

Vitamin D: A potent regulator of dopaminergic neuron differentiation and function

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Abstract

Vitamin D has been identified as a key factor in dopaminergic neurogenesis and differentiation. Consequently, developmental vitamin D (DVD) deficiency has been linked to disorders of abnormal dopamine signalling with a neurodevelopmental basis such as schizophrenia. Here we provide further evidence of vitamin D's role as a mediator of dopaminergic development by showing that it increases neurite outgrowth, neurite branching, presynaptic protein re-distribution, dopamine production and functional release in various in vitro models of developing dopaminergic cells including SH-SY5Y cells, primary mesencephalic cultures and mesencephalic/striatal explant co-cultures. This study continues to establish vitamin D as an important differentiation agent for developing dopamine neurons, and now for the first time shows chronic exposure to the active vitamin D hormone increases the capacity of developing neurons to release dopamine. This study also has implications for understanding mechanisms behind the link between DVD deficiency and schizophrenia.

KEYWORDS

differentiation, dopamine, fluorescent false neurotransmitter, vitamin D

1 | INTRODUCTION

The aetiology of schizophrenia remains unknown. However, epidemiological data clearly indicate schizophrenia has neurodevelopmental origins (Weinberger, 2017). The evidence that schizophrenia is also a disorder of abnormal dopamine (DA) signalling would appear to be strong (McCutcheon et al., 2018). For some time now our group have been exploring the hypothesis that exposure to early developmental risk factors for schizophrenia may change how early

DA circuits are formed (Eyles, 2021a; Eyles et al., 2012). In turn, we believe that such early alterations may change how DA circuits function in the adult brain in a manner consistent with that observed in patients with schizophrenia.

We have shown that low levels of maternal vitamin D increase the risk of schizophrenia in offspring (Eyles et al., 2018; McGrath et al., 2010). We have also developed an animal model of this risk, developmental vitamin D (DVD) deficiency, in order to understand the neurobiology of this relationship (Eyles et al., 2003). In particular, we

Abbreviations: 1,25OHD, 1,25 dihydroxy Vitamin D₃; COMT, catechol-o-methyl transferase; DA, dopamine; DAPI, 4',6-diamidino-2-phenylindole; DIV, days in vitro; DMEM, Dulbecco's modified Eagles Medium; E14.5, embryonic day 14.5; FBS, foetal bovine serum; MAO-A, monoamine oxidase; NA, noradrenalin; PVDF, polyvinylidene fluoride; RA, all-trans-retinoic acid; SH-SY5Y/VDR⁺, human neuroblastoma cell line over-expressing the vitamin D receptor; SV2C, synaptic vesicle glycoprotein 2C; SynD, synapse detector (automatic cell analysis program); Syp1, synaptophysin-1; TH, tyrosine hydroxylase; VDR, vitamin D receptor.

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have focused on the consistent mechanistic links we have observed between vitamin D levels and the differentiation of DA neurons.

To explore the gene-regulatory actions of vitamin D on DA neurons, we have utilised SH-SY5Y cells in which the vitamin D receptor (VDR) is over-expressed (SH-SY5Y/VDR+) (Cui, Pertile, et al., 2015). SH-SY5Y cells can be readily differentiated into dopamine-like neurons via the use of all-trans retinoic acid. VDR over-expression in this cell line primes the cells for response to the vitamin D ligand, 1,25 dihydroxy vitamin D (1,25OHD) and has highlighted numerous molecular targets for vitamin D, that is DA specification factors (Cui et al., 2010), enzymes involved in DA turnover (Pertile et al., 2016) and regulation of dopaminergic neurotrophic factor receptors (Pertile et al., 2018, for review, see Cui et al., 2021). Moreover, we have confirmed many of these molecular mechanisms *in vivo* by showing that removing maternal vitamin D produces opposite effects in DVD-deficient mesencephalic DA neurons (Luan et al., 2018; Pertile et al., 2018).

Despite having knowledge of how certain early molecular factors are directly genomically targeted by 1,25OHD in immediately postmitotic DA neurons, we have no knowledge of how this hormone affects later maturation processes such as axonal elongation, synapse formation and DA release. Here, we have examined 1,25OHD's effects on neurite outgrowth in three culture systems; TH+ SH-SY5Y/VDR+ cells, mesencephalic DA neurons in culture and mesencephalic/striatal explant co-cultures. We have also considered the effects of 1,25OHD on synapse formation and distribution by examining the expression and distribution of a presynaptic protein believed to be important in DA release, SV2C (synaptic vesicle glycoprotein C) in TH+ SH-SY5Y/VDR+ cells. SV2C is one of three isoforms of the synaptic vesicle (SV2) family. The isoforms, SV2A, SV2B and SV2C, are integral vesicular membrane proteins (Buckley & Kelly, 1985), which are thought to be involved in neurotransmitter release. In contrast to SV2A and SV2B, SV2C is richly distributed throughout brain regions containing a high density of dopaminergic neurons and within their striatal projection targets (Dardou et al., 2011). The precise function of SV2C is unclear, although evidence suggests that it has a role in vesicular trafficking (Lezzi et al., 2005) and calcium-dependent exocytosis (Schivell et al., 2005). We also assessed whether such effects of vitamin D were more general by examining similar outcomes in synaptophysin (Syp-1 a widely used presynaptic marker) containing TH+ cells.

