RESEARCH

Vitamin D is crucial for maternal care and offspring social behaviour in rats

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Abstract

Early life vitamin D plays a prominent role in neurodevelopment and subsequent brain function, including schizophrenic-like outcomes and increasing evidence for an association with autism spectrum disorder (ASD). Here, we investigate how early life vitamin D deficiency during rat pregnancy and lactation alters maternal care and influences neurodevelopment and affective, cognitive and social behaviours in male adult offspring. Sprague–Dawley rats were placed on either a vitamin D control (2195 IU/kg) or deficient diet (0 IU/kg) for five weeks before timed mating, and diet exposure was maintained until weaning of offspring on postnatal day (PND) 23. MRI scans were conducted to assess brain morphology, and plasma corticosterone levels and neural expression of genes associated with language, dopamine and glucocorticoid exposure were characterised at PND1, PND12 and 4 months of age. Compared to controls, vitamin D-deficient dams exhibited decreased licking and grooming of their pups but no differences in pup retrieval. Offspring neurodevelopmental markers were unaltered, but vitamin D-deficient pup ultrasonic vocalisations were atypical. As adults, males that had been exposed to vitamin D deficiency in early life exhibited decreased social behaviour, impaired learning and memory outcomes and increased grooming behaviour, but unaltered affective behaviours. Accompanying these behavioural changes was an increase in lateral ventricle volume, decreased cortical FOXP2 (a protein implicated in language and communication) and altered neural expression of genes involved in dopamine and glucocorticoid-related pathways. These data highlight that early life levels of vitamin D are an important consideration for maternal behavioural adaptations as well as offspring neuropsychiatry.

Introduction

The quality of early life environment is a powerful determinant of adult health outcomes, including brain function (Cottrell & Seckl 2009). The abundant expression of vitamin D receptors within the foetal brain (Eyles *et al.* 2011) highlights the critical importance of vitamin D for optimal neurodevelopment and subsequent function. Prominent focus has been placed on this in the context of epidemiological associations between early life vitamin D deficiency and increased incidence of offspring schizophrenia (McGrath *et al.* 2004, 2010).

In animal models of early life vitamin D deficiency, there is substantial overlap with changes in brain systems commonly associated with schizophrenia (Eyles *et al.* 2009), including striking changes in lateral ventricle size (Eyles *et al.* 2003, Hawes *et al.* 2015) and marked changes in the dopamine (DA) system (Kesby *et al.* 2006, 2009, Cui *et al.* 2010, 2013). However, the generic role that vitamin D has in brain development and function likely means that vitamin D deficiency associates with many other neuropsychiatric outcomes.

We have recently shown that vitamin D deficiency in mice increases placental glucocorticoid transfer and increases fetal brain glucocorticoid exposure, which is known to alter adult behavioural outcomes (Holmes & Seckl 2006, Wyrwoll & Holmes 2012). A further key determinant of adult behaviour is the quality of maternal care experienced in early life. Pregnancy and lactation necessitate marked changes in the maternal brain to facilitate offspring care (Bridges 2016). In rats, the time spent licking and grooming pups has a potent effect on epigenetic modifications and neuroendocrinology in the offspring brain, which result in altered adult behaviours (Weaver *et al.* 2004). Whether vitamin D levels contribute to these maternal behaviours is unclear. Furthermore, vitamin D deficiency also reduces forkhead box protein P2 (FOXP2)-immunoreactive cells in the mouse foetal cortex (Hawes *et al.* 2015). FOXP2 is known to be important in human speech and language (Fisher & Scharff 2009) but whether the changes seen in FOXP2 in rodent models of vitamin D deficiency associate with altered vocalisations is unknown. Thus, vitamin D likely has key roles to play in many pathways that are integral for offspring neurodevelopment and brain function.

There is emerging evidence for associations of vitamin D deficiency with autism spectrum disorder (ASD) (Fernell *et al.* 2010, 2015, Vinkhuyzen *et al.* 2018), which is characterised by qualitative impairments in social interaction and communication and a restricted range of activities and interests. Reduced vitamin D exposure during early life is also associated with individual behaviours that comprise the ASD phenotype, such as language difficulties (Whitehouse *et al.* 2012). Furthermore, vitamin D supplementation during pregnancy in a mouse model of maternal immune activation prevents the development of autism-like behaviours in the adult offspring (Vuillermot *et al.* 2017). However, the purported link between early life vitamin D status and ASD is yet to be comprehensively investigated in experimental models.

Here, we utilise a Sprague–Dawley rat model of vitamin D deficiency during gestation and lactation to assess maternal care, offspring neurodevelopmental markers, ultrasonic vocalisations and adult behavioural outcomes including social, cognitive and affective-like behaviours. Magnetic resonance imaging (MRI) scans were conducted to assess offspring brain morphology. Plasma corticoisterone levels and gene expression associated with language, dopamine and glucocorticoid exposure were characterised. Our data indicate that early life vitamin D deficiency in rats elicits changes in maternal care, while offspring exhibit altered ultrasonic vocalisations, social interaction and cognition, with associated changes in gross brain structure, FOXP2 immunostaining and dopaminergic gene expression in neural tissue.

Materials and methods

Animals

Sprague–Dawley rats were supplied by the Animal Resources Centre (ARC, Murdoch, Western Australia). All animal procedures were conducted with approval of the Animal Ethics Committee of the University of Western Australia (RA/3/100/1333). Rats were maintained in environmentally controlled rooms with a 12:12 light/darkness cycle (lights on at 07:00 h) and food and water were supplied *ad libitum.* Five-week-old female Sprague–Dawley rats were provided with vitamin D-deficient (0 IU/kg, $n=15$) or vitamin D control (2195 IU/kg, $n=15$) diets (Specialty Feeds, Glen Forrest, Western Australia) for a minimum of five weeks before mating. Deficient diets were supplemented with 25 g/kg calcium, and caloric content was controlled to ensure similar energy intake (deficient: 15.3 MJ/kg; control: 15.8 MJ/kg). Female rats were mated randomly with Sprague–Dawley males consuming the control diet and then housed separately. Litter size was standardised to five males at postnatal day (PND) 1. At weaning, all offspring were fed the control diet and housed with littermates with a minimum of two animals per cage to prevent social isolation effects.

Maternal care

Maternal behaviour assessment was conducted as detailed in Capone *et al.* (2005). Briefly, from PND3–8, maternal care was recorded using a Webcam (Logitech C270) for 1 h using iSpy software (www.ispyconnect.com) at 06:00, 10:00, 13:00, 17:00 and 20:00 h. Behaviour of the dam (licking pups, nursing, self-grooming, drinking, eating, digging, rearing, walking and resting away from nest) was

scored for 20 s every four minutes of the recording (AVI Explorer, © 2015, NJ Yates). The observer was blind to maternal diet. At PND7 after the 06:00-h recording, pup retrieval testing was conducted. The dam and pups were placed in separate cages (with the pups incubated at 32°C) for 15 min. The dam was then removed from her cage, one pup was placed in each cage corner and the dam returned, with time taken to retrieve pups timed. If after 10 min all pups had not been retrieved, testing stopped and the pups returned.

