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VITAMIN D REGULATES TYROSINE HYDROXYLASE EXPRESSION: N-CADHERIN A POSSIBLE MEDIATOR

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Abstract—Vitamin D is a neuroactive steroid. Its genomic actions are mediated via the active form of vitamin D, $1,25(OH)_2D_3$, binding to the vitamin D receptor (VDR). The VDR emerges in the rat mesencephalon at embryonic day 12, representing the peak period of dopaminergic cell birth. Our prior studies reveal that developmental vitamin D (DVD)-deficiency alters the ontogeny of dopaminergic neurons in the developing mesencephalon. There is also consistent evidence from others that $1,25(OH)_2D_3$ promotes the survival of dopaminergic neurons in models of dopaminergic toxicity. In both developmental and toxicological studies it has been proposed that $1,25(OH)_2D_3$ may modulate the differentiation and maturation of dopaminergic neurons; however, to date there is lack of direct evidence. The aim of the current study is to investigate this both *in vitro* using a human SH-SY5Y cell line transfected with rodent VDR and *in vivo* using a DVD-deficient model. Here we show that in VDR-expressing SH-SY5Y cells, $1,25(OH)_2D_3$ significantly increased production of tyrosine hydroxylase (TH), the rate-limiting enzyme in dopamine synthesis. This effect was dose- and time-dependent, but was not due to an increase in TH-positive cell number, nor was it due to the production of trophic survival factors for dopamine neurons such as glial-derived neurotrophic factor (GDNF). In accordance with $1,25(OH)_2D_3$'s anti-proliferative actions in the brain, $1,25(OH)_2D_3$ reduced the percentage of dividing cells from approximately 15–10%. Given the recently reported role of N-cadherin in the direct differentiation of dopaminergic neurons, we examined here whether it may be elevated by $1,25(OH)_2D_3$. We confirmed this *in vitro* and more importantly, we showed DVD-deficiency *decreases* N-cadherin expression in the embryonic mesencephalon. In summary, in our *in vitro* model we have shown $1,25(OH)_2D_3$ increases TH expression, decreases proliferation and elevates N-cadherin, a potential factor that mediates these processes. Accordingly all of these findings are reversed in the developing brain in our DVD-deficiency model. Remarkably our findings in the DVD-deficiency model phenocopy those found in a recent model where N-cadherin was regionally ablated from the mesencephalon. This study has, for the first time, shown that vitamin D directly modulates TH expression and strongly suggests N-cadherin may be a plausible mediator of this process both *in vitro* and *in vivo*. Our findings may help to explain epidemiological data linking DVD deficiency with schizophrenia.

Key words: $1,25(OH)_2D_3$, tyrosine hydroxylase, dopamine, development, developmental vitamin D (DVD)-deficiency, proliferation, N-cadherin, vitamin D receptor.

INTRODUCTION

Vitamin D is a steroid hormone and exerts diverse physiological functions that extend beyond its classical role in calcium and bone homeostasis (Jones et al., 1998). Several lines of evidence indicate vitamin D is important for both developing and adult brains (Eyles et al., 2009, 2010, 2013). In particular, our recent findings reveal the role that vitamin D may play in developing dopaminergic neurons (Cui et al., 2010, 2013; Eyles et al., 2014). Based on epidemiological findings, prenatal vitamin D deficiency has been identified as a risk factor for disorders associated with abnormalities in dopamine signaling. A Danish case–control study has shown that neonatal vitamin D status was significantly associated with the subsequent risk of schizophrenia (McGrath et al., 2010). The latest meta-analysis of all published observational studies that have measured vitamin D levels in schizophrenic patients confirms a strong association between vitamin D deficiency and schizophrenia (Valipour et al., 2014). Low levels of vitamin D have also been detected in patients with Parkinson's disease (Newmark and Newmark, 2007; Evatt et al., 2008, 2011; Knekt et al., 2010). Outcomes from a randomized clinical trial of vitamin D supplementation in patients with Parkinson disease is eagerly awaited (Suzuki et al., 2013).

A model of depleting female Sprague–Dawley rats of vitamin D before conception until birth has proved useful for investigating the neurobiological effects of vitamin D deficiency during brain development. One consistent finding from this developmental vitamin D (DVD)-deficient

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Abbreviations: BSA, bovine serum albumin; DOPAC, dihydroxyphenylacetic acid; DVD, developmental vitamin D; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GDNF, glial-derived neurotrophic factor; HPLC, high-pressure liquid chromatography; HVA, homovanillic acid; PBS, phosphate-buffered saline; PVDF, polyvinylidene fluoride; RA, retinoid acid; RXR, retinoid X receptor; SN, substantia nigra; TH, tyrosine hydroxylase; VDR, vitamin D receptor; VDRE, vitamin D responsive element.

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model is an alteration in dopamine signaling. Although vitamin D levels return to normal by the 2nd post-natal week, the adult offspring display enhanced sensitivity to the locomotor inducing effects of the dopamine releasing agent, amphetamine (Kesby et al., 2010), and also to the dopamine receptor antagonist, haloperidol (Kesby et al., 2006). When the neonatal brains from this model are examined, DVD-deficient offspring have reduced expression of the dopamine-degradation enzyme catechol-O-methyl transferase (COMT) which converts the dopamine metabolite dihydroxyphenylacetic acid (DOPAC) to homovanillic acid (HVA). Accordingly the ratio of DOPAC/HVA is reduced indicating dopamine turnover is abnormal in the neonatal forebrain (Kesby et al., 2009). At earlier developmental stages, DVD-deficient embryos display a reduced expression of the crucial dopamine specification factors, Nurr1 and p57kip2, in mesencephalon (Cui et al., 2010). Finally DVD-deficiency has also very recently been shown to down-regulate TH gene and protein expression in the embryonic brains of female mice (Hawes et al., 2015). In summary, these findings strongly suggest that vitamin D modulates the orderly early development of dopaminergic systems.

