VITAMIN D RECEPTOR EXPRESSION IN THE EMBRYONIC RAT BRAIN

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SUMMARY

We are interested in determining whether low maternal vitamin D_3 affects brain development *in utero*. Whilst the vitamin D receptor (VDR) has been identified in embryonic rat brains, the timing and magnitude of its expression across the brain remains unclear. In this study we have quantitated VDR expression during development as well correlated the timing of its appearance with two vital developmental events, apoptosis and mitosis. Brains from embryonic rats (embryonic days 15 – 23) were examined. We show that the well-described increase in apoptotic cells and decrease in mitotic cells during development correlates with the appearance of the VDR in brain tissue. Given that vitamin D_3 regulates mitosis and apoptosis in non-neuronal tissue we speculate that the timing of VDR expression in embryonic brain may directly or indirectly mediate features of neuronal apoptosis and mitosis.

KEYWORDS: Vitamin D_3 receptor VDR; mitosis; apoptosis; brain development

INTRODUCTION

There is a growing recognition of the many important roles played by 1,25 dihydroxyvitamin D_3 in the adult central nervous system (9, 21). In addition, our group has recently shown that vitamin D plays a role during brain development in the rat (7). Compared to control neonates, the offspring of vitamin D deplete mothers had different shaped brains (longer but not wider) with larger lateral ventricles. At the cellular level, throughout the brain the offspring of vitamin D depleted mothers had more mitotic cells accompanied by a trend level reduction in apoptosis. These findings were entirely consistent with the known prodifferentiating, pro-apoptotic properties of vitamin D in other tissues and cell lines (6, 11, 12, 19, 23, 26).

The vitamin D_3 receptor (VDR) is a member of the highly conserved nuclear receptor family that also includes the sex hormones, glucocorticoids, thyroid hormone and retinoic acid (18), factors that are also known to impact on brain development through transcriptional activation and/or repression of target genes (5, 31, 45). Little is known about the role of the VDR during brain development. While the VDR has been identified in the adult brains of the rat (32, 36, 37), the hamster (25) and in humans (38), to date only one study has examined the VDR in embryonic brain tissue. In that study, Veenstra et al (39) used qualitative methods to show the expression of the VDR in various regions of the rat CNS on embryonic days 12, 15, 18 and 21. In particular, this paper commented on the association between the presence of the VDR in differentiating zones and mitotic activity in these regions. However, this association was not formally assessed (it was apparently based on visual impression), and no comment was made on the association between VDR and apoptosis.

Based on our findings linking low prenatal vitamin D and altered brain development (7) we are currently exploring potential mechanisms of action underlying this effect. In this current study, our aim was to chart the expression of VDR in whole embryonic brain at various developmental stages using quantitative measures, and to measure mitotic and apoptotic activity at these same time points.

MATERIALS AND METHODS

Animals: Female Sprague-Dawley rats were mated at 10 weeks. The sperm plug date was then defined as embryonic day zero (E0). Six dams were sacrificed at each gestational period; 15, 17, 19, 21 or 23 (birth) days gestation by CO_2 inhalation. Uteruses were removed, embryos decapitated and brains either fixed for histology or prepared for protein or RNA analysis as described below.

Histology: From each litter a number of brains were fixed in 4% paraformaldehyde for 2 hours followed by cryoprotection in 0.32M sucrose for 1 week. Frozen 42μm coronal sections were obtained on an IEC Minotome Plus cryostat from at least one embryo from each litter and mounted onto Superfrost Plus (Menzel Glaser, Germany) coated slides.

Cell counts: At E23, sections were selected at the anterior portion of the decussation of the corpus callosum. At the earlier embryonic ages, sections at equivalent bregma were selected. Choice of this region enabled cell counts to be performed in cortical cingulate, basal ganglia and hypothalamic regions. These brain areas were chosen as they represent disparate circumscribed brain regions readily identified at all embryonic periods. As we observed no asymmetry in the distribution of cells, the results were reported as a mean of both hemispheres.

Apoptosis: Sections were stained with 1μM Hoechst 33258 (Sigma B-1782) and both total cell number and cells containing an apoptotic nucleus were counted in single fields. Apoptotic cells were defined as cells with nuclei that were fragmented or highly condensed.

