Vitamin D Deficiency in BALB/c Mouse Pregnancy Increases Placental Transfer of Glucocorticoids

Dijana Tesic, Jazmin E. Hawes, Graeme R. Zosky, and Caitlin S. Wyrwoll

School of Anatomy, Physiology and Human Biology (D.T., J.E.H., C.S.W.), The University of Western Australia, Perth 6009, Australia; and School of Medicine (G.R.Z.), University of Tasmania, Hobart 7000, Australia

The prevalence of vitamin D deficiency in pregnancy is increasing and implicated in adverse consequences for the health of offspring in later life. The aim of this study was to determine whether vitamin D deficiency increases fetal exposure to glucocorticoids, which are known to alter fetal development and result in adverse adult health outcomes. Female BALB/c mice were placed on either a vitamin D control (2195 IU/kg) or deficient (0 IU/kg) diet for 5 weeks before and during pregnancy. Maternal serum, placentas and fetal brains were collected at embryonic day 14.5 or 17.5 for morphological and gene expression analysis. Vitamin D deficiency during pregnancy increased maternal corticosterone concentrations and reduced placental weight. Maternal vitamin D deficiency decreased placental expression of 11β-hydroxysteroid dehydrogenase type II, which inactivates glucocorticoids thereby protecting the fetus from inappropriate glucocorticoid exposure. There was a corresponding increase in placental and fetal expression of the highly glucocorticoid-sensitive factor glucocorticoid-induced leucine zipper. Furthermore, placental expression of the angiogenic factor vascular endothelial growth factor-A was reduced in vitamin D-deficient pregnancies, with a corresponding decline in fetal capillary volume within the placenta. Overall, we show that prenatal vitamin D deficiency leads to an increase in maternal corticosterone, alterations in genes indicative of increased fetal glucocorticoid exposure and impairment in placental vascular development. Thus, the long-term adverse health consequences of vitamin D deficiency during early development may not just be due to alteration in direct vitamin D-related pathways but also altered fetal glucocorticoid exposure. **(*Endocrinology* 156: 3673–3679, 2015)**

During fetal life the maternal milieu can induce changes in development that have long-term impact on later health and disease. One environmental factor of increasing interest is the significance of maternal vitamin D status. The prevalence of maternal vitamin D deficiency is common and increasing (1) and has been implicated in adverse offspring health outcomes such as neuropsychiatric disorders and asthma (2–4).

The mechanisms underlying gestational vitamin D deficiency and poor offspring health outcomes are currently uncertain. The vitamin D receptor is abundantly expressed in both placental and fetal tissues (5–7) and thus deficiency of vitamin D will impact on vitamin D receptor-related gene transcription. But could there be other underlying mechanistic changes additional to "direct" vitamin D related pathways? One possible mechanism is alteration in fetal exposure to glucocorticoids as a consequence of vitamin D deficiency, although little has been published to this effect. The interaction of vitamin D on glucocorticoids has been proposed in models of asthma and hippocampal cell culture (8, 9), whereas one study in rats has shown that maternal vitamin D deficiency did not alter basal or restraint stress glucocorticoid levels in the dams or adult offspring, although area under the curve was increased (10). However, more extensive studies are required to conclusively demonstrate whether maternal vitamin D deficiency exposes the fetus to higher levels of glucocorticoids.

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Abbreviations: E, embryonic day; FC, fetal capillary; GILZ, glucocorticoid-induced leucine zipper; GR, glucocorticoid receptor; HPA, hypothalamic-pituitary-adrenal; 11β-HSD2, 11β-hydroxysteroid dehydrogenase type II; LZ, labyrinth zone; 25(OH)D, 25-hydroxy vitamin D; VEGF, vascular endothelial growth factor.

