). Post hoc analysis indicated that juvenile rats in 6.0 IU/g groomed significantly less compared to those in 0 IU/g ($^{*}p < .05$). (B) Ratio of entries into open arms/total entries in the EPM in juveniles as a function of vitamin D dosage administered to mothers. Deficient vitamin D diet resulted in a marginal increase in anxiety as measured by and ratio of open entries to total in juvenile offspring ($F_{(4,54)} = 2.135$, $p = .090$). Rats with 3.3 IU/g maternal vitamin D diet were the most likely to enter the open arms ($F_{(4,54)} = 2.135$ $p = .045$, 1T) $^{*}p < .05$. (C) Grooming frequency of pups in the EPM in adulthood in adults as a function of vitamin D dosage administered to mothers. There were no significant group effects, $F_{(4,53)} = .023$, $p = .999$. (D) Ratio of entries into open arms/total entries in the EPM in adults as a function of vitamin D dosage administered to mothers. There were no significant group effects, $F_{(4,53)} = 1.17$, $p = .336$.

locomotion in the lowest vitamin D groups (0 and 1.5 IU/g) which showed marginal differences from the control group (3.3 IU/g). In general, the animals showing the most clearly adapted responses on these measures were those in the middle dosage 3.3 IU/g group which represents the dosage that is present in usual rat chow. Further, with the exception of social behaviors, effects seen in most of the behaviors tested do not persist into adulthood. For social behaviors, however, DVD deficiency results in alterations in some social behaviors that occurred during the juvenile period and alterations in others (i.e., playfighting) in adulthood. Finally, the current study shows that vitamin D_3 excess also exerts negative effects both on growth and on some behaviors, suggesting that high concentrations may in fact be toxic.