In the adult brain, vitamin D has been recognized as a neuroprotective agent for dopaminergic neurons. Multiple studies have shown that treatment with the active form of vitamin D, $1,25(OH)_2D_3$, protects against the adverse effects of the well-known dopaminergic toxin, 6-hydroxydopamine (6-OHDA) (Wang et al., 2001; Smith et al., 2006; Cass et al., 2012, 2014). Cass et al. showed that long-term $1,25(OH)_2D_3$ treatment increases the level of glial-derived neurotrophic factor (GDNF) in the damaged striatum. In addition to its well-known trophic actions on dopaminergic neurons, GDNF has also been found to be able to restore evoked dopamine levels after 6-OHDA-induced striatal damage (Cass and Manning, 1999). It was postulated that GDNF at least partially contributed to the protective effect of $1,25(OH)_2D_3$ in these animal models (Smith et al., 2006; Cass et al., 2014). Interestingly, although it has been suggested that tyrosine hydroxylase (TH), a rate limiting enzyme for dopamine synthesis, might be directly modulated by $1,25(OH)_2D_3$ this was never established. $1,25(OH)_2D_3$ treatment before or after local cerebral 6-OHDA administration partially restores TH protein and TH-immunoreactive fibers in the striatum and substantia nigra (SN) as well as increases GDNF protein (Sanchez et al., 2009). $1,25(OH)_2D_3$ has also been shown to increase TH-positive cell number in the SN of 6-OHDA-lesioned mice (Kim et al., 2006). Although the authors of these last two studies stated that $1,25(OH)_2D_3$-induced alterations in GDNF or microglia were responsible for its neuroprotective effects on dopaminergic neurons, they also could not rule out a more direct effect on TH expression. To date only one study has shown that $1,25(OH)_2D_3$ can directly increase the expression of TH mRNA but this was in adrenal medullary cells (Puchacz et al., 1996).

$1,25(OH)_2D_3$ exerts its genomic functions via the vitamin D receptor (VDR). The VDR is a member of a larger nuclear steroid receptor family, well-known for their actions in the developing brain. The VDR is expressed as early as embryonic day (E) 12 in developing rat mesencephalon (Veenstra et al., 1998). We confirm this and have shown punctate VDR staining in the nucleus of dopaminergic neurons at this age (Cui et al., 2013). The time window from E12 to E15 represents the critical period for the birth of dopaminergic neurons in Sprague–Dawley rats (Gates et al., 2006). DVD-deficiency during this period leads to a reduction in mesencephalic expression of both early dopaminergic specification factors, Nurr1 and p57kip, and more mature dopaminergic neuronal markers such as TH (Cui et al., 2010). It would therefore appear that vitamin D signaling is strategically positioned to affect the ontogeny of developing dopaminergic neurons.

$1,25(OH)_2D_3$ inhibits cell proliferation and promotes differentiation by multiple mechanisms. In cancer cells that express the VDR this has been frequently linked to the expression of E-cadherin (Palmer et al., 2001; Ordonez-Moran et al., 2008; Lopes et al., 2012). E-cadherin is only transiently expressed in developing diencephalon and mesencephalon between E9.5 and E15 (Shimamura and Takeichi, 1992) and we could not detect it in developing mesencephalon by western blot (data no shown). However the classical cadherin family includes E-, N-, P-, and R-cadherin members. N-cadherin is a neural adhesion molecule that plays important role in neurogenesis and of particular interest to our group, dopamine neuron development (Sakane and Miyamoto, 2013). Therefore we have investigated the expression of N-cadherin both in our *in vitro* and *in vivo* models.

Although evidence from adult and developing brain indicates that $1,25(OH)_2D_3$ modulates TH expression either in developing or adult dopamine neurons subjected to neurotoxin exposure there is no evidence that $1,25(OH)_2D_3$ can directly modulate TH expression or promote dopamine neuron differentiation. In addition, the underlying mechanisms behind this process remain opaque. We therefore have generated an *in vitro* model where the rat VDR is expressed in SH-SY5Y cells to examine this question. SH-SY5Y cells have been widely used to study dopamine neuronal differentiation and dopamine neuron survival in cell-based models of Parkinson's disease (Xie et al., 2010). Next, we have also used mesencephalon from our DVD-deficient rat embryos to see if the developmental absence of vitamin D reverses these processes in the developing brain. We aimed to clarify whether: (1) $1,25(OH)_2D_3$ directly regulates TH expression; (2) $1,25(OH)_2D_3$ regulates the differentiation and maturation of dopamine neurons, and (3) suggest potential mechanisms involved in these processes.

EXPERIMENTAL PROCEDURES

Cell line

Human SH-SY5Y cells were purchased from Sigma–Aldrich (MO, USA). Cells were cultured in Dulbecco's Modified Eagles Medium (DMEM)/F12 supplemented with 10% fetal calf serum (Invitrogen Life technology Australia, Victoria, Australia), L-glutamax (Invitrogen), 100 U/ml penicillin and 0.1 mg/ml streptomycin (Sigma).

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After stable transfection, the VDR-transfected cells were cultured in the presence of geneticin (Invitrogen) at a concentration of 500 μg/ml.

Cloning of VDR

Total RNA from adult rat kidney was prepared using RNeasy Plus mini kit (Qiagen, Hildon, Germany) and cDNA synthesized using SuperScript® III First-Strand Synthesis System (Invitrogen) according to the manufacturer's protocol. 0.2 μM of full–length rat VDR primers (Forward: 5' CTACTCACCCTGCTCCTTCAG 3', Reverse: 5' GTCAGGAGATCTCATTGCCG 3') and 1U Phusion Hot Star II DNA Polymerase (Thermo Scientific, IL, USA) were employed for PCR. The PCR products were sequenced and the full-length VDR inserted into pTarget vector (Promega Corporation, MI, USA).