Prenatal overexposure to glucocorticoids is known to profoundly alter fetal structure and function and thus "program" increased risk of cardio-metabolic and psychiatric disorders in later life (11). Although glucocorticoids are highly lipophilic and thus able to readily diffuse across the placenta, fetal glucocorticoid levels remain significantly lower than maternal levels throughout gestation, indicative of a placental "barrier" protecting the fetus from the harmful effects of glucocorticoid overexposure. This barrier results from high placental expression of 11β-hydroxysteroid dehydrogenase type II (11β-HSD2), which converts glucocorticoids from their active form (cortisol in humans, corticosterone in rodents) to their biologically inactive forms (cortisone and 11-dehydrocorticosterone, respectively) (12). In rodents, the abundant expression of placental 11β-HSD2 then markedly declines at the end of gestation, presumably to facilitate fetal maturation in preparation for birth (13, 14). Extensive investigations in animal models have shown that bypass, inhibition, down-regulation or removal of feto-placental 11β-HSD2 recapitulates programmed health outcomes, with similar outcomes in human populations (15).

This aim of this study was to investigate whether vitamin D deficiency alters fetal exposure to glucocorticoids in BALB/c mice. Maternal serum, placentas and fetal heads were collected at embryonic day (E)14.5 and E17.5 and assessed for glucocorticoid-related parameters. These included maternal corticosterone levels, placental and fetal gene expression of factors including 11β-HSD2 and glucocorticoid-induced leucine zipper (GILZ), which is highly regulated by glucocorticoids (16), as well as stereological assessments of placental morphology.

Materials and Methods

Animals

All studies were performed according to animal health and welfare guidelines and approved by the Telethon Kids Institute Animal Ethics Committee. Mice were housed in rooms with a 12-hour:12-hour ambient UV-B-free light:dark cycle with ad libitum food and water. Three-week-old female BALB/c mice (ARC) were provided with vitamin D-deficient (0 IU/kg) or vitamin D control (2195 IU/kg) diets (Specialty Feeds) for 5 weeks before mating. Deficient diets were supplemented with 25-g/kg calcium (which maintains serum calcium levels between the 2 diets) (17), and the caloric content was manipulated (wheat starch content 34.0 g/100 g deficient vs 37.8 g/100 g replete) to ensure similar energy intake (deficient, 15.3 MJ/kg; control, 15.8 MJ/kg). Female mice were mated randomly with 1 of 20 vitamin D control BALB/c males. The morning on which a vaginal plug was identified was termed E0.5, and dams were then singly housed.

Tissue collection

Tissue collections were conducted before 9 AM at E14.5 (when placental 11β-HSD2 is normally abundant) and E17.5 (when placental 11β-HSD2 expression is down-regulated) with term being 20 days of gestation. Dams were euthanased ip with an overdose of ketamine (800 mg/kg; Troy Laboratories) and xylazine (40 mg/kg; Troy Laboratories). Maternal blood was obtained via cardiac puncture, to determine maternal serum vitamin D and corticosterone levels. Uterine horns were removed and placed on ice. Placentas and fetuses were dissected from the uterus and weighed. At both gestational time points, placentas were randomly selected and either fixed in 4% paraformaldehyde for sterological assessments or dissected into labyrinth zone (LZ) and junctional zone and snap frozen in liquid nitrogen for subsequent gene analyses. Fetal heads were collected at E14.5, whereas at E17.5, the fetal brains were removed from the skull. Brains were snap frozen in liquid nitrogen for gene analysis studies. Fetal tails were sampled for gender typing using male-specific sex-determining region Y primers. Maternal adrenals were fixed, subsequently trimmed of fat, weighed, and expressed relative to maternal body weight.

Maternal vitamin D analysis

Maternal vitamin D status was examined using a 25-hydroxy vitamin D (25(OH)D) enzyme immunoassay according to the manufacturer's instructions (Immunodiagnostic Systems). This assay quantifies total metabolite 25(OH)D (D2+D3).

Maternal corticosterone analysis

Maternal corticosterone levels were determined using a mouse corticosterone ELISA according to the manufacturer's instructions (ALPCO).

Placental immunohistochemistry and stereology

Placental immunohistochemistry and sterological assessments were conducted as detailed previously (18). Zonal fractions (% placental volumes) were determined for decidua basalis, spongiotrophoblast zone, LZ, and chorion. Volumes associated with maternal blood spaces and fetal capillaries (FCs) were also determined. Assessments were only conducted on E17.5 placentas. Measures were adjusted for tissue shrinkage by measurement of average maternal erythrocyte diameter before and after tissue processing. All measurements were performed blind, and intraobserver error was less than 5%.