Assessment of neurodevelopment markers

To assess markers of neurodevelopment in early postnatal life, negative geotaxis, righting reflex and timing of eye opening were assessed from PND3 to PND14 as previously detailed (Holmes *et al.* 2006).

Ultrasonic vocalisations

At PND12, ultrasonic vocalisations of the pups were assessed. A pup from each litter was removed from the dam and placed within an aerated clean Perspex box situated in a sound proof room. The Perspex box contained a warming pad to maintain pup body temperature. Ultrasonic vocalisations (ultrasonic vocalisation) were recorded for 5 min. The pup was then returned to the litter for 5 min before a subsequent 5 min re-isolation period to record vocalisations. Ultrasonic vocalisations were acquired using a bat detector (Batbox Baton, Batbox Ltd.) linked to a laptop and recorded using Audacity (© 2016 Audacity Team). After the second recording, pups were killed and tissues were collected for subsequent analyses. Sound recordings were analysed using custom-made software (Sound Spectrum Analyser, © 2015, NJ Yates) for sound intensity, frequency and duration.

Adult offspring behavioural assessments

Adult behavioural testing was performed during the light period between 07:00 and 14:00 h at four months of age. When appropriate, behavioural tests were recorded using a Webcam (Logitech C270) using iSpy software. Analysis of the videos was performed offline using the Anymaze tracking system (Stoelting, IL, USA). All animals were habituated to the testing room for a minimum of 30 min prior to each test. Where applicable, testing setups were counterbalanced. Between tests, all apparatus were thoroughly cleaned with 70% ethanol to eliminate odour cues. All assessments were conducted blind to treatment.

The assessments tested motor function (using a rotarod and open field), social behaviours (social interaction test), anxiety-like behaviours (open field and elevated plus maze), grooming behaviour, learning and memory (object recognition and object-in-place) and anhedonia (sucrose preference test). Specific details of these tests can be found in the Supplementary Materials and methods (see section on supplementary data given at the end of this article).

MRI scans

At the completion of behavioural assessments, offspring were scanned using MRI to assess brain morphology. The rats were scanned under general anaesthesia (~1.5% isoflurane in 100% medical oxygen, supplied at ~1.5 L/min) using a Bruker BioSpec 94/30 US/R magnet ($B_0 = 9.4$ T), Avance III HD Console, BGA-12SHP gradients, ParaVision 6.0 software, 86 mm H-1 quadrature transmit-only volume coil and a rat brain H-1 quadrature surface receive coil (Bruker BioSpin GmbH, Ettlingen, Germany). MRI slices were examined for each animal using ITK-SNAP software. Further information on the scanning parameters and volume analysis is included in the Supplementary Materials and methods.

Tissue and blood collection

Offspring tissue collections were conducted at PND1, PND12 and four months of age. One male offspring from each litter was decapitated at PND1, the brains dissected from the skull and snap-frozen whole for subsequent gene analysis. At PND12, the pups assessed for ultrasonic vocalisations were decapitated and whole heads were fixed for later histological analysis. Blood was collected at weaning from the dams and one offspring from each litter to assess maternal and offspring vitamin D status. Blood samples were prepared and assessed for vitamin D status and corticosterone using LC/MS/MS as previously described (Clarke *et al.* 2013, Wharfe *et al.* 2016). At approximately four months of age, after the behavioural investigations and MRI scans, offspring were anaesthetised (Isoflurane/nitrous oxide) for blood and tissue collections. Brains were dissected into cortex, hippocampus, mesencephalon and cerebellum and snap-frozen for subsequent gene expression analysis.

Immunohistochemistry

Briefly, whole fixed PND12 brains were transferred to 0.1 M sodium phosphate buffer (PBS), 30% sucrose for

five days prior to sectioning on a cryostat. A set of brain sections (40 μm thick, 200 μm apart) were immunostained and quantified for FOXP2 (1:200; N-16, sc-21069, Santa Cruz Biotechnology) or tyrosine hydroxylase (TH, 1:2000; NCL-TH, Novocastra, Leica Microsystems) with relevant primary and secondary antibody controls as previously described (Hawes *et al.* 2015).

Real-time qRT-PCR

Total RNA was isolated from tissue samples and transcribed to cDNA as previously described (Tesic *et al.* 2015). Analyses of rat mRNA expression levels of genes encoding for dopamine receptor D1 (*Drd1*), dopamine receptor D2 (*Drd2*), glutamic acid decarboxylase (*Gad1*), *N*-methyl D-aspartate receptor subtype 2B (*Grin2b*), glucocorticoid receptor (*Nr3c2*), 11β-hydroxysteroid dehydrogenase type 1 (*Hsd11b1*), 11β-hydroxysteroid dehydrogenase type 2 (*Hsd11b2*), glucocorticoid-induced leucine zipper (*Tsc22d3*), and the reference genes *Ppia*, *Sdha* and *Tbp* were performed by real-time PCR on the Rotorgene 6000 system (Corbett Research, Sydney, Australia). Primer pairs for all genes (Table 1) were purchased as QuantiTech primers with the exception of *Ppia*, *Sdha* and *Tbp*, 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. Melt-curve analysis showed a single PCR product for each gene, which was confirmed by gel electrophoresis and DNA sequencing. Standard curves were generated with 10-fold serial dilutions of gel extracted (QIAEX II; Qiagen) PCR products and relative gene expression were analysed using the Rotorgene 6000 software. All samples were run in duplicate and normalised against *Ppia*, *Sdha* and *Tbp* using the GeNorm algorithm (Groves *et al.* 2013).

Statistical analysis

Statistical analyses were performed using R (version 3.2.2) and R Studio (version 0.99.482) and/or GraphPad Prism (version 7.03). All data were assessed for violations of normality and homogeneity of variance. In general *t*-tests were used to assess differences between groups, with the exception of ultrasonic vocalisation total call number (Kurskal–Wallis ranked-sum test, violations of equal variance and normality), ultrasonic call frequencies (two-way ANOVA) and open field testing (repeated-measures two-way ANOVA) due to multifactorial study design. Maternal care from PND3–8 was analysed using

Table 1 Qiagen name, primer sequences and melting temperature (T_m) for analysis and reference genes.