Transfections

The pTarget-VDR construct was transfected into human SH-SY5Y cells using Lipofectamine 2000 (Invitrogen) in serum free Opti-MEM (Invitrogen) in 6-well plates. 18 h after transfection, the serum-free medium was replaced with normal growth medium. 48 h later cells were passaged at a 1:10 dilution into fresh growth medium in the presence of 2 mg/ml geneticin. The medium was changed every 3 days. Three weeks later, geneticin-resistant single-cell colonies were selected and expanded. VDR over-expression was confirmed by western blot (Data not shown). Long-term maintenance of stable transfectants was performed in the presence of 0.5 mg/ml geneticin. All experiments with transfected cells were conducted within 10 passages.

$1,25(OH)_2D_3$ treatment

VDR-expressing SH-SY5Y cells were seeded into 24-well plates at a concentration of 6×10^4 cells per well. Cells were allowed to settle for 2 days and then induced to differentiate in DMEM/F12 with 1% FCS in the presence of 10 μM retinoic acid (RA)(Sigma) for 7 days. Then $1,25(OH)_2D_3$ (Calcitriol, Calbiochem, Life Technology), was applied at varying concentrations in serum-free medium with B27 supplement (Invitrogen) and cells cultured for another 7 days. The medium containing these factors was changed every 3–4 days. This treatment regime is depicted in Fig. 2.

DVD-deficient rat model

Briefly, female Sprague–Dawley rats were fed with a diet depleted of cholecalciferol but with normal calcium and phosphorous. The rats were housed under a 12-h light/dark cycle using incandescent lighting free of ultraviolet radiation in the vitamin D action spectrum (290–315 nm). After 6 weeks, serum vitamin D depletion was confirmed prior to mating ($25(OH)D_3$ < 0.34 ng/ml) (Eyles et al., 2011). The resulting dams were housed under these conditions until the birth of pups. Control animals were kept under standard lighting conditions and supplied with standard rat chow containing vitamin D. Both vitamin D deplete dams and offspring remained normocalcemic. This model has been described in detail elsewhere (Eyles et al., 2009). The embryonic (E18) brains were dissected and frozen on dry ice for western blot. All experiments were conducted with the ethical approval of the University of Queensland.

Western blot

VDR-expressing SH-SY5Y cells were collected in lysis buffer (150 mM NaCl, 20 mM HEPES, 2 mM EDTA, pH7.4) containing protease inhibitor cocktail (Roche Diagnostics Australia, castle Hill, NSW, Australia). Protein concentrations were determined using a micro BCA assay (Peirce, Bonn, Germany) and 5 μg of total protein per lane was loaded. Proteins were resolved by SDS–PAGE using 4–12% NUPAGE Bis-Tris gels (Invitrogen) before being transferred to polyvinylidene fluoride (PVDF) membranes at 400 mA for two hours. The PVDF membranes were blocked in 5% non-fat milk and then probed with primary antibodies, anti-VDR antibody (D-6, 0.2 μg/mL) (Santa Cruz, TX, USA), anti-TH (0.1 μg/mL) (Millipore, Darmstadt, Germany), or anti-N-cadherin (1:2500, Millipore) at 4 °C overnight and then incubated with horseradish peroxidise (HRP)-labeled goat anti-mouse secondary antibody (1:5000, Cell Signalling Technology, MA, USA) for one hour. Transferred protein bands were visualized using standard chemiluminescence techniques (Thermo Scientific Pierce). Membranes were then washed and reprobed with anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibody (1:50,000, Millipore) as a loading control. The films were scanned and the intensity of the bands assessed using Quantity One software (Bio-Rad Laboratories Pty., Ltd. Life Science, Australia).

Real time PCR

Cells were collected and RNA was isolated and cDNA synthesis was performed as described above. The following primers were used: TH (Forward, 5′-GCAGTT CTCGCAGGACATTG-3′ and reverse, 5′-TGGATGCGT GAGGCATAGC-3′), GDNF (Forward, 5′-CTGGGCTATG AAACCAAGGA-3′ and reverse, 5′-GACAGGTCATCATC AAAGGC-3′) and GAPDH (Forward, 5′-TTTACATGTTT CCAATATGATTCCAC-3′ and reverse, 5′-TTGTCATAC TTCTCATGGTTCACAC-3′). Real time PCR was performed on LightCycler 480 system (Roche Diagnostics, Castle Hill, Australia). The PCR conditions were as follows: a denaturation step at 95 °C for 10 min followed by amplification for 40 cycles (95 °C for 10 s, and 60 °C for 1 min). The relative expression of genes of interest was normalized to GAPDH as a housekeeping gene using the comparative threshold method.

Stereological cell counting

Cells were grown on coverslips. On day 7 of $1,25(OH)_2D_3$ treatment, cell culture medium was replaced with serum free medium containing 20 μM of 5-ethynyl-2′-deoxyuridine (EdU), and 3 h later, EdU incorporation was detected using Click-iT EdU Imaging kit (Invitrogen)