Real-time quantitative reverse transcription-PCR

Total RNA was isolated from tissue samples using the QIAGEN extraction protocol (QIAGEN) as per manufacturer's instructions. RNA was quantified using the NanoDrop (ND-1000) spectrophotometer and RNA (1 μg) was reverse transcribed using mouse Moloney leukemia virus reverse transcriptase and random hexamers (Promega). The resultant cDNAs were purified using an ultraclean PCR spin kit (MoBio Laboratories, Inc) as per manufacturer's instructions.

Analyses of mRNA expression levels for genes encoding for 11β-hydroxysteroid dehydrogenase I (*Hsd11b1*), 11β-HSD2 (*Hsd11b2*), GILZ (*Tsc22d3*), glucocorticoid receptor (GR) (*Nr3c1*), and vascular endothelial growth factor (VEGF)-A (*Vegfa*), and the reference genes *Ppia*, *Sdha*, and *18s* were performed by real-time PCR on the Rotorgene 6000 system (Corbett Research). Primer pairs for all genes (Table 1) were purchased as

Table 1. PCR Conditions

Gene	QuantiTech Name or Primer Sequence	Tm (°C)
Hsd11b1	Mm_Hsd11b1_1_SG	55
Hsd11b2	Mm_Hsd11b2_SG	55
Nr3c1	Mm_Nr3c1_1	55
Vegfa	Mm_Vegfa_1_SG	55
GILZ	F, 5-GGTGGCGGTCTATCAGCTGCAC-3′	85
	R, 5′-CCTCCACCTCCTCTCTCACAGCG--3′	
Sdha	F, 5′-TGGGGCGACTCGTGGCTTTC-3′	85
	R, 5′-CCCCGCCTGCACCTACAACC-3′	
Ppia	F, 5′-AGCATACAGGTCCTGGCATC-3′	83
	R, 5′-TTCACCTTCCCAAAGACCAC-3′	
18s	Rn18s	84

QuantiTech name, primer sequences, and melting temperature (Tm) for analysis and reference genes. *Hsd11b1*, 11β-HSD1; *Hsd11b2*, 11β-HSD2; *Tsc22d3*, GILZ; *Nr3c1*, GR; *Sdha*, succinate dehydrogenase complex, subunit A; *Ppia*, peptidylprolyl isomerase A; F, forward; R, reverse; bp, base pair.

QuantiTech primers with the exception of *Ppia* and *Sdha*, which were designed using Primer-BLAST (http://www.ncbi.nlm.nih.gov). Primer pairs were designed to span introns to prevent amplification of product from genomic DNA. Standard curves were generated with 10-fold serial dilutions of gel extracted (QIAEX II; QIAGEN) PCR products and relative gene expression was analyzed using the Rotorgene 6000 software. All samples were run in duplicate and normalized against *Ppia*, *Sdha*, and *18s* using the GeNorm algorithm (19).

Statistical analysis

All data are expressed as the mean ± SEM, with each litter representing an n of 1. Three-way ANOVA (age, treatment, and sex as independent variables) and subsequent two-way ANOVAs were used to examine data where appropriate (GraphPad Prism 5 for Mac OS X). Where no interaction was observed, a Fisher's Least Significance Difference test was used post hoc. When a significant interaction was observed after two-way ANOVA, separate analyses were conducted using 2-tailed unpaired Student's *t* tests.

Results

Maternal outcomes

As previously reported (20), dams placed on the vitamin D-deficient diet had significantly lower levels of 25(OH)D than controls. Maternal diet did not alter weight gain trajectories over gestation or average litter size, but fetal head size and crown-rump length were reduced (20). Although food intake was not measured, the lack of difference in maternal weight gain between the 2 groups in this study or in earlier studies is suggestive of limited differences in food intake.

Vitamin D deficiency decreases placental weight

Fetal weight was not altered by maternal diet or fetal sex, although crown-rump length was reduced (20). Although also unaffected by fetal sex, vitamin D deficiency significantly decreased placental weight by 10% at E14.5 and 14% at E17.5 ($P < .01$) (Figure 1).