Gene	Qiagen name or primer sequence	Catalogue no. or T_m (°C)
Drd1	DRD1	QT00386631
Drd2	DRD2	QT01081990
Gad1	GAD1	QT00194600
Grin2b	GRIN2B-(NMDA subunit)	QT00184793
Nr3c2	Nr3c	QT00195251
Hsd11b1	HSD11-B1	QT00107303
Hsd11b2	HSD11-B2	QT00252609
Tsc22d3	TSC22D3	QT00404075
Sdha	F, 5′-TGGGGCGACTCGTGGCTTTC-3′	60
	R, 5′-CCCCGCCTGCACCTACAACC-3′	60
Tbp	F, 5′-GGCTGCTCGTTTTG-3′	62
	R, 5′-TGGGGAGGCCAAGCCCTGAG-3′	62
Ppia	F, 5′-AGCATACAGGTCCTGGCATC-3′	60
	R, 5′-TTCACCTTCCCAAAGACCAC-3′	60

Drd1, dopamine receptor D1; *Drd2*, dopamine receptor D2; F, forward; *Gad1*, glutamic acid decarboxylase; *Grin2b*, *N*-methyl D-aspartate receptor subtype 2B; *Hsd11b1*, 11β-hydroxysteroid dehydrogenase type 1; *Hsd11b2*, 11β-hydroxysteroid dehydrogenase type 2; *Nr3c2*, glucocorticoid receptor; *Ppia*, peptidylprolyl isomerase A; R, reverse; *Sdha*, succinate dehydrogenase complex, subunit A; *Tbp*, TATA box binding protein; *Tsc22d3*, glucocorticoid-induced leucine zipper.

two-way ANOVA as occasional time points were missing, which prevented repeated-measures analysis.

Results

Vitamin D deficiency alters maternal care and offspring ultrasonic vocalisations but not neurodevelopmental markers

Assessment of maternal care from PND3 to 8 revealed that vitamin D deficiency caused variation in the percentage of total time spent licking and grooming pups (Fig. 1A, $F (1, 105) = 7.544$, $P < 0.01$, two-way ANOVA), but there was no effect of day or an interaction between diet and postnatal day. When comparing the percentage of total time spent licking and grooming over the entire duration of PND3–8, vitamin D-deficient dams spent 27% less time grooming their pups in comparison to controls (Fig. 1B, $t (21) = 2.85$, $P < 0.01$, *t*-test). Pup retrieval parameters did not significantly alter between control and vitamin D-deficient dams (Fig. 1C, $t (20) = 1.614$, $P = 0.1223$, *t*-test).

At PND12, ultrasonic call rates were increased in vitamin D-deficient pups during the first period of maternal separation in comparison to controls (Kruskal–Wallis rank sum test, $\chi^2 = 6.89$, $df = 1$, $P < 0.01$; Fig. 2A) although a difference between controls and vitamin D-deficient pups was not apparent in the second separation
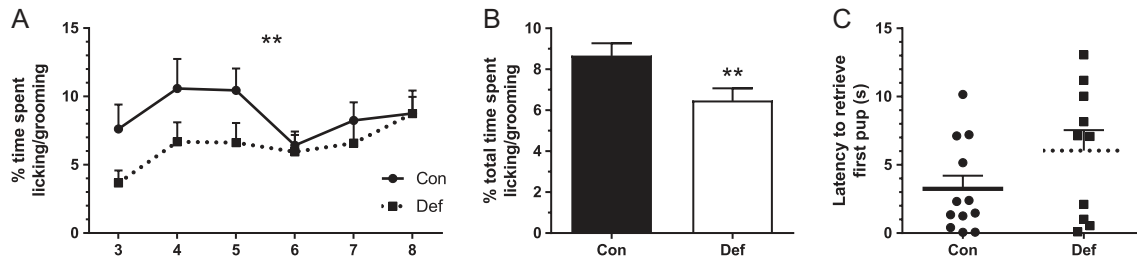
Figure 1
Vitamin D levels altered maternal care parameters in the first postnatal week. (A) Maternal vitamin D deficiency had a significant effect on the amount of time the dam spent licking and grooming her pups, but there was no effect of day or interaction between diet and day. (B) Combining all the data points from PND3 to 8 demonstrates the extent to which vitamin D deficiency decreased total licking and grooming from PND3 to 8. (C) At PND7 there were no differences in the latency for control or vitamin D-deficient dams to retrieve their pups during the pup retrieval test. Bars indicate mean + s.e.m. (*n* = 12 control dams and 10 vitamin D-deficient dams). Significant differences; two-way ANOVA or *t*-test, *P < 0.05, **P < 0.01.

(Kruskal–Wallis rank sum test, $\chi^2 = 1.085$, $df = 1$, $P > 0.05$; Fig. 2A). The lack of difference in the second separation between the two experimental groups is due to a 170% increase in call rates from the control pups between the first and second maternal separations (Kruskal–Wallis rank sum test, $\chi^2 = 3.98$, $df = 1$, $P < 0.05$), which was not evident in the vitamin D-deficient pups (5% increase, Kruskal–Wallis rank sum test, $\chi^2 = 0.05$, $df = 1$, $P > 0.05$). The distribution of call peak frequencies was unchanged in both experimental groups in the first and second recording (two-way ANOVA; Fig. 2B and C). Due to the directional and easily attenuated nature of ultrasonic vocalisations, the intensity of calls was not suitable for analysis.

We then examined whether changes in ultrasonic vocalisations were accompanied by alteration in neural tissue expression of FOXP2, a gene in part implicated in speech and language development (Fisher & Scharff 2009). FOXP2 protein immunostaining was reduced by 43% in the cortex of vitamin D-deficient male brains at PND12 compared to controls ($P < 0.05$; Fig. 3A, B, C and D). No significant differences in FOXP2 were distinguished in the caudate putamen, amygdala, post

thalamic nucleus or post thalamic group (all $P > 0.05$, data not shown). We also investigated TH levels at PND12, but no differences were detected in TH immunostaining in either the substantia nigra pars compacta or ventral tegmental area of control and vitamin D-deficient brains (all $P > 0.05$; Fig. 3E, F and G).

Maternal vitamin D diet allocation had no effect on routine measures of offspring neurodevelopment (timing of eye opening, negative geotaxis, righting reflex) or other gross parameters such as gestation length, litter size, offspring sex ratio or offspring weight (Table 2). At weaning, maternal and offspring serum 25(OH) D3 and 3-epi-25(OH)D3 concentrations reflected diet consumption (Table 2).