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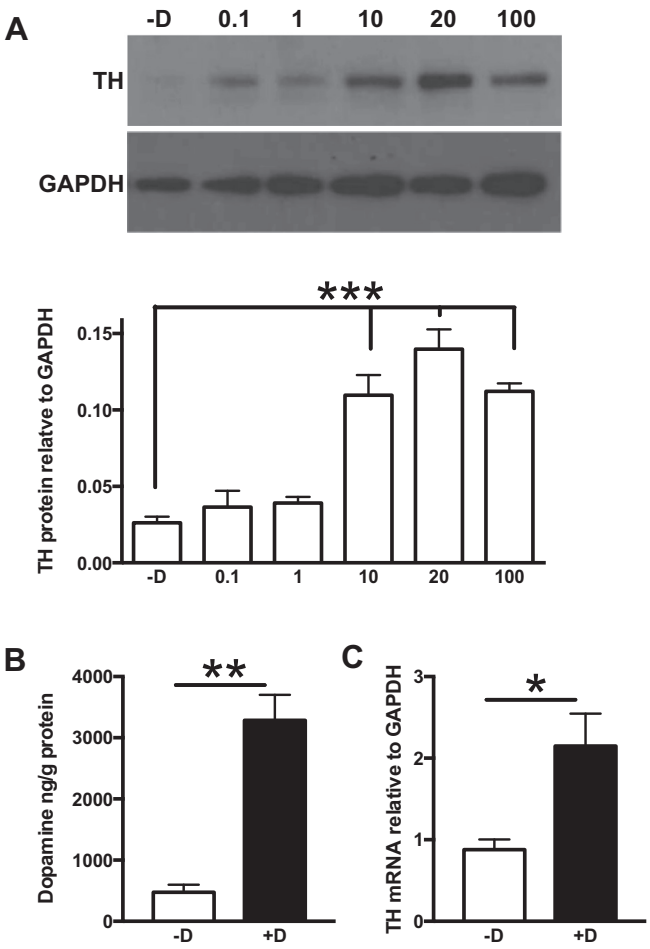

Fig. 1. TH expression is modulated by $1,25(OH)_2D_3$. VDR-expressing SH-SY5Y cells were treated with retinoid acid (RA) (10 μM) for 7 days and then $1,25(OH)_2D_3$ for 7 days. $1,25(OH)_2D_3$ increases (A) TH protein in a dose-dependent manner [0.1–100 nM; $F(5,18) = 27.82$, $p < 0.001$, $n = 4$] relative to vehicle (−D 0.04% ethanol); (B) dopamine synthesis ($t = 6.448$, $n = 3$, $^{**}p < 0.01$) and (C) TH mRNA ($t = 3.056$, $n = 4$, $^{*}p < 0.05$). Data = mean ± SEM.

according to manufacturer's instructions. Briefly, cells were fixed and rinsed twice with phosphate-buffered saline (PBS) containing 3% bovine serum albumin (BSA, Sigma) and incubated with 150 μL EdU-Click reaction-mix for 30 min at room temperature, and then rinsed with 3% BSA/PBS. Hoechst 33342 was used to stain the nuclei to count all cells. The coverslips were mounted on glass slides with fluorescence mounting medium (DAKO, Glostrup, Denmark). EdU and Hoechst 33342-positive cells were counted using stereoinvestigator software and unbiased stereological methods.

Flow cytometry to determine TH-positive cell number

Cells were collected before differentiation (Undifferentiated cells), 3 and 7 days after RA treatment, and then 1, 3 and 7 days after $1,25(OH)_2D_3$ (20 nM) (Fig. 2A). Ethanol (0.04%) was used as vehicle. Cells were directly collected in lysis buffer for western blot or digested by 0.05% trypsin (Invitrogen) and fixed as above. After fixation, cells were blocked with 3% BSA and incubated with a rabbit monoclonal anti-TH antibody (1:4000) (Abcam) for 30 min. After being washed with PBS, cells were incubated with Alexa 488 anti-Rabbit antibody (Invitrogen) for 20 min. All incubations were done on ice. The cells were then stained with Hoechst 33342 and then subjected to flow cytometry.

Measuring dopamine levels

VDR-expressing cells were seeded in a 24-well plate and treated with RA for 7 days and then $1,25(OH)_2D_3$ for 7 days. Cells in each well were collected in 100 μL of lysis buffer containing 0.1 M perchloric acid and 50 ng/ml deoxypinephrine (DE) used as an internal standard. 50 μL of cell lysate was centrifuged at 13,000 rpm for 10 min and 35 μL of supernatant were filtered by a 0.45 μM polypropylene syringe filter (Agilent Technologies, Inc., CA, USA) and injected directly into a high-pressure liquid chromatography system consisting of an autosampler, an isocratic HPLC pump (Model 1100, Agilent Technologies, Inc.), a Sunfire C18 column, 4.6 mm × 150 mm, 5 μM; (Waters Corporation, MA, USA) and a Coulochem III (ESA Laboratories, Inc., MA, USA) electrochemical detector. The mobile phase consisted of a 10% acetonitrile/75 mM potassium dihydrogen phosphate buffer containing 25 μM EDTA