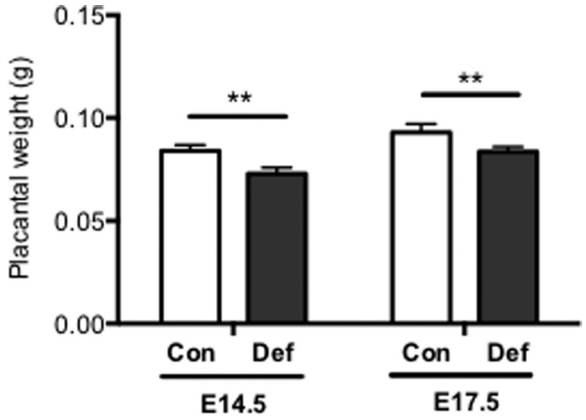

Figure 1. Placental weights after prenatal vitamin D deficiency. Values are the mean ± SEM (n = 6–8 per group). There was a significant treatment effect, with lighter placentas after vitamin D deficiency at both E14.5 and E17.5 (**, $P < .01$; two-way ANOVA).

Vitamin D deficiency increases circulating maternal corticosterone at E17.5

Serum corticosterone levels increased by over 2-fold from E14.5 to E17.5 ($P < .001$) (Figure 2A). Furthermore, consumption of a vitamin D-deficient diet significantly increased serum corticosterone by 0.8-fold at E14.5 and 0.6-fold at E17.5 ($P < .05$) (Figure 2A). There were corresponding changes in relative adrenal weight (Figure 2B) with vitamin D deficiency significantly increasing relative adrenal weight at both E14.5 and E17.5 ($P < .05$).

Vitamin D deficiency alters placental gene expression

Hsd11b2

As expected, gene expression of *Hsd11b2*, the enzyme that inactivates glucocorticoids was markedly reduced by over 90% from E14.5 to E17.5 ($P < .001$) (Figure 3A). A reduction in *Hsd11b2* expression was also apparent at E14.5 but only in vitamin D-deficient male placentas (32% reduction, treatment-sex interaction $P < .05$) (Figure 3A).

Hsd11b1

Gene expression patterns of *Hsd11b1*, which predominantly act to convert glucocorticoids to their active form, showed the well-established up-regulation over gestation, with over a 9-fold increase between E14.5 and E17.5 ($P < .001$) (Figure 3B). There were no effects of fetal sex or vitamin D deficiency on placental *Hsd11b1* expression.

Tsc22d3

The gene expression of *Tsc22d3*, which encodes for the highly glucocorticoid-sensitive factor GILZ, mostly cor-

