1,25-Dihydroxyvitamin D_3 induces nerve growth factor, promotes neurite outgrowth and inhibits mitosis in embryonic rat hippocampal neurons

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Abstract

There is an accumulation of evidence implicating a role for vitamin D_3 in the developing brain. The receptor for this seco-steroid is expressed in both neurons and glial cells, it induces nerve growth factor (NGF) and it is a potent inhibitor of mitosis and promoter of differentiation in numerous cells. We have therefore assessed the direct effect of vitamin D_3 on mitosis, neurite outgrowth, as well as NGF production as a possible mediator of those effects, in developing neurons. Using cultured embryonic hippocampal cells and explants we found the addition of vitamin D_3 significantly decreases the percentage of cultured hippocampal cells undergoing mitosis in conjunction with increases in both neurite outgrowth and NGF production. The role of vitamin D_3 during brain development warrants closer scrutiny.
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In recent years our understanding of the role of vitamin D_3 in the adult central nervous system has increased dramatically. In a recent review, Garcion and colleagues [6] have compiled the robust literature implicating calcitriol in brain functioning. The vitamin D receptor (VDR) is present in the brain, as are several of the key enzymes involved in the production of vitamin D_3 from its precursors (suggesting paracrine and autocrine properties for this hormone). In addition, vitamin D_3: (a) induces various neurotrophic factors and their receptors; (b) is involved in neuroprotection and immunomodulation via various pathways; and (c) has pro-apoptotic properties in glioma cell lines [6].

There is much less evidence for the role of this vitamin in brain development. Veenstra et al. [19] used qualitative methods to demonstrate that the VDR is expressed 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 increased density of the VDR in differentiating zones and increased mitotic activity in these regions. Recently our group has examined the impact of prenatal vitamin D_3 depletion on brain development in the rat [5]. Compared to control neonates, the offspring of vitamin D_3 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_3 depleted mothers had more mitotic cells accompanied by a trend level reduction in apoptosis. These findings are entirely consistent with the known prodifferentiating and pro-apoptotic properties of vitamin D_3 in other tissues and cell lines [6]. In addition, we found that offspring of vitamin D_3 depleted dams had reduced nerve growth factor (NGF), a finding that is also consistent with previous in vitro work demonstrating that vitamin D_3 is a potent inducer of this neurotrophin [14,17,20].

Recently it was shown that the VDR was distributed throughout rat hippocampus [8]. We have therefore cultured embryonic hippocampal neurons in order to establish whether vitamin D_3 affects neuronal development. We were interested in three questions related to the prodifferentiating properties of the hormone. Does the addition of vitamin D_3 to the cultures: (a) lead to reduced mitotic activity; (b) increased neurite outgrowth; and (c) increased production of NGF?

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Hippocampi were dissected from foetuses at embryonic day 18 (E18) from timed pregnant Sprague–Dawley rats. To examine mitosis, hippocampal cells were prepared by trypsinization and mechanical dissociation according to the methods described by Banker and Cowan [3]. Cell suspensions were plated at a density of 800 cells/mm^2 onto poly-L-ornithine coated glass coverslips in 16 mm diameter wells in 'neurobasal media' supplemented with 2% B27 growth additives (Gibco BRL), 0.5 mM glutamine, 0.1% bovine serum albumin (BSA) and 25 μM glutamate. After 3 h this media was replaced with the same media without glutamate containing either 0.1% ethanol (vehicle) or 100 nM vitamin D$_3$ in ethanol. Every 6 h (for 36 h) 2 mM Bromodeoxyuridine (BrdU) was added to a new set of coverslips. After 6 h, cells were fixed in 4% paraformaldehyde. To observe BrdU stained cells, coverslips were rinsed with phosphate-buffered saline (PBS) pH 7.4, incubated in 2 M HCL at 37 °C for 30 min, neutralised in borate buffer, pH 8.6 followed by PBS. Endogenous peroxidases were blocked with 0.6% hydrogen peroxide for 30 min at room temperature. After washing in PBS cells were incubated in blocking solution (2% BSA, 10% Horse Serum, 5% non-fat milk powder, 0.1% Triton X-100 in PBS) overnight. The following day, cells were incubated for 1 h in mouse monoclonal Anti-BrdU antibody (Sigma) diluted 1:200 in blocking solution. After rinsing with PBS, the cells were exposed to biotin conjugated horse anti-mouse secondary antibody diluted 1:200 in PBS for 1 h. The cells were subsequently rinsed in PBS and incubated for 1 h with avidin-biotin peroxidase complex reagent (Vector Laboratories Inc). Cells were then washed and immuno-reactive cells visualized with 3,3,diaminobenzidine (Sigma). Bias was eliminated by photographing four fields at the same position on each cover slip. Mitotic cells positively labelled

for BrdU were counted and expressed as a percentage of total cell number.