In summary, we provide further evidence of vitamin D's role as a mediator of dopaminergic development by showing that it increases TH+ neurite outgrowth in SH-SY5Y/VDR+ cells that have been differentiated into DA-like cells, mesencephalic DA neurons in culture and mesencephalic/striatal explant co-cultures. We also present the first evidence to suggest that vitamin D may alter the expression and distribution of synaptic vesicle proteins. We also show that vitamin D increases DA synthesis and directly visualises drug-mediated DA release from TH+ cells using a recently developed false fluorescent neurotransmitter for DA (FFN200) (Pereira et al., 2016). Taken together our findings suggest vitamin D is an important neurosteroid in DA neuron ontogeny and function.

2 | MATERIALS AND METHODS

2.1 | SH-SY5Y/VDR+ cell culture

A rat vitamin D receptor (VDR) was stably transfected into SH-SY5Y cells (Sigma-Aldrich, Australia. #94030304) as previously described (Cui, Pertile, et al., 2015; Pertile et al., 2016). Briefly, cells were cultured and seeded in (Dulbecco's modified Eagles Medium (DMEM)/F12 (Life Technologies, Australia, #11320-033)), 10% heat-inactivated foetal bovine serum (FBS) (Life Technologies, #A3840001), 1% L-Glutamax 100X (Life Technologies, #35050-061), 100 Unit/ml penicillin and 0.1 mg/mL streptomycin (Sigma, #P43333), containing 0.5 mg/mL G418-geneticin (Life Technologies, #15710064) and incubated at 37°C, 5% CO₂. One day later, cells were differentiated using serum-free DMEM/F12 supplemented with B27 supplement (Life Technologies, #17504-044) for 2 days and then treated with 25 µM of all-trans retinoic acid (RA) (Sigma, R2625) for 4 days, followed by either vehicle, 25 µM RA or 20 nM 1,25OHD (Calcitriol, Calbiochem, Millipore, #509721) for a further 3 days. 1,25OHD was dissolved in DMSO and then diluted in ethanol (Sigma, E7023). This meant the vehicle control was 0.002% DMSO +0.04% ethanol.

For immunocytochemistry, SH-SY5Y/VDR+ cells were seeded on coverslips coated with poly-D-lysine (Sigma, #P7886) and poly-L-ornithine (Sigma, #P4957) at a density of 10 × 10⁴ cells/well. After treatment cells were fixed with 4% paraformaldehyde (Sigma, #441244) for 20 min, washed and kept in PBS at 4°C until use. For measurements of DA levels, cells were seeded on 24 well plates at a density of 10 × 10⁴ cells/well.

Initial experiments showed SH-SY5Y/VDR+ cells were insufficiently developed to release the FFN200 marker at DIV10 but were at DIV 15. Additionally, to investigate the effects of vitamin D treatment on DA release from DA-like cells, cultures were transfected with TH-GFP AAV1 virus (a gift from Kwang-Soo Kim, Addgene plasmid #80336; <http://n2t.net/addgene:80336>; RRID: Addgene_80336) at DIV4 to identify dopamine-like TH+ cells during live imaging. Cells were cultured on 35 mm Dish glass bottom dishes (No. 1.5 Coverslip, 10mm Glass Diameter, MatTek Corporation, #P35G-1.5-10-C). To investigate the chronic effects of vitamin D after 7 days, the medium was replaced with either 20 nM 1,25OHD or vehicle control for another 7 days. To investigate the acute effects of vitamin D, 25 µM RA treatment was continued until DIV15 when the cells were perfused with either 20 nM 1,25OHD or vehicle. Timelines are outlined in Figure S1.

SH-SY5Y cells were chosen as they are routinely used for investigations of dopamine function. However, SH-SY5Y cells are listed as a commonly misidentified cell line by the International Cell Line Authentication Committee (ICLAC; <http://iclac.org/databases/cross-contaminations/>).

We did not conduct external cell line authentication from Promega or any other service companies. SH-SY5Y cells are the only cell line cultured in our laboratory, with spatially separated hoods and solutions dedicated only to this cell line. Therefore, this cell line

is unlikely to be contaminated by other cell lines. In addition, we closely monitor/observe SH-SY5Y cell growth rates, cell morphology and adhesion and spreading phenotype. After retinoic acid/vitamin D differentiation, tyrosine hydroxylase, the dopamine neuronal marker, is always dramatically increased compared to undifferentiated cells. The average passage number is 20 and the maximum is 27.