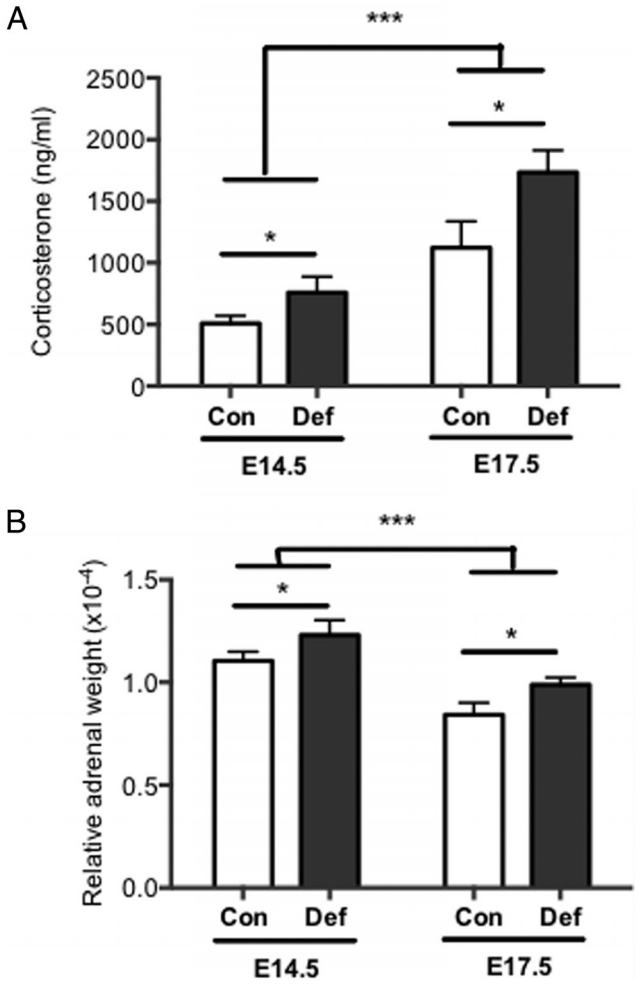

Figure 2. Maternal serum corticosterone concentrations and relative adrenal weight after prenatal vitamin D deficiency. Values are the mean ± SEM (n = 5–8 per group). There was a significant gestational effect in increasing (A) serum corticosterone concentrations and (B) decreasing relative adrenal weight (*, $P < .05$; two-way ANOVA). After vitamin D deficiency serum corticosterone concentrations and adrenal weight increased at E14.5 and E17.5 (*, $P < .05$; two-way ANOVA).

responded in a reciprocal manner with placental *Hsd11b2* expression patterns. Thus, between E14.5 and E17.5, the gene encoding for GILZ was up-regulated by at least 1.5-fold, and vitamin D deficiency also increased expression in both sexes but only at E14.5 by 1.2-fold (time-treatment interaction $P < .05$) (Figure 3C).

Vegfa

Gene expression patterns of *Vegfa*, which is a potent angiogenic factor, increased by 1.5-fold between E14.5 and E17.5 ($P < .001$) (Figure 3D). Additionally, there was a diet effect ($P < .05$), with *Vegfa* decreased in both male and female vitamin D-deficient fetuses at E14.5 and E17.5.

Nr3c1

The expression of the gene encoding GR was unaltered by gestation day, sex, and maternal diet (data not shown).

Vitamin D deficiency increases fetal head gene expression of GILZ

Tsc22d3

Similar to placental *Tsc22d3* expression, between E14.5 and E17.5 in the fetal head, *Tsc22d3* expression was up-regulated by over 2-fold ($P < .05$) and vitamin D deficiency increased expression by at least 1.2-fold at both E14.5 and E17.5 in both sexes ($P < .05$) (Figure 4). No change was seen in the expression of genes encoding for *Hsd11b1*, *Hsd11b2*, or *Nr3c1* in the fetal heads (data not shown).

Vitamin D deficiency decreases LZ and FC volumes

After the down-regulation of placental weight as a consequence of maternal vitamin D deficiency there was a corresponding, albeit small, decrease in the relative LZ volume of vitamin D-deficient placentas by 29% ($P < .05$) (Table 2). This was accompanied by a 36% decrease in the volume of FCs within the LZ of vitamin D-deficient placentas ($P < .05$) (Table 2). The relative volume of the other placental zones (decidua, junctional zone, and chorionic plate) remained unaltered (Table 2). There was no differential effect of fetal sex on placental morphology.

Discussion

Maternal vitamin D deficiency in BALB/c mice elevated maternal corticosterone levels and reduced placental *Hsd11b2* at E14.5 in male fetuses. Accompanying these changes was an up-regulation in the expression of the gene encoding for GILZ in the placenta and in the fetal brain in both sexes. In addition, both male and female vitamin D-deficient placentas exhibited a reduction in *Vegfa* expression and a decline in LZ and FC volume. These alterations in the maternal, placental, and fetal compartments highlight that maternal vitamin D deficiency increases glucocorticoid parameters in pregnancy and alters placental morphology.