Early life vitamin D deficiency impairs adult social-like behaviours, cognition and grooming behaviours but not affective behaviours

In the social interaction test, adult offspring exposed to vitamin D deficiency in early life spent 22% less time in the chamber containing the social cue compared to
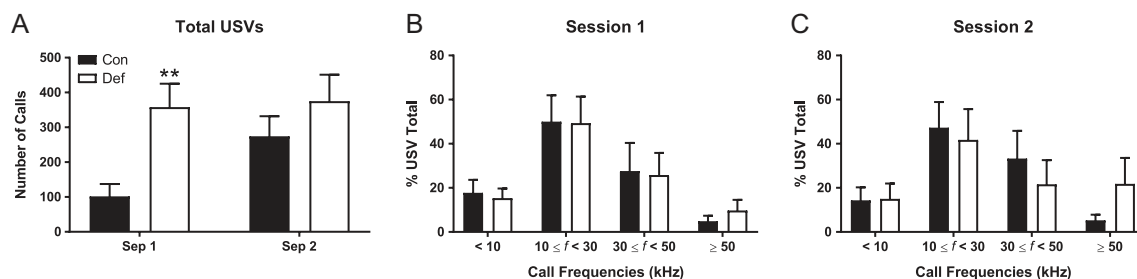
Figure 2
Ultrasonic vocalisations (USV) of PND12 pups upon 5-min repeated maternal separation. (A) Ultrasonic vocalisation total call number was markedly increased in vitamin D-deficient pups for the first (Sep 1), but not the second (Sep 2) maternal separation period. For each separation period, the calls were also classified based upon the peak frequency of the call (*f*), shown in panels (B) and (C). However, these did not differ. Bars indicate mean + s.e.m. (*n* = 8 for both control and vitamin D-deficient groups). Significant differences: Kurskal–Wallis, **P < 0.01.
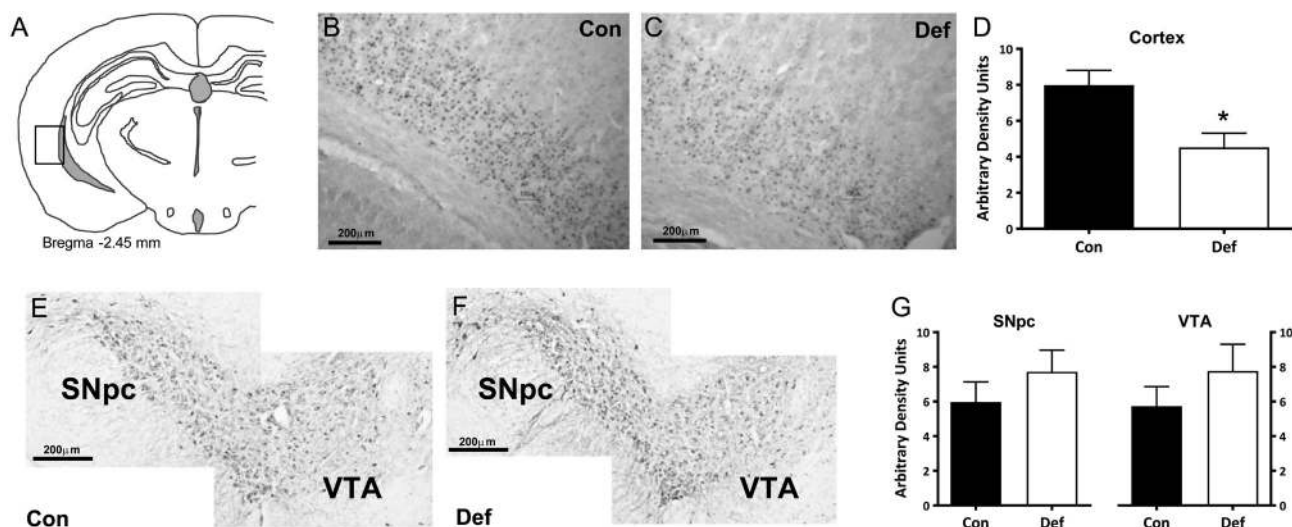
Figure 3
FOXP2, but not TH, immunolabelling was decreased in vitamin D-deficient pups in comparison to control at PND12. FOXP2 immunolabelling and analysis is shown in panels (A, B, C and D). (A) Coronal schematic representation of region of interest for immunohistochemical labelling of FOXP2 in the cortex of PND12 animals. The box represents the region shown in example (B) control and (C) vitamin D-deficient images of FOXP2 immunolabelling in the cortex and was used for (D) semi-quantitative immunointensity analysis. Tyrosine hydroxylase (TH) immunolabelling and analysis is shown in panels (E, F and G). Example substantia nigra pars compacta (SNpc) and ventral tegmental area (VTA) immunolabelling in (E) control and (F) vitamin D-deficient animals. (G) Semi-quantitative analysis revealed no differences in TH immunointensity. Bars show mean + s.e.m., $n = 6$–8 per diet group. Significant differences: *t*-test, *$P < 0.05$.

control offspring ($P < 0.05$, Student's *t*-test; Fig. 4A). This lack of specific interest in the social cue was further demonstrated by the vitamin D-deficient offspring spending 26% less time directly around the social cue cage in comparison to controls ($P < 0.05$, Student's *t*-test; Fig. 4B). Overall exploration of the maze (as measured by total distance travelled in the maze) did not differ between control or vitamin D-deficient offspring (35.65 ± 3.40 m and 35.00 ± 3.00 m respectively).

In the object recognition test, control and early life vitamin D-deficient offspring exhibited similar discrimination ratios for novel object recognition (1.77 ± 0.30 and 1.63 ± 0.32, respectively; Fig. 4C). However, the more complex object-in-place task revealed cognitive differences. Offspring that had been exposed to vitamin D deficiency in early life were unable to discriminate between the objects that had swapped positions and those that had remained in the same position as in the sample phase. In comparison, control littermates showed discrimination by investigating the moved objects longer than the objects that were in their original position ($P < 0.05$, Fig. 4D).

Sucrose preference testing indicated that there was no indication of anhedonia with control and vitamin D-deficient offspring showing equal preference for sucrose (Fig. 4E). In the open field testing, on the standard testing days or days with restraint stress, there were no differences between the groups effects or interactions with testing day

on total distance travelled (Fig. 4F), ratio of time spent in the centre compared to periphery (Fig. 4G) or any other outcome measured (all $P > 0.05$, repeated-measures 2-way ANOVA). In rotarod assessments, offspring showed no difference in motor coordination (time to fall in control offspring: 120 ± 58 s; vitamin D deficient: 110 ± 22 s). Assessment for anxiety-like behaviours using the elevated plus maze was unsuccessful on the test day as neither group explored the open arm extensively. The reason for this is uncertain; experimental parameters had been earlier optimised and tested to ensure appropriate elevated plus maze exploration. However, grooming behaviours were scored during this test and suggest increased restricted and repetitive behaviour in vitamin D-deficient offspring, which groomed for longer, but with similar grooming frequency over the five-minute test period (grooming duration: Kruskal–Wallis, $P < 0.05$, Fig. 4H; grooming frequency: Kruskal–Wallis, $P > 0.05$, Fig. 3I).