To examine neurite outgrowth freshly dissected hippocampi were sliced into 150 μm sections using a McIlwain chopper (Mickle Laboratory Engineering Company Ltd., Gomshall, Surrey, UK). Explants were allowed to attach to poly-L-ornithine coated 35 mm plates in a minimal volume of the same glutamate containing media described above for 3 h. Explants were cultured in the same media without glutamate in the presence of either 0.1% ethanol (vehicle) or 1 nM, 10 nM or 100 nM vitamin D$_3$. Neurite extension was measured from the edge of the explant to the tip of the outgrowth at the same position from nine independent explants/treatment group every 12 h for 96 h. Whilst there was some small variation in the size of the explants that adhered to the tissue culture plates, outgrowth rates did not correlate with explant size. Pilot data showed that neurite outgrowth was optimised at the 10 nM concentration (mean outgrowth over all time periods for control, 1 nM, 10 nM and 100 nM was 0.46, 0.53, 0.63 and 0.58 mm respectively). Thus, the main experiment examined the control and 10 nM conditions only. The various outcome measures were analysed using a repeated measures ANOVA based on Proc Mixed (SAS Version 8) with data examined for group, time and group × time effects.

NGF content was assessed in disassociated cells using a commercial ELISA (Promega WI). NGF could not be measured directly in the explants. The experimental design required explants to be plated at low density (three explants/35 mm tissue culture well) in order to ensure axonal outgrowth over 4 days would not be affected by contact with processes from neighbouring explants. Due to the low plating density, the concentration of NGF in these cultures was below the sensitivity range of the assay and
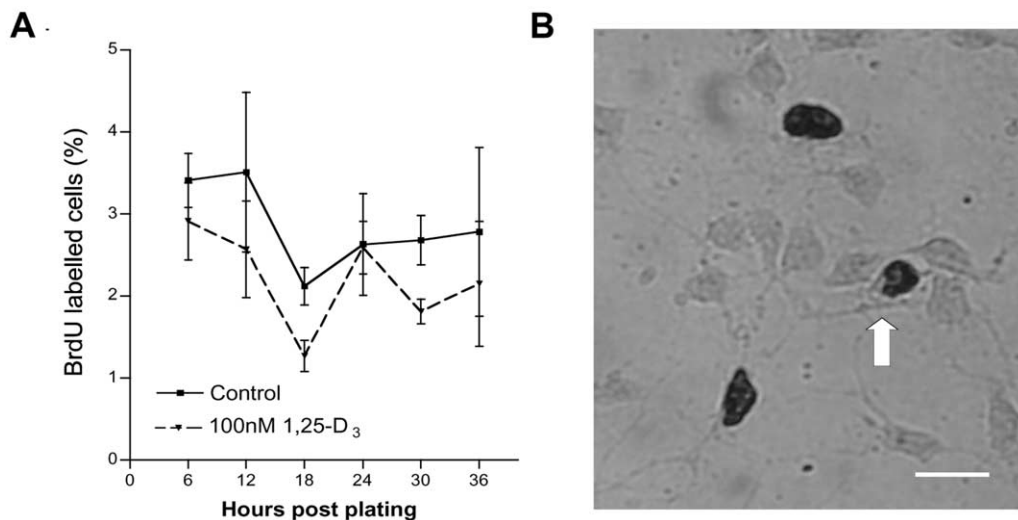

Fig. 1. The addition of 100 nM vitamin D$_3$ significantly decreases the number of mitotic hippocampal cells in culture. (A) BrdU labelled cells are expressed as percentage of the total cell number. These experiments were repeated twice and the same findings were generated. (B) A mitotic cell (indicated by arrow) exhibiting characteristic immunohistochemical nuclear staining for BrdU (36 h in vitro). Bar = 20 μm.

therefore the effect of vitamin D_3 on NGF production was investigated in disassociated cells. Briefly, cells were again exposed to either 0.1% ethanol (vehicle) or 100 nM vitamin D_3 in ethanol. Cells were harvested every 24 h for 4 days by removing media, washing in warm balanced saline solution followed by lysing in 200 μl of 20 mM Tris–saline buffer pH 6.8 containing 10% glycerol, a protease inhibitor cocktail (Boehringer Mannheim) with 1 mM phenylmethyl-sulfonyl fluoride and 0.5 mM sodium vanidate. Lysed cells were immediately frozen. The protein concentration of homogenates was estimated using a commercial Bradford assay (Biorad CA). Free levels of NGF were assessed using commercial ELISA (Promega WI). Specific protein concentrations were expressed relative to total protein concentration.