These data show for the first time that maternal vitamin D deficiency is likely to expose the developing fetus to higher levels of glucocorticoids. Thus, our observation of elevated maternal corticosterone accompanied by a reduction in placental *Hsd11b2* in vitamin D-deficient male fetuses at E14.5 would presumably increase the placental transfer of glucocorticoids. In female fetuses, *Hsd11b2* was not significantly reduced but the data were variable and suggestive of a decline. Indeed, we observed in both sexes an up-regulation of the gene encoding for GILZ, which is highly sensitive to glucocorticoids (16) in both the vitamin D-deficient placentas and fetal brains. Although this study has not looked at transport of glucocorticoids

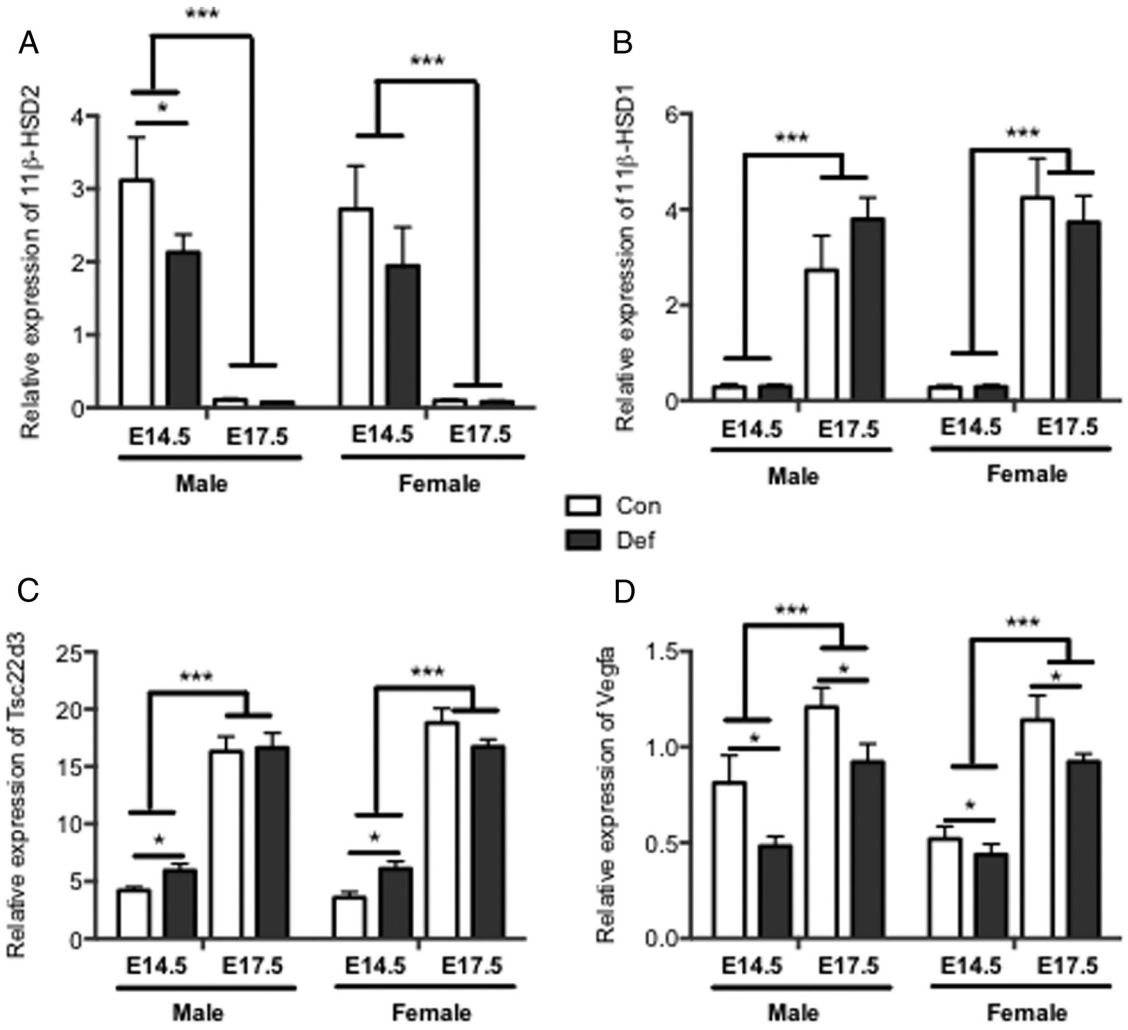

Figure 3. Prenatal vitamin D deficiency alters gene expression in the placenta. A, *11β-HSD2* mRNA expression decreased between E14.5 and E17.5. At E14.5, after vitamin D deficiency, male placentas exhibited reduced *11β-HSD2* expression. B, *11β-HSD1* mRNA expression was unchanged by treatment but higher E17.5 in comparison with E14.5. C, *Tsc22d3* mRNA expression increased between E14.5 and E17.5. At E17.5, after prenatal vitamin D deficiency, *Tsc22d3* expression was increased in both male and female E14.5 placentas. D, *Vegfa* mRNA increased between E14.5 and E17.5 and decreased by vitamin D deficiency in both male and female placentas. Values are the mean ± SEM (n = 6–8 per group). *, $P < .05$, three-way ANOVA; ***, $P < .001$, three-way ANOVA.

across the placenta, previous work has demonstrated that in mice transplacental glucocorticoid passage closely reflect the normal mRNA expression patterns of *Hsd11b2* (21). By E17.5, when *Hsd11b2* is normally lowly expressed in the rodent placenta (13, 14) there is no evidence for differential placental *Hsd11b2* expression in vitamin D-deficient fetuses in comparison with controls. However, fetal brain GILZ levels remain elevated