Adult lateral ventricle volume and neural gene expression are altered by early life vitamin D deficiency

MRI scans revealed that vitamin D deficiency in early life enlarged lateral ventricle size markedly by 140% at four months of age in comparison to controls ($P < 0.05$; Fig. 5). In contrast, early life vitamin D status did not alter

Table 2 General pregnancy characteristics, vitamin D serum concentrations (25(OH)D3 and 3-epi-25(OH)D3) in dams and offspring at time of weaning, postnatal weight gain, neurodevelopmental markers and plasma corticosterone concentrations of control and vitamin D-deficient offspring.

	Control	Vitamin D deficient
Pregnancy characteristics		
Gestation length (days)	22.5 ± 0.2	22.1 ± 0.1
Litter size	12.4 ± 1.1	11.1 ± 1.0
Gender ratio (M:F)	1.2 ± 0.2	1.2 ± 0.2
Postnatal weight (g)		
PND1	7.5 ± 0.4	7.4 ± 0.3
PND12	33.8 ± 2.2	37.1 ± 0.9
PND21	70.3 ± 2.9	72.7 ± 2.8
PNW16	510.3 ± 16.6	518.4 ± 20.5
Neurodevelopment		
Eye opening (PND)	13.8 ± 0.2	14.4 ± 0.2
Negative geotaxis (s)	21.0 ± 2.8	20.7 ± 2.6
Righting reflex (s)	2.2 ± 0.4	1.8 ± 0.3
Vitamin D serum concentration (nmol/L)		
Dams		
25(OH)D3	35.42 ± 1.69	3.33 ± 0.62***
3-Epi-25(OH)D3	21.46 ± 6.53	0.82 ± 0.23***
Offspring		
25(OH)D3	23.85 ± 1.89	1.63 ± 0.13***
3-Epi-25(OH)D3	20.04 ± 6.65	0.39 ± 0.24***

Maternal vitamin D status had no effect on gestation length, litter size, or offspring gender ratios. Furthermore, there was no effect of early life vitamin D deficiency on offspring weight or measures of neurodevelopment at any time point measured (PND1, PND12, PND21 or PNW16). At weaning, maternal and offspring serum 25(OH)D3 and 3-epi-25(OH)D3 concentrations reflected diet consumption with both dams and offspring exhibiting a marked reduction in 25(OH)D3 and 3-epi-25(OH)D3 if consuming a vitamin D-deficient diet. Vitamin D deficiency did not alter offspring plasma corticosterone at any time point. Values are the mean ± S.E.M. ***$P < 0.001$; *t*-test. PND, postnatal day; PNW, postnatal week.

cerebellum, hippocampus, corpus callosum or total brain volume (all $P > 0.05$).

Changes were observed at PND1 in genes associated with dopamine signalling and GABA synthesis. The expression of *Gad1*, which encodes for a GABA synthesis enzyme, showed a 48% decrease in the whole brains of vitamin D-deficient animals ($P < 0.05$; Fig. 5D), while the expression of dopamine receptor *Drd2* was decreased by 42% in the vitamin D-deficient pups in comparison to controls ($P < 0.05$; Fig. 5E). At four months of age, gene expression was analysed in dissected brain regions, with only discrete changes observed. Thus, of the genes analysed, *Drd2* was decreased by 44% in the cerebellum of offspring exposed to vitamin D deficiency in early life in comparison to control ($P < 0.05$; Fig. 5F) and *Grin2b* expression was decreased by 48% in the mescencephalon of vitamin D-deficient offspring ($P < 0.01$; Fig. 5G).

Glucocorticoid-related genes are only altered at PND1 while offspring plasma corticosterone levels did not vary

Expression of genes involved in glucocorticoid-related pathways was also assessed and changes were only evident at PND1. Thus, whole-brain *Hsd11b2*, which converts active glucocorticoids to their inactive form, was decreased by over 50% in vitamin D-deficient offspring in comparison to control ($P < 0.05$; Fig. 6A). The glucocorticoid receptor, *Nr3c2*, was also decreased by vitamin D deficiency (34%, $P < 0.05$; Fig. 6B) while *Tsc22d3* expression, an indication of glucocorticoid receptor activation, was increased by over 2.5-fold ($P < 0.05$; Fig. 6C). Offspring corticosterone concentrations did not vary at PND1, weaning or four months of age (Fig. 6D).

Discussion

Early life vitamin D deficiency in male rats resulted in a phenotype characterised by atypical pup ultrasonic vocalisations and impairments in adult social, memory and learning behaviours as well as increased grooming. This was accompanied by a marked increase in lateral ventricle volume, altered neural proteins implicated in language development and differential neural expression of genes involved in neurotransmitter signalling and increased early life glucocorticoid exposure. Furthermore, the quality of maternal care was decreased in dams consuming a vitamin D-deficient diet. These data demonstrate that vitamin D deficiency in early life influence multiple pathways that are critical for optimal neural development and adult brain function. Of particular interest is that aspects of the behavioural phenotype are suggestive of features of ASD, including communication differences, lack of interest in social interaction, restricted and repetitive behaviour and changes in brain development.