We found that the addition of 100 nM vitamin D_3 significantly decreased the percentage of hippocampal cells incorporating BrdU over the 36 h period studied ($F = 5.65$, df 1,14, $P = 0.03$; Fig. 1).This is consistent with the action of this vitamin in non-CNS cells. The anti-mitotic properties of vitamin D_3 were first recognised in a broad range of cancer cells, including those from breast cancer, prostate cancer, myeloid leukemia, neoplastic cell lines and colon carcinomas (for details see the review by Garcion et al. [6]). Consequently, pharmacological analogues of vitamin D_3 have been targeted as candidates for anti-cancer drugs. Vitamin D_3 has also been shown to regulate mitosis in many non-cancerous cells. For example, O'Connel and co-workers showed in primary cultures of cardiac myocytes that treatment with vitamin D_3 inhibited proliferation by over 50% after 4 days [15].

The molecular mechanisms for vitamin D_3's anti-proliferative effects are only now being unravelled. Vitamin D_3 has been shown to impede mitosis by decreasing the expression of G_1/S and G_2/M cellular gatekeeper components such as cyclins D1, B1 and retinoblastoma protein pRb in promyelocytic cells [9]. Additionally vitamin D_3 has been shown to upregulate the anti-mitotic cyclin-dependent kinase inhibitors p21 and p27 in a related cell line [16]. As the temporal and spatial cascades required for brain development are sensitive to alterations in the rates of mitosis, the role of vitamin D_3 in orderly brain development warrants closer scrutiny. Indeed, our whole animal experiments in the rat [5] confirm that in the absence of vitamin D_3, mitosis is increased and brain shape is altered.

This study also shows for the first time that the addition of 10 nM vitamin D_3 accelerates neurite outgrowth (Fig. 2). As expected explant outgrowth increased with time in culture. However the addition of vitamin D_3 to explants resulted in a significantly faster rate of outgrowth compared to the control condition ($F = 5.44$, df 1,89, $P = 0.02$). Although a mechanism of action for this response remains unclear, vitamin D_3 mediated up-regulation of certain neurotrophins would appear a likely candidate. As can be seen in Fig. 3, the addition of vitamin D_3 was associated
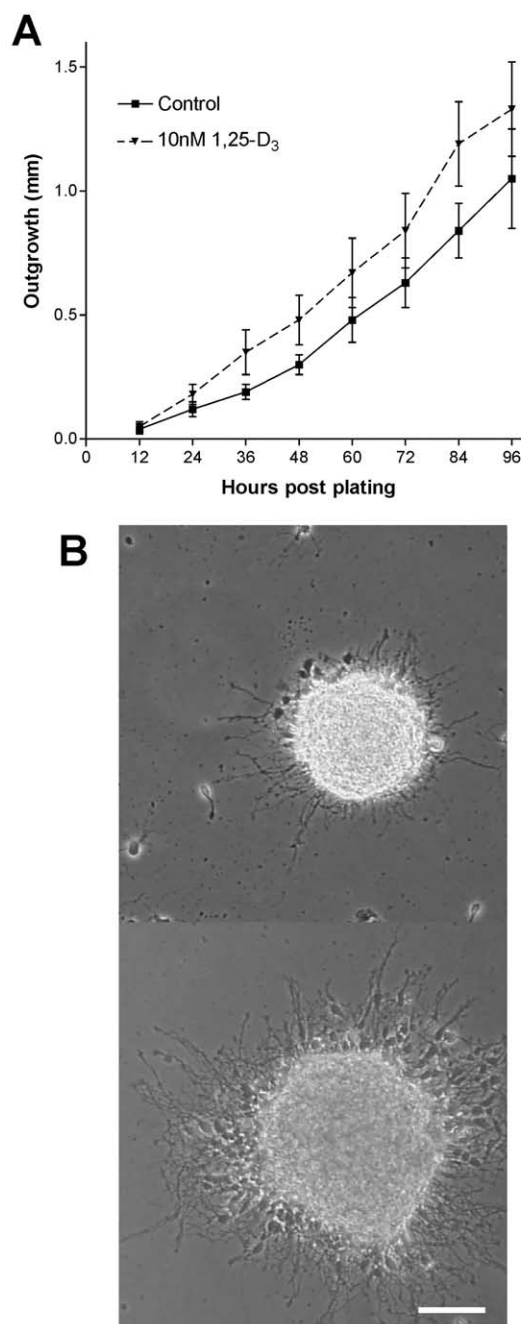