in the vitamin D-deficient fetal brains. The levels of corticosterone in fetal tissues and circulation in this model are currently unknown and further investigation is required. Furthermore, although vitamin D deficiency does elevate maternal glucocorticoid levels the underlying mechanism has yet to be ascertained. Further investigation is required to assess maternal ACTH, GR, and mineralocorticoid receptor, although the observed enlargement of the adrenal gland in the vitamin D-deficient dams is consistent with hypothalamic-pituitary-adrenal (HPA) axis activation.

The implications of increased fetal glucocorticoid exposure as a consequence of maternal vitamin D deficiency are considerable. Exposure to high glucocorticoid levels too early in development has long-term ramifications for the future health outcomes of the offspring both in terms of cardiometabolic and neuropsychiatric disease (15). Of note is the extreme sensitivity of the developing brain to glucocorticoids, which are fundamental for brain maturation as glucocorticoids initiate terminal maturation, remodel axons and dendrites, and determine programmed cell death (22). These alterations can lead to altered brain morphology, neurotransmitter levels (including dopamine-related pathways) as well as memory, learning and affective behaviors (23–25). This closely parallels the

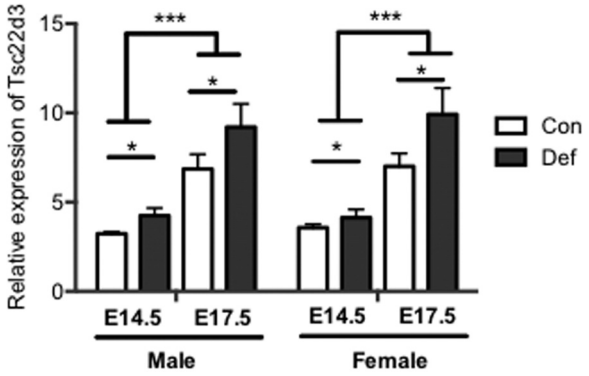

Figure 4. Expression of *Tsc22d3*, the gene encoding for GILZ in the fetal brain. *Tsc22d3* mRNA expression increased between E14.5 and E17.5 and was also up-regulated by vitamin D deficiency in both male and female fetuses. Values are the mean ± SEM (n = 6–8 per group). *, $P < .05$, three-way ANOVA; ***, $P < .001$, three-way ANOVA.

abundant adverse effects of vitamin D deficiency, which range far beyond their well-established skeletal effects. Thus, vitamin D deficiency has been implicated in neural disorders such as schizophrenia, cognitive and affective behaviors (26–28).