Variation in maternal care has a profound influence on offspring neurodevelopment and neuropsychiatry (Curley & Champagne 2016). Therefore, the reduction in maternal licking and grooming in dams consuming a vitamin D-deficient diet is a likely important contributor to the adult offspring behavioural phenotype that results from early life vitamin D deficiency. Extensive work has highlighted the crucial role of maternal care in epigenetic modifications in the offspring brain and the optimal development of neural circuitry, including neuroendocrine systems such as the hypothalamic–pituitary–adrenal (HPA) axis and dopaminergic pathways
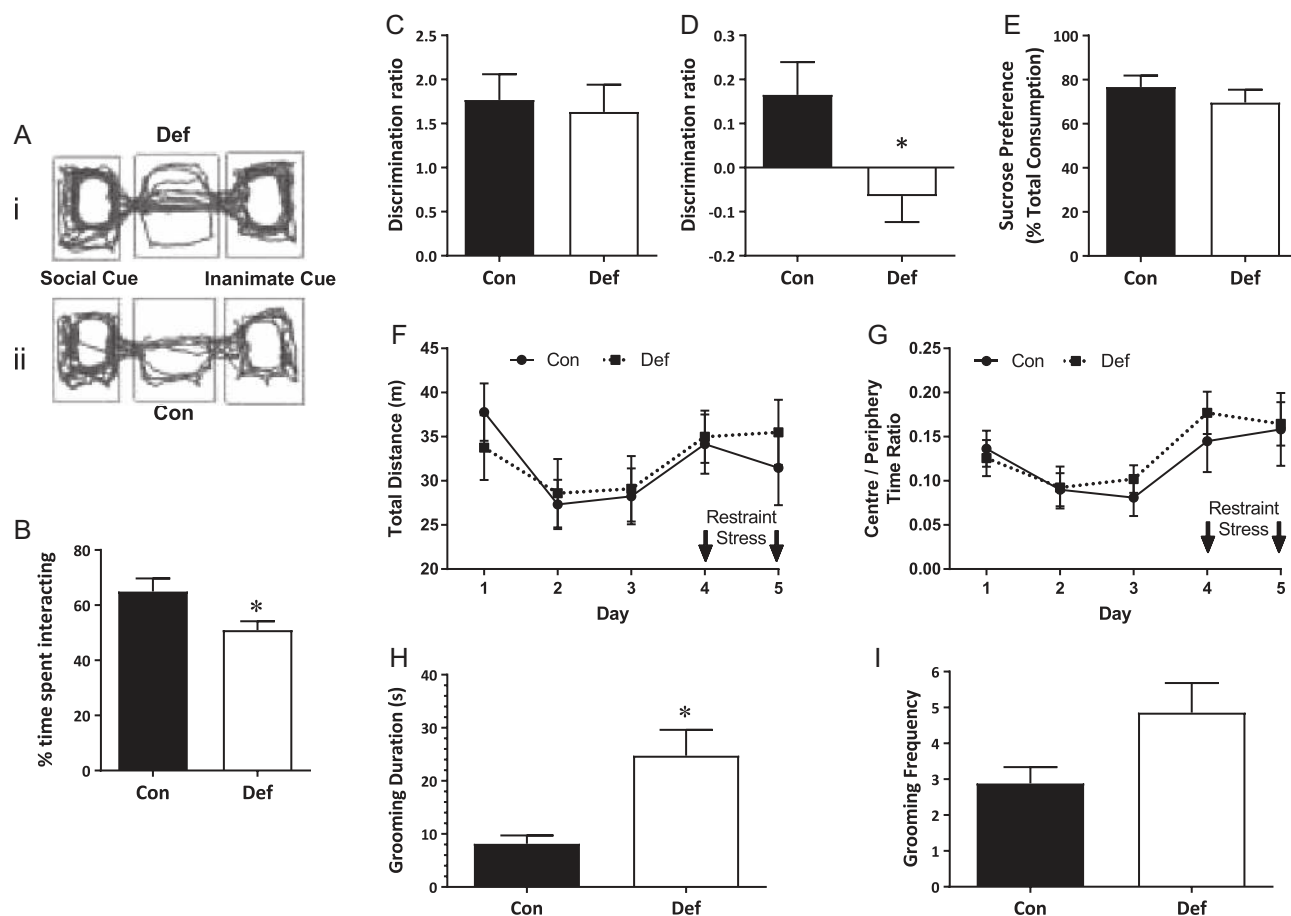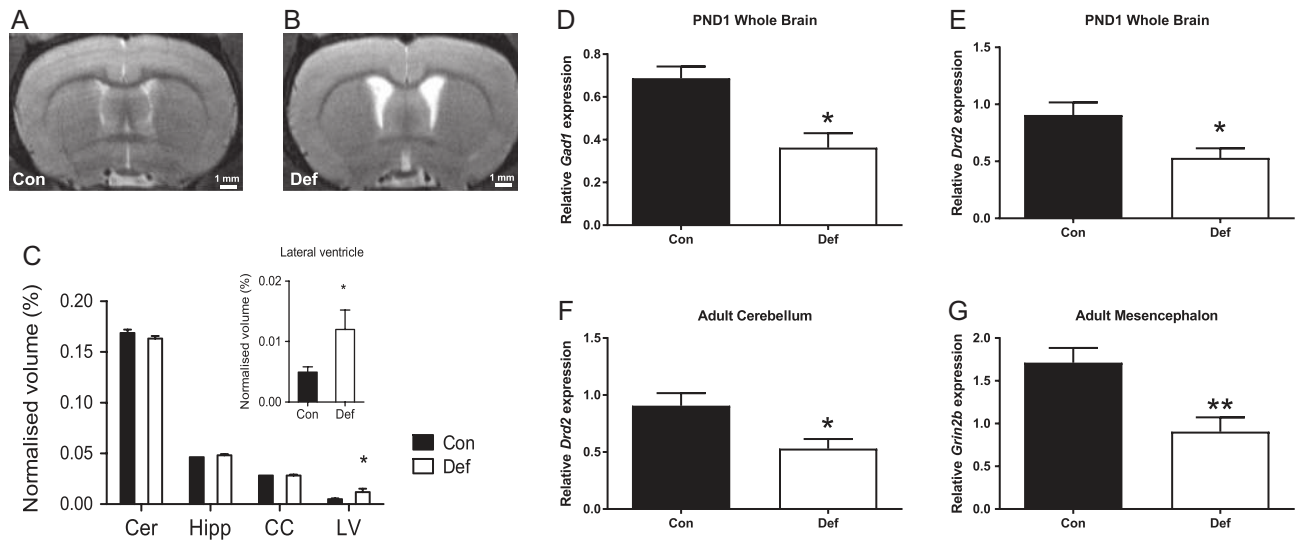
Figure 4

Adult social and cognitive behaviours were decreased animals exposed to early life vitamin D deficiency in comparison to control. Social interaction test: (A) example exploration traces percentage of time spent in the social cue chamber containing the novel rat for (i) vitamin D-deficient offspring and (ii) control offspring. Social preference was quantified by (B) percentage of time socially interacting in the social cue chamber. Cognitive outcomes: (C) novel object recognition discrimination ratio after 1 h separation, and (D) object-in-place discrimination ratio. Sucrose preference test assessment of anhedonia revealed no differences (E) as assessed by preference for sucrose solution over water. Open field test outcomes were unaltered between the groups during standard testing days (days 1–3) and following restraint stress (days 4 and 5), (F) total distance travelled and G) centre/periphery exploration time ratio. While exploration of the elevated plus maze could not be assessed, grooming behaviour was scored. Early life vitamin D deficiency increased total grooming duration (H) but not grooming frequency (I). Bars represent mean + s.e.m., $n = 10–15$ per diet group. Significant differences: *t*-test or Kruskal–Wallis, *$P < 0.05$.