Fig. 2. Vitamin D_3 (10 nM) significantly increased the rate of neurite outgrowth from hippocampal explants. (A) Each point and bar represent the mean $\pm$ SEM from nine explants. These experiments were repeated twice and the same findings were generated. (B) Representative explants cultured without vitamin D_3 (top image), and in the presence of 10 nm vitamin D_3 (bottom image) (48 h in vitro). Bar = 10 μm.

with a substantial increase in the production of NGF ($P < 0.001$).

Vitamin D_3 is one of the most potent promoters of both NGF protein and mRNA yet reported [14,17,20]. The 'halo' of nerve fibres that radiate from hippocampal explants was described by Levi-Montalcini and her laboratory, when they first discovered the neurotrophic effects of NGF almost 40 years ago [10]. More recently, some of the molecular
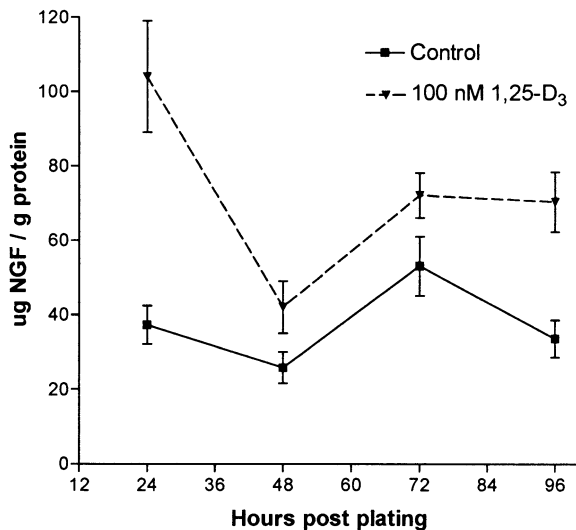
Fig. 3. Addition of vitamin D_3 (100 nM) increased the amount of NGF protein produced in hippocampal neurons. Free levels of NGF are expressed relative to total protein concentration of cell homogenates. These experiments were repeated twice and the same findings were generated.

mechanisms behind this effect were elucidated by Brann and co-workers [4]. This group showed that NGF stimulated outgrowth in primary hippocampal neurons was mediated through a ceramide signalling pathway downstream from NGF binding to the low affinity neurotrophin receptor p75 receptor. Vitamin D_3 responsive elements are also present in the promoter region of the p75^{NTR} gene [11] and vitamin D_3 regulates p75^{NTR} expression in glioma cells [2,11]. Thus in the presence of basal levels of NGF this could represent a complimentary mechanism for the observed vitamin D_3 mediated increase in hippocampal neurite outgrowth. Finally, our whole animal experiments demonstrate that in the absence of vitamin D_3, NGF protein was decreased in the brain of the neonatal rat [5].

Whether vitamin D is able to affect neuronal differentiation in vivo remains to be clarified. While the concentrations of vitamin D_3 used in this study were higher than the levels we have detected in adult rats raised on standard rat chow (vitamin D_3 level $= 0.4 \pm 0.1$ nM), (mean $\pm$ standard deviation, $n = 12$), it is known that vitamin D_3 levels in humans are significantly higher during pregnancy and peak in the third trimester [1,18]. The human placenta expresses the enzyme required to synthesize vitamin D_3, a feature thought to be related to the increased calcium requirements during pregnancy [1].

Concerning the relevance to in vivo brain development, it should also be noted that NGF production is likely to be low prenatally. The expression profiles of the neurotrophins and their receptors in the developing rat brain were mapped by Ip and co-workers, who showed almost undetectable levels of NGF until just preceding birth with increasing levels in the immediate post natal stage [7], equivalent to the period studied here. However, NGF is not the only neurotrophin that

is regulated by vitamin D_3. For example both NT-3 and GDNF are up-regulated by vitamin D_3 [12,13] and NT-3 is abundant in brain at this embryonic period [7].

We have shown that calcitriol acts as a potent differentiation agent in rat hippocampal cultures as assessed by a reduction in mitosis and increased neurite outgrowth. In addition, vitamin D_3 induces NGF, a neurotrophin known to affect the processes examined in this study. The role of vitamin D_3 during brain development warrants closer scrutiny.

Acknowledgements

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