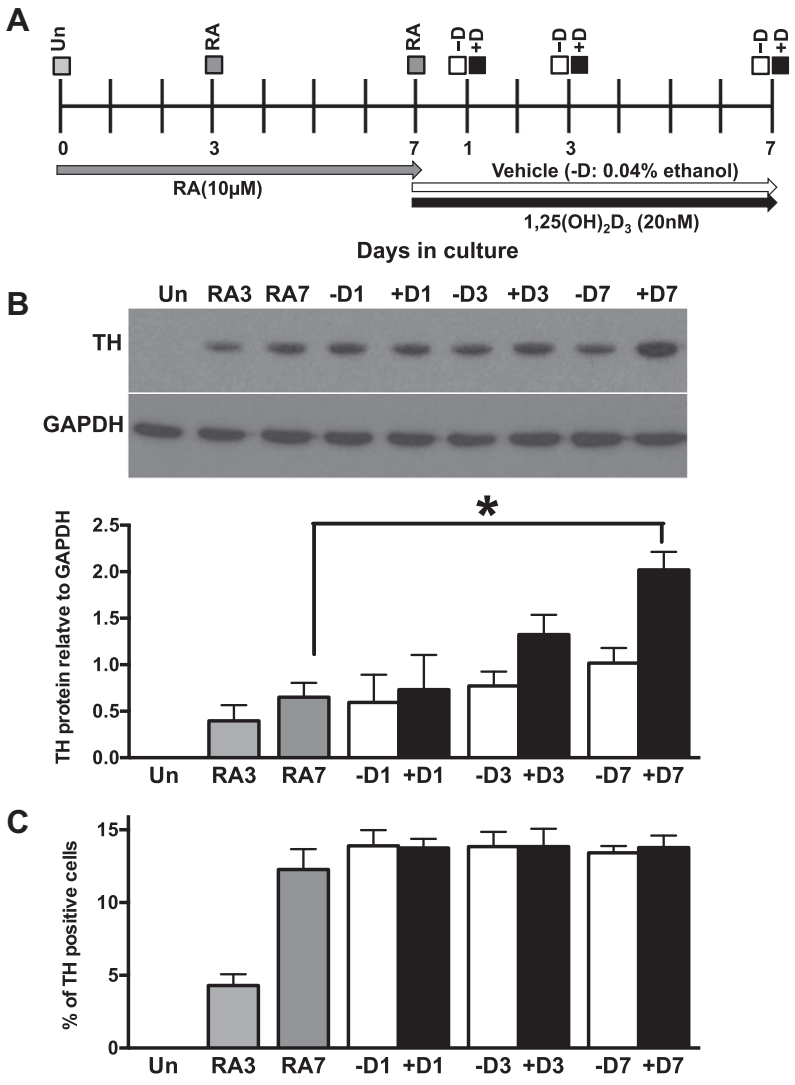

Fig. 2. TH expression is modulated by $1,25(OH)_2D_3$ in a time-dependent manner. (A) VDR-expressing SH-SY5Y and wild type SH-SY5Y cells were harvested at 3 and 7 days after RA (10 μM) treatment (RA3 and RA7) or at 1, 3 and 7 days after 20 nM $1,25(OH)_2D_3$ (+D1, +D3, +D7) or after vehicle treatment (−D1, −D3, −D7). (B) In VDR-expressing SH-SY5Y cells $1,25(OH)_2D_3$ treatment for 7 days significantly increases TH expression compared to levels after 7 days of RA treatment ($F(8,18) = 7.29$, $n = 3$, $^*p < 0.05$). (C) $1,25(OH)_2D_3$ did not change the percentage of TH-positive cells ($n = 4$). Data = mean ± SEM.

and 1.7 mM octane sulfonic acid adjusted to pH 3 with phosphoric acid delivered at 1 mL/min. Detector settings were as follows: conditioning cell (Model 5020, ESA Laboratories, Inc., MA) +350 mV; analytical cell (Model 5014B, ESA Laboratories, Inc., MA) with the first and second electrodes maintained at −150 and +250 mV respectively. Data were quantified by calculating peak-height ratios for each specific analyte relative to an internal standard, DE. Data were processed with Chemstation software (Rev B.01.03, Agilent Technologies, Inc.). Samples were corrected for dilution and expressed as nanogram per gram (ng/g) protein.

Statistical analysis

Unpaired *t*-tests were used to analyze the effects of $1,25(OH)_2D_3$ on dopamine production; TH mRNA expression; GDNF expression; dividing or total cell number and N-Cadherin expression. For the effects of $1,25(OH)_2D_3$ concentration or the effects of $1,25(OH)_2D_3$ over time on TH protein production, statistical analyses were performed by an analysis of variance (ANOVA) followed by post hoc tukey's tests. Significance level was set at $p < 0.05$.

RESULTS

Effect of $1,25(OH)_2D_3$ on TH expression and activity *in vitro*

$1,25(OH)_2D_3$ significantly increased TH protein in VDR-expressing SH-SY5Y cells in a dose-dependent manner (Fig. 1A, $F(5,18) = 27.82$, $p < 0.001$, $n = 4$). The optimal $1,25(OH)_2D_3$ dose proved to be 20 nM. This dose was used in all further cell-based experiments. Dopamine, the product of TH, was also increased 10-fold in cell lysates (Fig. 1B, $t = 6.448$, $p < 0.01$,

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$n = 3$). TH mRNA was also significantly enhanced by $1,25(OH)_2D_3$ (Fig. 1C, $t = 3.056$, $p < 0.05$, $n = 4$).

Next we examined the time-dependent nature of TH protein expression in VDR-expressing SH-SY5Y cells. Cells were treated with RA for 7 days and $1,25(OH)_2D_3$ at 20 nM for 7 days with media changed on days 3 and 6. The cells were collected at 3 and 7 days after RA treatment and then 1, 3 and 7 days after vitamin D treatment (Fig. 2A). In undifferentiated cells, no TH protein was observed. TH protein increased with RA treatment. In VDR-expressing SH-SY5Y, the addition of $1,25(OH)_2D_3$ progressively further increased TH expression which became significant after 7 days of treatment compared to the last day of RA treatment (Fig. 2B, $F(8,18) = 7.29$, $p < 0.05$, $n = 3$; RA7 vs. +D7, $p < 0.05$). By contrast, the ethanol vehicle produced no further increase in TH. To investigate whether this $1,25(OH)_2D_3$ – induced increase in TH expression in VDR-expressing SH-SY5Y was due to an increase in TH production per se or TH-expressing cell number the percentage of cells expressing TH was determined using flow cytometry. We clearly showed that the use of RA increased TH-positive cell number; however $1,25(OH)_2D_3$ had no further effect (Fig. 2C). This suggests the $1,25(OH)_2D_3$ – mediated increase in TH levels reflects an increase in expression, rather than an increase in TH-positive cells.

$1,25(OH)_2D_3$ does not alter GDNF expression *in vitro*

GDNF is a neurotrophic factor for dopaminergic neurons and multiple lines of evidence have indicated that $1,25(OH)_2D_3$ may directly modulate GDNF expression (Naveilhan et al., 1996; Sanchez et al., 2002). In our differentiated cells the GDNF transcript was thus examined in the presence of $1,25(OH)_2D_3$ but no alteration was observed (Fig. 3, $t = 0.2843$, $p = 0.7857$, $n = 4$).