([Meaney *et al*. 1996](), [Weaver *et al*. 2004](), [Pena & Champagne 2015]()). Indeed, we and others find consistent evidence of early life vitamin D deficiency altering dopaminergic-related pathways in the adult brain ([Kesby *et al*. 2006](), [2009](), [Cui *et al*. 2010](), [2013](), [Hawes *et al*. 2015]()). Adult HPA axis parameters do not differ our model, although intracellular changes have not yet been properly characterised. Therefore, the degree to which the altered maternal behaviour in our current study contributes to the adult offspring phenotype is unclear and further cross-fostering studies are required to elucidate this. Furthermore, our experimental design meant that litter size was reduced to six male pups only, which is not standard in maternal behavioural assessments. This may

have skewed maternal behaviours and further work is required to clarify this point.

The brain-specific mechanisms underlying the reduction in licking and grooming behaviours in vitamin D-deficient mothers in our model are uncertain. Given that dopamine is important in the establishment of maternal behaviours ([Numan 2007]()), this could be a key area for further investigation. Indeed, the impact of vitamin D deficiency on dopaminergic pathways has been extensively characterised ([Kesby *et al*. 2006](), [2009](), [Cui *et al*. 2010](), [2013]()), although not in the context of maternal behaviours. Previous studies have reported an association between reduced maternal vitamin D levels during pregnancy and perinatal depressive symptoms,
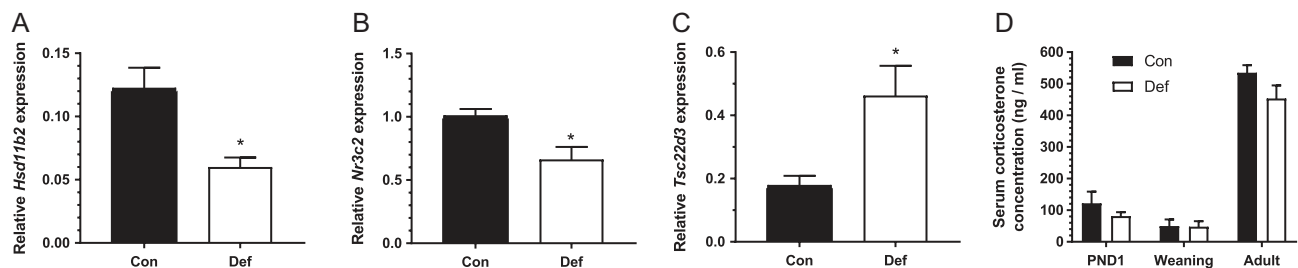
Figure 5

MRI volumetric analysis of brains from ~PNW16 control and vitamin D-deficient animals, and relative changes in gene expression. Example images illustrating the marked differences in lateral ventricle volumes of (A) control and (B) vitamin D-deficient animals at a comparable section level of the brain. (C) Volume of brain regions of the cerebellum (Cer), hippocampus (Hipp), corpus callosum (CC) and lateral ventricles (LV) normalised to total brain volume. Early life vitamin D deficiency caused a downregulation in some genes involved in dopaminergic pathways. Relative gene expression to house-keeping genes for whole brain on postnatal day 1 (PND1) for: (D) GABA synthesis enzyme, *Gad1*; (E) dopamine receptor type 2, *Drd2.* Adult (4 months old) brain regional gene expression for (F) dopamine receptor type 2, *Drd2*, in the cerebellum; and (G) NMDA receptor subunit, *Grin2b*, in the mesencephalon. Bars show mean+s.e.m., $n=8$ for both control and vitamin D-deficient groups. Significant differences: *t*-test, *$*P<0.05$, $**P<0.01$.

which may indicate that dopamine-related pathways underlie abnormal maternal behavioural outcomes in vitamin D-deficient pregnancies (Robinson *et al.* 2014, Vaziri *et al.* 2016, Williams *et al.* 2016).

Our current study is the first to identify increased ultrasonic vocalisation call rates during forced isolation in a rat model of vitamin D deficiency. This increased call rate was marked in the first maternal separation but not the second, likely due to the maximum threshold of call rates already having being reached. Another possibility may be that vitamin D-deficient pups have impaired auditory detection and are unable to monitor their vocalisations, although more research is required to clarify this

possibility. Interestingly, removal of vitamin D receptor in zebra fish elicits impaired inner ear development (Kwon 2016). Our observations differ from previous assessments of ultrasonic vocalisations in vitamin D-deficient rat pups, which did not reveal any alteration at PND0 (Burne *et al.* 2011). However, this previous study and our own, only characterise a snapshot of ultrasonic vocalisations over the neonatal period. Ultrasonic vocalisations follow a discrete ontogenetic profile of call rates peaking in the first few days and then diminishing over the remainder of the first two weeks of life in mice and rats (Schwarting & Wöhr 2012). Therefore, a thorough characterisation of the ontogenetic profile of control and vitamin D-deficient

Figure 6

Early life vitamin D deficiency altered some genes involved in glucocorticoid pathways. In postnatal day 1 (PND1) animals, vitamin D deficiency caused a reduction in (A) *Hsd11b2* and (B) *Nr3c2* levels in the whole brain. There was an (C) increase in *Tsc22d3* expression in vitamin D-deficient animals, and (D) no changes in serum corticosterone levels, at PND1, weaning, and in adulthood (4 months). Bars show mean+s.e.m., $n=8$ for both control and vitamin D-deficient groups. Significant differences: *t*-test, *$*P<0.05$.

pup ultrasonic vocalisations will be useful to understand our current observations.

The significance of the increased call rates may be related to an anxious phenotype in vitamin D-deficient pups, possibly reflecting their exposure to a lower quality of maternal care. Ultrasonic vocalisations are proposed to reflect communication (Ehret 2005, Hofer *et al.* 2002). In the current study, the altered ultrasonic vocalisations at PND12 were accompanied by a decrease in cortical FOXP2 levels. In rodents, FOXP2 is closely linked with ultrasonic vocalisation production and is important in maternal care interactions such as pup retrieval (Bowers *et al.* 2013); however, a decrease in FOXP2 is normally associated with reduced ultrasonic vocalisations. Thus, FOXP2 homozygous and heterozygous knockouts, disruption and heterozygous mice on PND8, and heterozygous nonsense FOXP2-mutant mice on PND4 all show reduced call rates (Shu *et al.* 2005, Fujita *et al.* 2008, Gaub *et al.* 2010). The incongruence between ultrasonic vocalisation call rate and FOXP2 levels in our model is currently unclear, but the decreased FOXP2 is consistent with results of our earlier investigations in a mouse model of vitamin D deficiency (Hawes *et al.* 2015). FOXP2 has roles beyond communication and is implicated in neurogenesis and neuroplasticity; therefore, there may be additional implications for the FOXP2 reductions elicited by early life vitamin D deficiency.