Mitosis: Sections adjacent to those used to count apoptotic nuclei were used to assess mitosis. This was achieved by immunohistochemical visualisation of cells in "S" or the synthetic phase of the cell cycle using an antibody directed against proliferating cell nuclear antigen (PCNA), anti-Mouse PC10 clone (Dako Denmark). Although PCNA can also be an indicator of DNA repair in mature animals, almost all immunoreactive cells in embryos will be dividing cells. Sections were antigen

retrieved in 10mM EDTA (pH 7.5) by boiling in a 600-watt microwave oven. Endogenous peroxidase activity was quenched with 0.3% H_2O_2 in methanol. Non-specific binding was excluded by incubation in 10% horse serum and 2% bovine serum albumin and the primary antibody (1:200) in the above blocking solution was then added. Cells containing PCNA were detected by incubation with an anti-mouse rat-preadsorbed biotinylated secondary antibody (1:200, Vector Laboratories Inc. CA). This immunospecific interaction was amplified with Vectastain® ABC reagents (Vector Laboratories Inc. CA) and visualised using diaminobenzidine as the chromogen. Slides were counterstained with thionine to allow counting of total cell number. Both immunoreactive and total cell number were counted in two adjacent, non-overlapping fields.

Western blots for PCNA and VDR: Embryonic brains were immediately dispersed into 1mL of cold 0.32M sucrose containing a protease inhibitor cocktail (Boehringer Mannheim, Germany) and 1mM phenylmethylsulfonyl fluoride by sonication. Protein suspensions were immediately placed on dry ice and stored at -80°C. Samples were subjected to SDS-polyacrylamide gel electrophoresis using 12 % and 9.8% gels for PCNA and the vitamin D receptor (VDR) respectively. Sample loadings for both proteins were the same. Gels were transferred onto PVDF membranes (Immobilon™-P Transfer Membranes, Millipore Corporation, MA) and the band of interest identified using the anti-Mouse PC10 clone for PCNA (1:500) or the N-20 rabbit polyclonal VDR antibody (1:500, Santa Cruz Biotechnology, CA). Blots were then exposed to HRP-conjugated anti-mouse or anti-rabbit antibodies (Zymed Laboratories Inc., CA) followed by chemiluminescence (Amersham Pharmacia Biotech, England). Densitometry was then used to obtain a qualitative measure of protein level.

RT-PCR: Total RNA was extracted from brains, following the single step method of Chomczynski and Sacchi (4). Genomic DNA was removed from the sample using DNaseI (Invitrogen, CA), and a single stranded cDNA template subsequently synthesised using Superscript™II RNase H-Reverse Transcriptase (Invitrogen, CA). Reverse transcriptase polymerase chain reactions were undertaken for the VDR with glutaraldehyde 3-phosphate dehydrogenase (GAPDH) as a reference gene. Primer sequences for the VDR were obtained from Fraga et al (8). GAPDH primers were designed as follows: Forward: GATGC TGGTG CTGAG TATGT CG; Reverse: GTGGT GCAGG ATGCA TTGCT CTGA. Primers were obtained from Sigma Genosys (Australia). Genes were amplified for 33 cycles in reactions containing 1.0mM $MgCl_2$ for the VDR and for 25 cycles with 2.0mM $MgCl_2$ for GAPDH. Products were subsequently visualised on 1.8% agarose gels by staining with ethidium bromide.

The change in the main outcome was measured with MANOVAs and assessed for linear trend over time. All p values are two-tailed and significance level was $p < 0.05$.

RESULTS

VDR: The appearance of VDR protein and mRNA in embryonic brains followed a similar pattern across embryonic development. At E15 the presence of protein could be detected however the transcript could not. From this point a significant increase in both protein (linear trend $F = 127.2$ df 1, $p < 0.001$) and transcript (linear trend $F = 30.7$, df 1, $p < 0.001$) was observed with the most pronounced increase in both protein and transcript occurring between E19 and E21 (Figure 1).