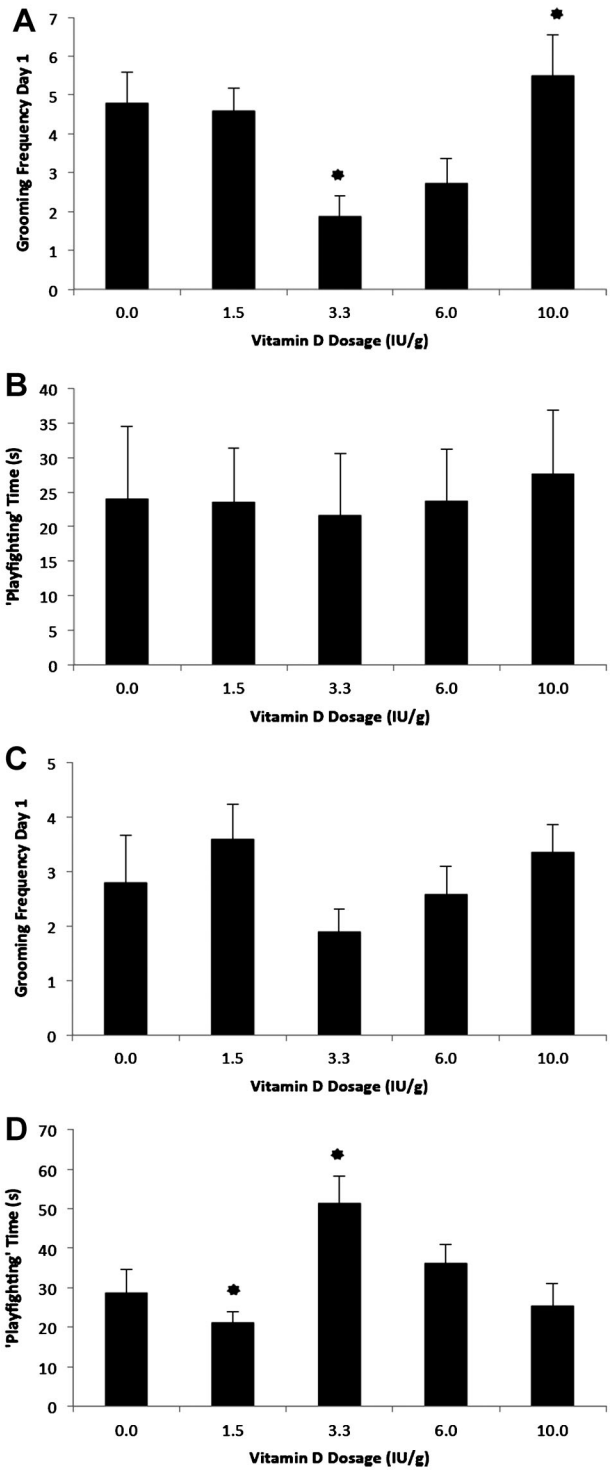


FIGURE 4 (A) Grooming frequency of pups during the social interactions in juveniles as a function of dosage of vitamin D administered to the mothers. $F_{(4,53)} = 3.452$, $p = 1.015$. (B) Social behavior as measured by the total duration of "playfighting" indicates no significant difference between vitamin D groups. $F_{(4,53)} = .066$, $p = .992$. (C) Social behavior in adults as measured by grooming frequency on Day 1 of test days indicates no difference between Vitamin D groups. $F_{(4,55)} = 1.223$, $p = .313$. (D) Deficient vitamin D levels in the mother's diet significantly reduced "playfighting" duration in the adult offspring. Similar effects were seen in groups that received the highest level of dietary maternal vitamin D. Post hoc analysis showed that the 1.5 IU/g group and the 10.0 IU/g group were significantly different from the 3.3 IU/g group ($F_{(4,55)} = 4.546$, $p = .003$).$^{*}p < .05$.

While there was a strong dosage effect of DVD levels on all body measurements, only the group that received the highest level of maternal vitamin D showed shorter body lengths, lower body, and brain weights compared to those on the regular diet (3.3 IU/g vitamin D_3 added). Groups receiving deficient doses of vitamin D_3 did not show decreased measurements in body weight, unlike what previous studies have shown (Brune, Féron, et al., 2004; O'Loan et al., 2007). While the biological basis for such effects is not immediately clear, we suspect that the anti-proliferative effects of vitamin D_3 contributed to the decreased body and brain weights in the heavily supplemented group, as VDRs are found throughout the brain as well as in various types of cells throughout the body including muscle, adipose tissue, and bone marrow (Norman, 2006; Prufer et al., 1999; Veenstra et al., 1998). We know based on the maternal food intake results that the effects on the offspring did not result from differential eating by the different groups of mothers during the first 10 days postpartum. However, mothers' eating could have affected pup growths after the first 10 days although we think this is unlikely. Since by the first 10 postpartum days, animals would have been on the diets for a month, we assume that any changes in mothers food intake that were vitamin D dependent would be revealed in the 10 days during which it was measured. Based on this rationale, we assume that any differences