$1,25(OH)_2D_3$ reduces cell proliferation *in vitro*

We have previously shown that $1,25(OH)_2D_3$ has potent anti-proliferative effects on primary hippocampal neurons (Brown et al., 2003). In line with our previous finding, $1,25(OH)_2D_3$ significantly reduced the number of dividing cells in culture by 30 per cent (Fig. 4A, $t = 5.195$, $p < 0.01$, $n = 4$). It is reasonable to expect that total number might therefore also be decreased; however, the total number of cells was not altered as assessed by unbiased stereological cell counting techniques (Fig. 4B).

$1,25(OH)_2D_3$ increases N-cadherin expression

N-cadherin is a neural adhesion molecule that plays important role in the neurogenesis of dopamine neurons (Sakane and Miyamoto, 2013). In our current study, $1,25(OH)_2D_3$ increased N-cadherin protein in VDR-expressing SH-SY5Y cells (Fig. 5A, $t = 2.476$, $p < 0.05$, $n = 4$). When we examined N-cadherin protein in the mesencephalon from DVD-deficient embryonic brains, accordingly, it was significantly reduced (Fig. 5B, $t = 3.304$, $p < 0.05$, $n = 3$).

DISCUSSION

There are several novel and important findings in this study. First, we provide unambiguous evidence that $1,25(OH)_2D_3$ promotes an increase in TH expression and dopamine in neuroblastoma cells transfected with the rodent VDR. This regulation is dose and time-dependent and does not reflect any change in TH cell number. Second, this increase in TH expression is not associated with increased trophic effects of GDNF at least in this culture system. Third, we show that $1,25(OH)_2D_3$ inhibits the proliferation of VDR-expressing SHSY-5Y cells. Fourth, we find that $1,25(OH)_2D_3$ enhances that expression of N-cadherin, a neural adhesion molecule important in dopaminergic neuron differentiation and maturation. Finally when these factors were investigated *in vivo* in developing neurons from our DVD-deficiency model, accordingly, we observe the reverse findings. A comparison of these findings from our *in vitro* model of $1,25(OH)_2D_3$ administration compared with current and previously published findings from our *in vivo* model of DVD-deficiency is presented in Table 1.

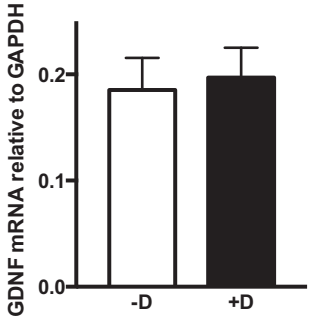

Fig. 3. $1,25(OH)_2D_3$ does not alter the expression of GDNF mRNA. VDR-expressing SH-SY5Y cells were induced to differentiate by addition of 10 μM RA for 7 days and then 20 nM $1,25(OH)_2D_3$ (+D) or vehicle (−D: 0.04% ethanol) for 7 days. RNA was isolated and GDNF expression was examined by real-time PCR ($t = 0.2843$, $p = 0.7857$, $n = 4$).

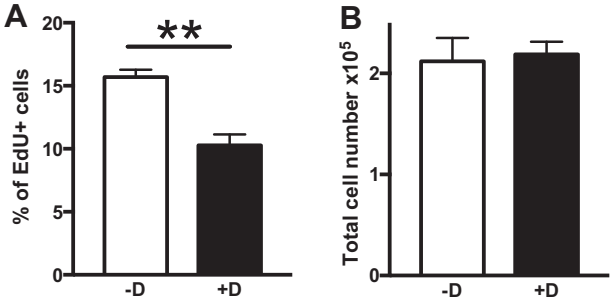

Fig. 4. The effect of $1,25(OH)_2D_3$ on proliferation. VDR-expressing SH-SY5Y cells were treated with RA (10 μM) for 7 days and then $1,25(OH)_2D_3$ (20 nM) (+D) or vehicle (−D: 0.04% ethanol) for 7 days. EdU was applied on day 7 of $1,25(OH)_2D_3$ treatment. 1, $25(OH)_2D_3$ (A) reduces EdU incorporation ($t = 5.195$, $n = 4$, $^{**}p < 0.01$), but (B) does not alter total cell number ($t = 0.2781$, $n = 4$, $p = 0.7841$). Data = mean ± SEM.

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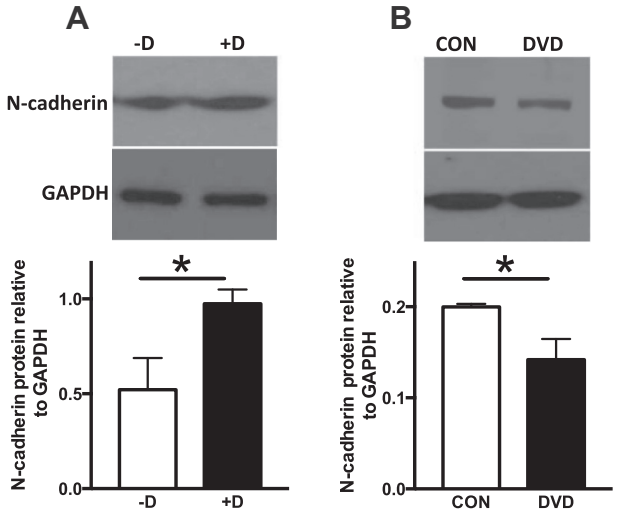

Fig. 5. N-cadherin is modulated by $1,25(OH)_2D_3$. (A) VDR-expressing SH-SY5Y were differentiated in the presence of RA (10 μM) for 7 days and then $1,25(OH)_2D_3$ (20 nM) (+D) or vehicle (−D: 0.04% ethanol) for 24 h. Cells were collected and subjected to western blot. $1,25(OH)_2D_3$ increases N-cadherin protein in VDR-expressing SH-SY5Y cells *in vitro* ($t = 2.476$, $n = 4$, $^*p < 0.05$) (B) DVD-deficiency reduces N-cadherin protein *in vivo* ($t = 3.304$, $n = 3$, $^*p < 0.05$). Data = mean ± SEM.