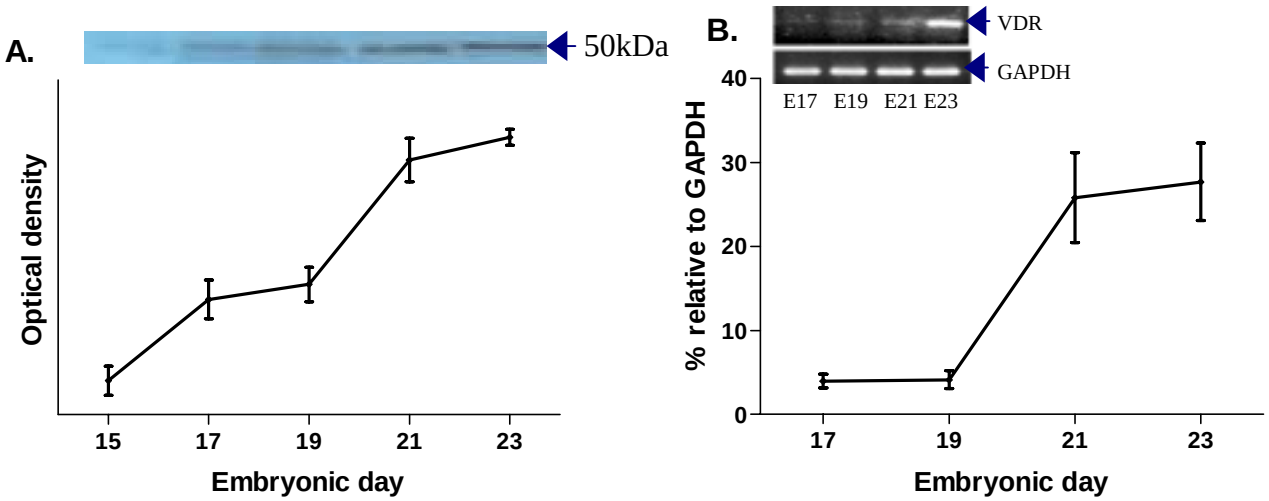

Figure 1.VDR expression in embryonic brain.
(A) Western blot for the VDR showing the 50kDa protein (n=6).
(B) RT-PCR analysis of VDR transcript (n=6).

Mitosis: Mitosis was assessed both regionally (via cell counts) and globally (measuring protein) utilising PCNA as the mitotic marker in both cases. Both cell count and protein data show a significant decline with increasing embryonic age over the time period (both variables show a significant linear trend, $p<0.001$). Regional variability was highest at the 2 earliest embryonic periods measured but from E19 onwards the rate of the decline in mitotic cells was similar in all three brain regions (Figure 2A). Pooling the cell count data produced a pattern of mitotic cell decline very similar to that seen by measuring the 36kD protein via western blot (Figure 2B).

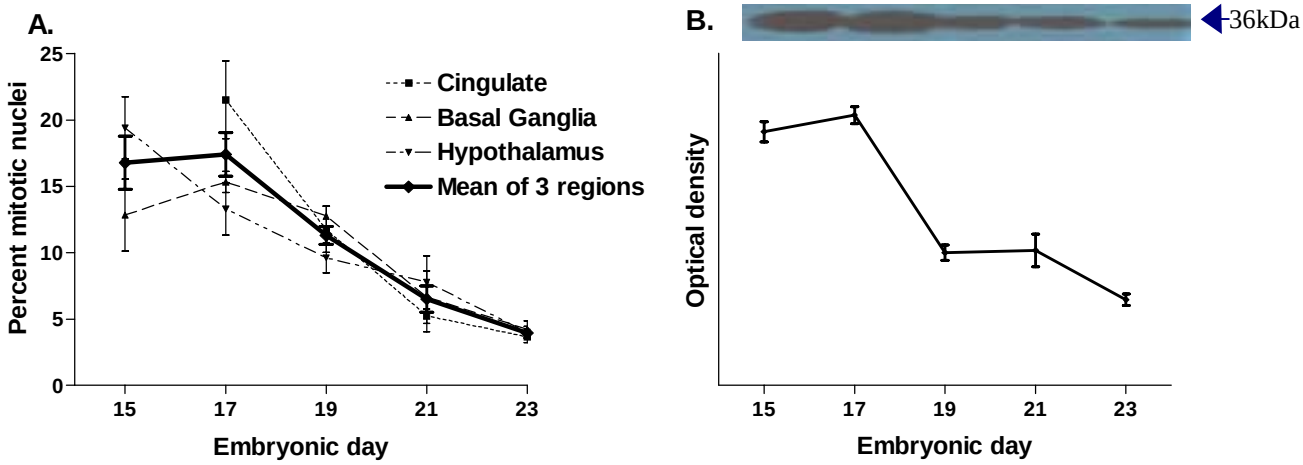

Figure 2. Mitosis in the embryonic brain
(A) Number of PCNA immunoreactive cells indicating that in each region examined, a similar decrease in mitotic cells with increasing age was observed (n=6).
(B) Western blot of PCNA showing a similar decline in the 36kDa protein with embryonic age (n=6)