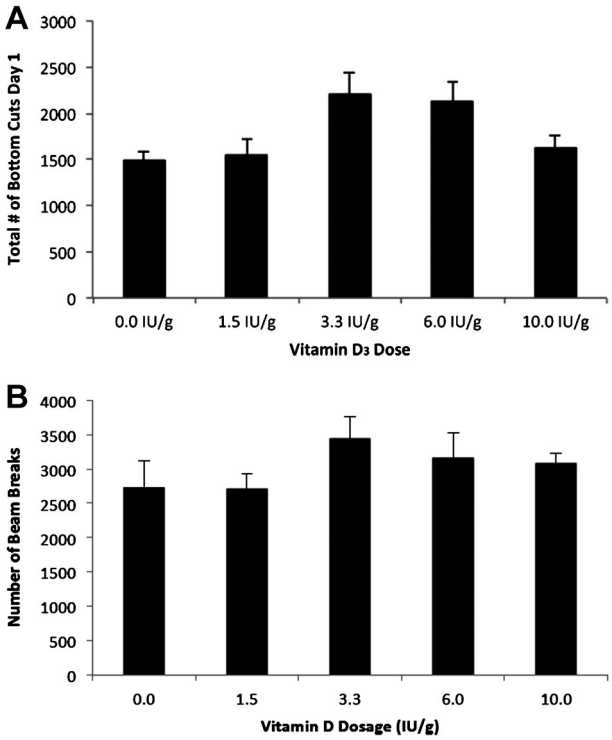


FIGURE 5 (A) Locomotor activity levels in juveniles as measured by total bottom beam breaks on the first day of testing in a locomotor activity box (mean ± *SE*). Mother's dietary vitamin D levels has a significant effect $F_{(4,56)} = 3.217$, $p = .021$. Post hoc analysis shows no significant difference between any particular pairs of groups. However, the juvenile offspring that received the control maternal vitamin D diet (3.3 IU/g) showed the highest level of activity. (B) Locomotor activity levels in adults as measured by average beam breaks across 3 days indicate no difference between vitamin D groups. $F_{(4,54)} = .845$, $p = .504$).

in serum vitamin D_3 levels in the offspring are a function of the concentration of vitamin D_3 in their mothers' diets and not a function of alterations in food consumption by their mothers. We can also infer from this that any adverse effects present in juvenile rats are a function of the level of vitamin D_3 in their systems, and not due to malnutrition induced by changed eating habits in the lactating dams.

The observation that dams that received a diet completely depleted of vitamin D_3 still produced pups with detectable levels of serum vitamin D_3 may not be surprising given that newborn rats depend primarily on the stored vitamin D_3 obtained in utero and other studies suggest that the dietary administration beginning 6 weeks prior to conception would be necessary to fully eliminate maternal vitamin D_3 (O'Loan et al., 2007). It is also worth noting that the litters in our study litters were culled to 12 pups from litter sizes of up to 16 pups, a procedure that may have amplified the amount of vitamin D_3 each pup received over what they would have received had the litter sizes been larger. Finally, the dams that received higher-than-normal dietary vitamin D_3 produced pups with serum vitamin D_3 levels that were less than expected by a linear relationship, which may suggest that there is a ceiling effect in which excessive levels of maternal vitamin D_3 are either not being transferred to the pups or are being metabolized by the pups. It is plausible that the inhibition results from a preventative mechanism given the physiological implications of excessive vitamin D_3 intake such as progressive weight loss, difficulty in movement and respiration, epistaxis, and subnormal body temperature in rats (Chavhan et al., 2011; Hathcock, Shao, Vieth, & Heaney, 2007 for review).

Increased grooming activity both in the EPM and in the social tests likely indicates increased levels of anxiety in maternal vitamin D_3 deficient juvenile offspring. Although grooming was not the central aspect of either test, it nevertheless has been interpreted as a sign of anxiety (van Erp, Kruk, Meelis, & Willekesens-Bramer, 1994). Similar to our findings, VDR null mutant mice show an increase in grooming behavior in multiple anxiety tests (Kalueff et al., 2004). There was a marked increase in grooming frequency at the 10.0 IU/g group that was very similar to the trends seen in physiological measures. While the reason for such an effect is unclear, this trend suggests possible adverse effects of excess DVD. However, this relationship was no longer seen in adulthood, suggesting that effects of transient DVD anomaly can be normalized by post-weaning rearing on a diet containing normal levels of vitamin D_3 (3.3 IU/g). Unfortunately, in the present design, we did not monitor estrous cyclicity of adult animals, where vitamin D effects were reduced or absent, and it is possible that our tests did not occur randomly with respect to the estrous cycle. This could have had an impact on our results given that there is evidence that emotional responsiveness and activity do change as a function of stage of estrous cycle (Mora, Dussaubat, & Díaz-Véliz, 1996). This issue would not be relevant in the case of testing of pre-pubertal animals who are not yet cycling.