$1,25(OH)_2D_3$ directly modulates TH expression in neuronal cells

In our *in vitro* model, SH-SY5Y cells were first pre-treated with RA. This promoted approximately 12% of these cells to differentiate and produce the dopaminergic marker TH. Subsequent treatment with $1,25(OH)_2D_3$ did not further increase the overall percentage of TH-positive cells in culture. However it clearly increased the production of not only the TH protein and its transcript but also its product dopamine, indicating this increase in TH is functional. The optimal $1,25(OH)_2D_3$ dose was 20 nM. The response relationship is best described as an "inverted U" dose response. This dose and this dose-relationship is entirely consistent with numerous previous studies examining the effects of this steroid *in vitro* (Kelly et al., 1985; Ryhanen et al., 1998; Toell et al., 2000; Chattopadhyay et al., 2003; Wu et al., 2007) .

Consistent with $1,25(OH)_2D_3$ having no effect on TH-positive cell number were the findings from unbiased cell counting indicating that $1,25(OH)_2D_3$ also had no effect on total cell number in culture. This further suggests that the $1,25(OH)_2D_3$ mediated increase in TH and dopamine results from $1,25(OH)_2D_3$ acting as a differentiation agent on TH-positive neurons to increase neuronal maturation and subsequent dopamine production rather than increasing TH cell number or survival (see below). The idea that $1,25(OH)_2D_3$ could promote aspects of neuronal maturation such as neurite outgrowth in primary neuronal cultures (Brown et al., 2003) as well as in neuroblastomas (Agholme et al., 2010) has been made previously. Only one previous study has reported that $1,25(OH)_2D_3$ may directly increase TH expression but this was in adrenal medullary cells (Puchacz et al., 1996). Unfortunately only TH mRNA levels were examined in that study, with neither TH protein nor TH-positive cell number examined.

A number of studies have shown that $1,25(OH)_2D_3$ can act as a neuroprotective agent for dopaminergic neurons (Wang et al., 2001; Cass et al., 2006; Smith et al., 2006; Sanchez et al., 2009; Cass et al., 2012, 2014). These data and the epidemiological findings linking low levels of vitamin D with increased risk of Parkinson's disease (PD) (Newmark and Newmark, 2007; Evatt et al., 2008, 2011; Knekt et al., 2010; Peterson et al., 2013) have led to a clinical trials of vitamin D in this disease. The initial findings are very encouraging; the patients on placebo continued to have a steady worsening on neurological outcomes whereas those who took vitamin D (1200IU/day) had no further progression in PD symptoms (Suzuki et al., 2013). These results strongly suggest that vitamin D status influences disease progression. Progression of PD symptoms is associated with the degeneration of dopaminergic neurons. As previously outlined, preclinical studies in neurotoxicity animal models of this disease confirm that $1,25(OH)_2D_3$ can restore dopamine levels in striatum and ameliorate dopamine neuron death induced by intra-cerebral administration of 6-OHDA (Wang et al., 2001; Smith et al., 2006; Sanchez et al., 2009; Cass et al., 2012, 2014). Cass et al. and Sanchez et al. have showed that vitamin D increases GDNF in the lesioned striatum indicating this trophic agent may be the mediating factor for vitamin D's neuroprotective actions on dopamine neurons. $1,25(OH)_2D_3$ has also been shown to reduce dopaminergic neuronal death induced by 6-OHDA in primary mesencephalic cultures by increasing GDNF expression (Orme et al., 2013). Therefore the evidence that GDNF acts as a trophic factor for DA neurons in the presence of toxic agents would appear strong. For these

Table 1. Vitamin D mediates alterations in N-cadherin, proliferation and TH expression: comparison between findings presented here where $1,25(OH)_2D_3$ is present *in vitro* with findings in the developing brain where vitamin D is removed from the maternal diet and compared with findings in which N-cadherin is regionally knocked out

	In vitro ($1,25(OH)_2D_3$)	*In vivo* (DVD-deficiency)	Regional N-cadherin knock-out (Sakane and Miyamoto, 2013)
N-cadherin	↑	↓	↓
Cell proliferation	↓	↑ (Eyles et al., 2003; Ko et al., 2004; Cui et al., 2007)	↑ (Dopaminergic progenitors)
TH	↑	↓ (Cui et al., 2010)	↓

reasons we also examined whether GDNF expression may be a mediating factor in our *in vitro* model. The clear absence of any effect on GDNF expression in our *in vitro* system suggests this factor may be more relevant to $1,25(OH)_2D_3$'s actions in dopaminergic systems in the presence of active toxicity.

$1,25(OH)_2D_3$ regulates the transcription of a variety of genes via its receptor, the VDR. After ligand binding, the VDR forms a heterodimer with the retinoid X receptor (RXR). This complex binds to a vitamin D response element (VDRE) in the regulatory region of the target gene (Haussler et al., 1998). Our results suggesting a direct effect on TH expression may possibly be mediated through this classical genomic function. Using the multi-genome analysis of positions and patterns of elements of regulation (MAPPER) search engine, we identified multiple putative VDREs within 10 kb from the 5'upstream DNA sequence for the TH promoter (Marinescu et al., 2005). Further investigations are warranted to explore whether the VDREs on the promoter of TH are functional via a technique such as chromatin immunoprecipitation (ChIP). Indeed, other members of the same nuclear steroid family as $1,25(OH)_2D_3$ such as RA and estrogen have been reported to modulate TH expression through their respective response elements, the retinoic acid responsive element (RARE) or the estrogen responsive element (ERE) within the RXR complex binding to the promoter of TH (Maharjan et al., 2005; Jeong et al., 2006). It is thus plausible that some similar mechanisms may be operating for $1,25(OH)_2D_3$.