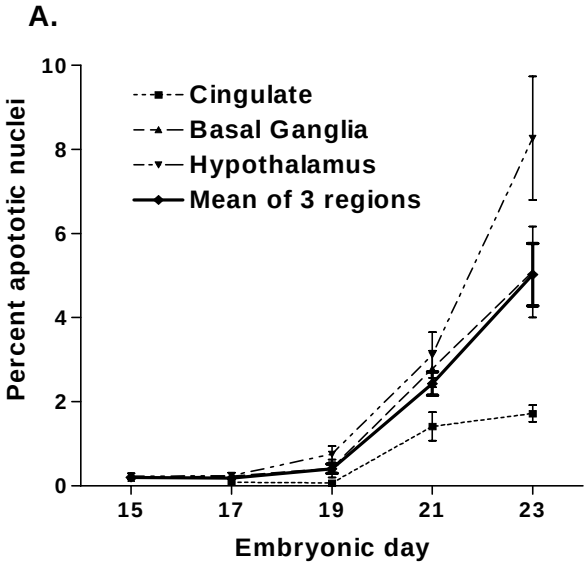

Figure 3. Counts of apoptotic nuclei in discrete brain regions showing that rate of apoptosis early in development is negligible and increases with embryonic age (n=6).

Apoptosis: As expected, the number of apoptotic cells increased significantly with embryonic age (test for linear trend, $p<0.001$), reflecting the fact that apoptosis occurs later in development (Figure 3). In fact, until E19, the number of cells with apoptotic nuclei appeared negligible. However from then on, a steep increase in the number of apoptotic cells was observed. The pattern of this increase was similar in all three brain regions investigated being most dramatic in the hypothalamus whilst a far more modest increase was noted in the cingulate region.

DISCUSSION

The expression of VDR protein and mRNA increases in the rat embryonic brain between days 15 and 23, a finding that is compatible with the qualitative findings of Veenstra et al (39). In addition, the timing of the expression of the VDR is coincident with increased apoptotic and decreased mitotic activity. While we cannot make any causal association between these findings, this study adds weight to the hypothesis that the VDR may either directly or indirectly mediate features of the neuronal apoptosis and cell cycle.

Although VDR mRNA and protein were not measured in discrete brain regions, the receptor for this vitamin appears to be widely distributed throughout the developing brain (39). Similarly, although mitosis and apoptosis occur at varying rates according to the brain region investigated, the general patterns remain remarkably similar (Figs 2A and 3). Therefore we have quantitated VDR message and actual protein in whole brain in order to gain general insight about its appearance across the brain with embryonic age.

Apart from the literature demonstrating the prodifferentiating and proapoptotic properties of vitamin D (as discussed above), vitamin D_3 has also been shown to down-regulate cyclin D1 (19, 29), cyclin E (35), cyclin C (30) and cyclin B1 (12). The vitamin also inhibits the expression of other key cell cycle proteins, particularly the retinoblastoma protein pRb (16, 19). It can also up-regulate cyclin dependent kinase inhibitors (CDKI), such as p27 and p21 (14, 44). In addition, vitamin D up-regulates the proapoptotic members of the family Bad and Bax and MEKK-1 whilst down-regulating the antiapoptotic members Bcl-2 and Bcl-xl (20, 22).

In addition to these effects, Vitamin D_3 may also affect brain development via its potent regulatory control over crucial neurotrophic agents such as NGF (1, 5, 13, 24, 28, 34, 42) and GDNF (27, 40). The data supporting a neurodevelopmental role for this vitamin, in combination with the timing of the VDR during brain development described here, lends weight to the hypothesis that vitamin D_3 is involved in normal brain development.

In light of their common mechanism of action via nuclear receptors, we propose that vitamin D_3 should be considered alongside vitamin A and thyroid hormone as important growth factor during brain development. It is likely that all 3 agents would exert an antimitotic, proapoptotic effect in developing brain tissue (3, 41, 43). Moreover there appears to be a similar temporal pattern in the expression of all three nuclear receptor hormones in the brain. α c-erbA, which gives rise to the dominant functional thyroid hormone receptor in the embryonic rat brain, is poorly expressed early in development but peaks by birth (2). Similarly, the transcript for the dominant retinoic acid receptor in the embryonic mouse brain (RARβ) also appears to increase with increasing embryonic age (33).

Many features that relate vitamin D and brain development remain unclear. For example, no gross brain changes have been noted in the VDR knock out mouse to date (15), suggesting that certain compensatory mechanisms are in place. In addition, our recent experiments have shown that the expression of neonatal brain VDR protein is not affected by maternal vitamin D depletion. This suggests that the VDR expression may be independent of the ligand, at least in the developing brain (7). While there is much to be learnt, we speculate that a closer inspection of the role of vitamin D in brain development may uncover new features of brain development in a fashion analogous to the discoveries related to the role of vitamin A and brain development (10, 17).

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