Little work has been conducted with the interaction of vitamin D on glucocorticoid levels and effects. Cultures of hippocampal neurons have shown that vitamin D alters GR and expression and function (8) and in asthma, calcitrol (the active metabolite of vitamin D) administration seems to beneficially modulate the clinical response to glucocorticoids in patients with steroid resistant asthma (9). Furthermore, hypocalcemia (a condition associated with low vitamin D efficacy) was associated with elevated cortisol levels in ruminants, potentially indicative of a response to stress induced by the low calcium levels (29). Of key relevance, in a rat model of early life vitamin D deficiency, Eyles et al (10) observed no alteration in baseline maternal corticosterone levels at E14.5 or adult offspring HPA axis function in response to restraint stress, but area under the curve was increased. This potentially reflects altered kinetics of corticosterone release (10) and thus is supportive of our current study. However, the relative gestational time point assessed in that rat model was slightly earlier in than our current mouse model so it may be that overt changes in circulating maternal glucocorticoids were missed by Eyles et al (10). Furthermore, placental 11β-HSD2 expression and indicators of fetal glucocorticoid exposure were not assessed so it is unclear if similar changes are occurring in this previous study as in our current study. The observation of unperturbed adult offspring HPA axis function also fits with some mouse models of prenatal glucocorticoid excess where overt perturbation in the HPA axis is not apparent but other pathways including catecholaminergic pathways (which are also vulnerable to vitamin D deficiency) are disrupted (23).

Table 2. Stereological Measures of Placentas From Control and Vitamin D-Deficient Fetuses at E17.5

	Con	Def
Placental fractions (% of total placental volume)		
Decidua basalis	21.9 ± 2.60	24.5 ± 1.02
Spongiotrophoblast	18.4 ± 3.07	23.7 ± 3.56
LZ	57.5 ± 1.97	40.6 ± 2.05[a]
Chorion	3.2 ± 0.50	4.1 ± 0.06
Absolute MBS volume (10^{-3} cm^3)	10.1 ± 1	11.2 ± 1
Absolute FC volume (10^{-3} cm^3)	15.6 ± 2	10 ± 1[a]

Values are the mean ± SEM (n = 6–8 per group). MBS, maternal blood space.

[a] Differences from Con (*t* test, $P < .05$).

Further enforcing the idea that vitamin D levels alter glucocorticoid parameters are our observed changes in placental morphology, with the reduction in LZ volume, FC volume, and *Vegfa* expression recapitulating (albeit to a lesser extent) that seen in rat and mouse models of glucocorticoid excess (18, 30). However, the consequent effects these placental changes have in this current vitamin D-deficient model are not as robust because normal fetal growth trajectories were not substantially altered, with only a mild reduction in crown-rump length of vitamin D-deficient fetuses (20), and thus placental function is still sufficient to maintain adequate fetal growth. Liu et al (31) obtained similar results of vitamin D deficiency impeding placental FC development in mice but this was accompanied by an increase in fetal weight. In comparison with other models of gestational glucocorticoid excess including maternal synthetic glucocorticoid administration (30), 11β-HSD2 gene removal (18), maternal exposure to elevated natural glucocorticoids (32, 33), and maternal dietary manipulations (21, 34), the fetal growth and placental phenotype of the vitamin D-deficient pups is not as overt. Indeed, the fold changes in corticosterone concentrations in vitamin D-deficient dams is substantially less than in these studies; this would presumably account, in part, for the more subtle changes observed with placental and fetal development. Thus, the implications of vitamin D deficiency and the resultant increased glucocorticoid exposure on fetal growth are currently unclear. There may be potential interaction with compensatory changes in fetal growth factors such as *Igf2*, but further investigation is required.

In summary, this study shows that maternal vitamin D deficiency alters maternal and placental glucocorticoid-related pathways that result in an increase in indication of fetal glucocorticoid exposure. This was also accompanied by a reduction in the vascular development of the placenta. Thus, the long-term adverse health consequences of vitamin D deficiency during early development may not just be

due to alteration in direct vitamin D-related pathways but also altered fetal glucocorticoid exposure.

Acknowledgments

Address all correspondence and requests for reprints to: Caitlin S. Wyrwoll, School of Anatomy, Physiology and Human Biology, M309, The University of Western Australia, Nedlands, Western Australia, 6009, Australia. E-mail: caitlin.wyrwoll@uwa.edu.au.

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