$1,25(OH)_2D_3$ and cell proliferation

The finding that $1,25(OH)_2D_3$ acts as a differentiation agent for neurons is no longer surprising (McGrath et al., 2001). Our results here indicating that $1,25(OH)_2D_3$ decreases the number of dividing neuroblastomas is consistent with our previous findings showing that $1,25(OH)_2D_3$ reduces BrdU incorporation in hippocampal progenitors (Brown et al., 2003). Further supporting evidence comes from the study of neurospheres which are formed when embryonic brain is dispersed and cultured under the appropriate conditions. This neurosphere number is directly proportional to number of dividing cells (Reynolds and Rietze, 2005) and we have shown that the addition of $1,25(OH)_2D_3$ to neurospheres in culture reduces neurosphere number (Cui et al., 2007). Furthermore, previous evidence from our DVD-deficient rat model is also consistent with these findings. In the developmental *absence* of vitamin D, DVD-deficient embryos have *increased* mitotic cells throughout the embryonic brain (Eyles et al., 2003; Ko et al., 2004) and *increased* neurosphere number (Cui et al., 2007). DVD-deficiency also alters the corresponding expression profile of genes regulating mitosis in the brain (Ko et al., 2004). In this later study results from pathway specific arrays showed that 48% of genes related to mitosis were up-regulated in the DVD-deficient developing brain. In addition, key cell-cycle modulators such as cyclins C and B, as well as the cyclin-dependent kinase inhibitor P21 cip1 are down-regulated in the DVD-deficient developing rat (Ko et al., 2004). These cyclins and cyclin-dependent kinase inhibitors play a key role on modulating cell cycle progression. These factors have also been shown to be altered by $1,25(OH)_2D_3$ in this cell line. Gumireddy et al. showed that $1,25(OH)_2D_3$ and its analogs regulate P21cip1 and reduce BrdU incorporation by 25–50% in SH-SY5Y cells (Gumireddy et al., 2003a,b). $1,25(OH)_2D_3$ and its analogs have also been found to inhibit the proliferation of human SH-SY5Y cells (Celli et al., 1999; Stio et al., 2001).

A reduction in cell division as shown here in our *in vitro* model may also be expected to produce a reduction in cell number. However using unbiased cell counting techniques the total cell number appeared to be normal. It is plausible that once cells have undergone substantial differentiation due to the exposure to both RA and $1,25(OH)_2D_3$ for 7 days each, $1,25(OH)_2D_3$ may act as a survival-promoting factor. This finding is alluded to by the data of Brewer et al., who showed that $1,25(OH)_2D_3$ protects hippocampal neuron against the toxicity of N-methyl-D-asparate (NMDA) or glutamate toxicity (Brewer et al., 2001). Alternately it is also possible that vitamin D reduces the programed elimination of cells in our model. This seems unlikely however as vitamin D is pro-apoptotic (Krishnan and Feldman, 2011). After 14 days in culture the percentage of dividing cells is only 15% in vehicle treated cells (Fig. 4). Given the variance in the estimation of total cell numbers it is unlikely a 5% reduction in cell number due to less dividing cells in vitamin D treated cultures would lead to a significant reduction in total cell numbers.

$1,25(OH)_2D_3$ – induced differentiation of dopaminergic neurons is possibly mediated by N-cadherin

Cadherins are cell adhesion molecules that are important in the formation and maintenance of diverse tissue. The classical cadherins E, P and N are expressed in distinct tissue types and display separate functions during development. For instance, E-cadherin is expressed in all epithelial tissue and contributes to the orientation of cell polarity in epithelium. N-cadherin is mainly expressed in neural tissue and muscles (Halbleib and Nelson, 2006). Vitamin D modulates the expression of cadherins in a tissue-dependent manor. $1,25(OH)_2D_3$ increases E-cadherin levels in colon carcinoma cells (Palmer et al., 2001) and in contrast, down-regulates N and P-cadherin in breast cancer cells (Pendas-Franco et al., 2007), Our result showed that $1,25(OH)_2D_3$ increased N-cadherin in neuroblastomas and DVD-deficiency reduced its level in embryonic mesencephalon.

N-cadherin is known not only to contribute to the maintenance of tissue structure but also to cortical neurogenesis (Zhang et al., 2010) and, of greater importance here, dopamine neuron differentiation. Regional specific loss of N-cadherin in mouse mesencephalon results in a significant expansion of dopamine progenitors due to a reduction in their exit from the cell cycle (Sakane and Miyamoto, 2013). Therefore less dopamine

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progenitors differentiate into dopaminergic neurons resulting in a marked reduction in the number of dopamine neurons in N-cadherin knockout mice. Our finding of increased N-cadherin expression by $1,25(OH)_2D_3$ *in vitro* is therefore consistent with the potential role this agent plays in differentiating dopamine neurons. This proposal is strengthened by our additional finding of a decrease in N-cadherin protein in developing mesencephalon from DVD-deficient embryos. In fact it would appear that DVD-deficiency phenocopies N-cadherin knockout animals in that there is reduced N-cadherin, increased proliferation and reduced TH (Table 1). One caveat to this proposal, however, is that we do not know whether N-cadherin is reduced specifically in developing dopaminergic neurons.

CONCLUSION

In this study we clearly demonstrate that $1,25(OH)_2D_3$ enhances TH expression in a VDR-expressing neuroblastoma cells and importantly this effect is reversed when vitamin D is removed from the developing brain. These findings strongly suggest that $1,25(OH)_2D_3$ is a direct differentiation agent for dopaminergic neurons. Schizophrenia is regarded as a neurodevelopmental disease and alterations in the early ontogeny of dopamine systems have been proposed to be a central etiological feature of the disease (Eyles et al., 2012). The direct modulation of TH by $1,25(OH)_2D_3$ as demonstrated here might help us to better understand the etiology for the epidemiological link between DVD-deficiency and schizophrenia.

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