There was an effect of vitamin D_3 dosage on juvenile activity level, that is, a reduction in locomotor activity level in the deficient groups as compared to those in the group that received regular diet. Our result is in contrast to previous findings in which the deficient offspring showed hyperlocomotion in the adulthood (Burne, Becker, et al., 2004; Eyles et al., 2006). The present results are, however, consistent with results based on VDR knockout mice in which the mutant mice showed decreased motor activity levels (Burne,

McGrath, Eyles, & Mackay-Sim, 2005; Kalueff et al., 2004).

The close association of vitamin D and calcium absorption may be informative in the interpretation of our data, as there was a significant group effect on serum calcium levels in the pups. Hence, the reduction in serum calcium levels may have disrupted musculoskeletal development and led to reduced activity levels. Notwithstanding, the largest reduction in calcium level was only at 11% and it is still possible that this effect can be attributed to dysregulation of the dopamine system as suggested for increased anxiety. Interestingly, there was a decline in activity level in groups that received higher level of DVD than the control. We speculate that overexposure to vitamin D_3 may have resulted in hypercalcemia, leading to a disruption in motor activity. At present, it is difficult to determine whether the effect was a result of brain anomaly resulting from DVD toxicity. In keeping with other behaviors, all locomotor anomalies disappeared in adulthood, suggesting that postnatal vitamin D_3 supplementation rescued any adverse behavioral effects.

Only social behavior showed a persistent, albeit subtle, effect in adulthood. The duration of social ''play-fighting'' was reduced in the deficient groups and in the heavily supplemented group. It is important to note that behaviors were changed in peri-pubertal rats and it is possible that DVD deficiency may selectively affect peri-pubertal behaviors only. While we cannot speculate on the neural correlates, alteration in social behavior in both the pre- and peri-pubertal periods are considered negative symptoms in the animal models of schizophrenia (Becker et al., 2003).

There is evidence that many of the outcomes reported here, including anxiety and locomotor activity, may result from alterations in dopamine function (Beninger, 1983; Koch, 1999; Serafim & Felicio, 2001). In fact, vitamin D has been implicated in the synthesis of dopamine (Baksi & Hughes, 1982) and the regulation of dopamine receptors and transporters (Kesby et al., 2010; Peeyush et al., 2010) throughout the limbic system and striatum (Walbert, Jirikowski, & Prufer, 2001), structures known to be important for emotion regulation, attention, and activity. Hence, dopamine dysfunction may well be the primary mechanism through which DVD affects behavior. Future research should be conducted to analyze whether the DVD deficiency of the mother affects dopamine receptors or function in the offspring's brain.

We found that high levels of vitamin D_3 may have adverse effects. To our knowledge, we are the first to examine the effects of high levels of vitamin D_3 on behavior in rats. It is difficult to determine whether the highest level given in the current study can be considered a toxic level, although levels given in this study are much lower than levels used in Chavhan et al. (2011). However, our experimental conditions ranged up to three times the amount given in the regular laboratory rat diet. While the exact mechanism by which the excess amount of vitamin D_3 can lead to an adverse behavioral outcome has yet to be studied, a recent study has found that there is a U-shaped relationship of prenatal vitamin D_3 level and risk of schizophrenia in humans (McGrath et al., 2010). Although the current study did not yield any direct evidence for a relationship between DVD deficiency and endophenotypes of schizophrenia, our behavioral results point to the possible dysregulation of the dopamine system. Future research investigating potential toxicity effects seems warranted.

The design of our study was unique primarily in that we investigated the effects of both deficient and excessive levels of vitamin D_3 in comparison to a normal control. This paradigm could have implications for other mammalian species (including humans), as (a) many mammalian offspring are naturally reared away from sunlight and dependent mainly on the milk for vitamin D supply in early postnatal development; and (b) DVD is still a large health problem for pregnant women and consequently their infants in many parts of the world (Holick & Chen, 2008; Raiten & Picciano, 2004). In this rat model, we have demonstrated that prolonged DVD deficiency from pregnancy and throughout lactation has real, but usually transient, effects on offspring behavior.

NOTES

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