We have shown that developmental vitamin D deficiency reduces social interaction (social interaction test) and attention switching (object-in-place) in male offspring, without generalised cognitive deficits (novel object recognition), anxiety-like behaviours, anhedonia or neurodevelopmental delay. In rodents, there is spontaneous preference for social interaction with a novel animal compared to an inanimate object (Moy *et al.* 2004, Ku *et al.* 2016). Mice that are vitamin D deficient until parturition (as opposed to weaning in our current study) have previously shown normal levels of social interaction when placed in an open field with another mouse (Harms *et al.* 2008). However, this approach does not give the test subject the opportunity to preferentially choose between social vs non-social activity. Furthermore, exposure to vitamin D deficiency solely during gestation gives limited context for human maternal vitamin D deficiency, given the relative altricial nature of rodent brains. We also found that vitamin D deficiency produced discrete cognitive changes in offspring. In simple unlearned memory tasks (object recognition), vitamin D-deficient animals perform normally. However, in the more demanding object-in-place testing, vitamin D-deficient offspring show a deficit

in change detection compared to controls. A previous study investigating prenatal vitamin D deficiency in rats also showed subtle changes in novelty detection, a lack of interest in exploration and a lack of habituation as shown in the hole-board habituation task (Becker *et al.* 2005). These findings are broadly consistent with vitamin D status in pregnant women negatively correlating with offspring attention switching (Whitehouse *et al.* 2013). While the elevated plus maze test was unsuccessful for assessing anxiety behaviours in the present study, it did enable us to demonstrate increased self-grooming (a measure of repetitive behaviour (for review see Kalueff *et al.* 2016)) in offspring that had been exposed to vitamin D deficiency in early life.

Changes in brain gene expression indicate that vitamin D plays an important role in neurotransmitter signalling and synthesis. In PND1 animals, the reduced expression of *Gad1* provides evidence for the role in vitamin D in GABA system regulation, which is supported by similar changes in adult mice, which are vitamin D deficient (Groves *et al.* 2013). We also observed reductions of *Drd2* expression in whole brain lysates on PND1 and in the cerebellum in adult vitamin D-deficient animals. This supports a restricted role for vitamin D in the development of dopaminergic systems in the brain (Cui *et al.* 2010) and is congruent with previous studies reporting that vitamin D deficiency does not necessarily result in widespread changes in dopamine receptor levels in male offspring, despite changes in dopamine turnover (Kesby *et al.* 2009, 2012). Limited differences were evident in the HPA axis, with changes at PND1 suggesting increased exposure of the neonatal brain to glucocorticoids. This supports previous work in a mouse model of vitamin D deficiency, which demonstrated increased placental transfer and exposure of the foetal brain to glucocorticoids in late gestation (Tesic *et al.* 2015). However, in this current study, there were no significant differences in offspring circulating corticosterone or HPA axis-related gene expression at other time points, which likely indicates that the adult HPA axis does not contribute to the behavioural phenotype.

Overall, the traits we have observed in male rats exposed to vitamin D deficiency in early life are suggestive of an ASD-like phenotype. While more extensive characterisation is required to confirm this, placing our data into the context of rodent models of ASD reveals some similarities in altered vocalisations, social, memory and learning parameters and grooming behaviours. Reductions in the initiation and maintenance of social interactions are common among children with ASD. Recent studies have also shown associations with maternal vitamin D

deficiency and poor language and social responsiveness (Vinkhuyzen *et al.* 2018) and poor attention switching (Whitehouse *et al.* 2013). Furthermore, change and novelty detection are impaired in children with autism (Bomba & Pang 2004) and adults show impairments in explicit (but not implicit) object memory location (Ring *et al.* 2015). Studies mostly identify reduced ultrasonic vocalisation in rodent models of ASD (Scattoni *et al.* 2009). However, Schmeisser *et al.* (2012) reported significantly increased call rate among female *ProSAP1/Shank2−/−* mice compared to wild-type controls during free social interactions. The communication difficulties observed in children and adults with ASD vary considerably between individuals, from those with language learning difficulties to those with pragmatic oddities, such as those with excessive talking (Whitehouse *et al.* 2010). We note that the epidemiological findings linking early vitamin D deficiencies with ASD traits in children (Vinkhuyzen *et al.* 2018) have reported a link with pragmatic language difficulties.

ASDs are commonly associated with changes in brain anatomy. While not a specific feature, enlarged lateral ventricles are common in autistic children along with increase of overall brain volume (Palmen *et al.* 2005, Vidal *et al.* 2008). The significant enlargement in lateral ventricle volume in our male rats exposed to vitamin D deficiency is consistent with an autism-like phenotype (Eyles *et al.* 2003) and the neurogenic role of vitamin D (Cui *et al.* 2007) documented in previous literature. However, because enlarged lateral ventricles are common in a variety of neurodevelopmental disorders, the direct implication this has for brain function is unclear. Furthermore, the enlargement of lateral ventricles is not overtly detrimental in our model, as the behavioural phenotype is not extreme. This implies that plasticity of the brain may compensate in part for early life vitamin D deficiency. Interpreting the changes in *Drd2* expression in the cerebellum of vitamin D-deficient animals is limited by the poor understanding of dopamine in the cerebellum, particularly with regard to autism (Fatemi *et al.* 2012). Similarly, interpreting the reduction in *GRIN2B* in the mesencephalon is difficult, but worth noting especially given the reported role of NMDA receptor dysfunction in autism (Lee *et al.* 2015), and the findings that GRIN2B alleles and *de novo* mutations are associated with schizophrenia and autism (Tarabeux *et al.* 2011).

Several phenotypic outcomes are most prominent in female vitamin D-deficient offspring. Therefore our study,

which was limited to phenotyping male rats, cannot detect changes in some dopamine-mediated behaviours and receptor levels, which are only altered in female offspring (Kesby *et al.* 2009). This may explain why we did not observe changes in TH staining in the SNpc, which occurs most prominently in female offspring in mice (Hawes *et al.* 2015).

Our results show that early life vitamin D deficiency elicits changes in maternal care, and in the offspring, alters ultrasonic vocalisations and induces adverse neuropsychiatric outcomes. These finding reinforce the importance of early life vitamin D for optimal neurodevelopment, but it can be attributed not only to direct effects of vitamin D on the developing brain, but also to indirect pathways such as maternal care and early life glucocorticoid exposure. Furthermore, the behavioural and anatomical phenotype of adult male offspring rats exposed to early life vitamin D exposure is broadly consistent with ASD-like traits. This indicates that vitamin D deficiency may play a mechanistic, and not purely associative, role in the developmental origins of ASD. Therefore, vitamin D level monitoring and appropriate supplementation should be a continually important consideration in antenatal care, due to implications for maternal behaviour, foetal neurodevelopment and later neuropsychiatric outcomes.

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