2.2 | Primary mesencephalic cell culture

Sprague–Dawley rat Dams were housed in groups of 3 and kept in standard macrolon cages with wire tops and had ad libitum access to water and food. They were killed by an intraperitoneal injection using Lethabarb (Pentobarbitone sodium solution at 325 mg/mL, 2 mL/dam) (Virbac, Australia, #3729556) according to standard operating procedures for the University of Queensland Ethics committee (LAB_011). The ventral mesencephalon of embryos was harvested at embryonic day 14.5. Within 1 hr of the dam's sacrifice tissue was dissociated in Papain mix (25 mL of 1 mg/mL DNaseI (Sigma, #11284932001) in Hanks Eagle Medium (Sigma, #H9394), 25 mL of HBSS medium and 50 mL of 2 mg/mL Papain stock (Sigma, #10108014001) in L-15 Medium (Sigma, #L5520) for 14 min total. The papain was neutralised with 1 volume of complete medium containing 5% FBS, cells were then filtered, centrifuged and pellet resuspended in 400 μ L charcoal-stripped (CS) complete media (DMEM)/F12, 5% CS-FBS (Invitrogen, #12676), 1% L-GlutaMAX 100X (Invitrogen)—each cell suspension contained tissue dissected from 2 embryos. About 100 μ L of cell solution was seeded onto glass coverslips pre-treated with poly-D-Lysine and 1 mg/mL laminin (Invitrogen, #23017015). After 1 hr 900 μ L neurobasal medium (Invitrogen, #21103), 2% B27-supplement (50X) and 0.5% N2-Supplement 100X (Invitrogen, #175020048) were added to each well. After three DIV cells were treated with either 20 nM 1,25OHD or vehicle control and cultured for a further 7 days and fixed in 4% PFA.

2.3 | Rat embryonic striatal-mesencephalic explant co-culture

Mesencephalic explants and their striatal axonal targets were cultured in close proximity (approximately the diameter of one explant apart, ~200–300 μ m) in a collagen matrix (660 μ L 3 mg/mL rat tail collagen (Invitrogen, #A10483-01), 100 μ L PBS 10X, 16.5 μ L 1 M NaOH, 223.5 μ L sterile H₂O) on coverslips as previously described (Schmidt et al., 2012). Then, 500 μ L of the neurobasal medium, containing either 20 nM 1,25OHD or vehicle control, was added once collagen was set. Explants were cultured for 48 h before being fixed in 4% PFA for 1 h. Mesencephalons were harvested from at least six embryos from each of the three pregnant Sprague–Dawley rats for use in primary and explant cultures. All studies in animals were approved by the University of Queensland animal ethics committee (ethics number QBI/296/18).

2.4 | Immunohistochemistry

Fixed SH-SY5Y/VDR+ cells were first preincubated for 30 min with PBS containing 0.1% triton-X100 (Sigma, #T8787) and 10% of normal goat serum (NGS) (ThermoFisher Scientific, #16210064). The cells were then incubated with primary antibodies: sheep anti-TH (1:100, Novus, #NB300-110) and/or rabbit anti-SV2C (1:300, Synaptic Systems, #119203) or Syp-1 (1:500, Invitrogen, #PA1-1043) for 1 h in buffer containing 1% NGS. Coverslips were washed three times with PBST buffer (PBA with 0.1% triton X100), then incubated with the appropriate secondary antibodies either goat anti-rabbit Alexa-fluor 488 (ThermoFisher Scientific, #A-11008) or goat anti-sheep Alexa-fluor 555 (Thermo Scientific, #A32727) for 1 h at room temperature. Cells were incubated with DAPI (1:500) (Sigma, #D9542) for 5 min to visualise cell nuclei. Coverslips were then mounted on slides with a fluorescence-mounting medium (Agilent Technologies, Victoria, Australia, #S3023).

Fixed mesencephalic cells and explants were stained as previously described (Schmidt et al., 2012). Briefly, cells and explants were blocked for 2 h (blocking buffer: PBST+ 0.5% BSA+0.05% sodium azide) and then incubated with sheep anti-TH (1:100, Novus) diluted in blocking buffer for 24 h, washed 5 \times 1 h in PBS and incubated with the appropriate secondary antibody in blocking buffer for 12 h, followed by a 6 \times 1 h washes in PBS, prior to staining with DAPI for 15 min, washing 3 \times 15 min in PBS and mounting in a fluorescence medium.

2.5 | Analysis of fixed cells and tissue

Images of fixed TH+ SH-SY5Y/VDR+ cells were captured using an LSM 510 META inverted confocal microscope equipped with a Plan-Apochromat 63x/1.40 Oil DIC M27 lens and ZEN software. For the TH+ SH-SY5Y cells to minimise selection bias, the observer was blinded to treatment conditions and the first 10–15 TH+ cells with suitable morphology (i.e. an obvious axon and a growth cone) encountered when scanning the coverslip in a systematic randomised manner were imaged. Two coverslips from each treatment were imaged. All experiments were replicated twice. In TH+ SH-SY5Y/VDR+ cells, analysis of neurite length, synapse number and localisation and synaptic marker intensity was performed using Synapse Detector software (Schmitz et al., 2011; Timms et al., 2002) (Figure S2).

For E14.5 primary mesencephalic cultures and explants, images were acquired using a Spinning Disk Confocal (Discovery) microscope equipped with a 60 \times magnification lens and NIS software. Z-stack images were acquired using an interval of 0.3 μ m, a maximum intensity projection was then performed using FIJI software, and cells were analysed using SynD software. Two coverslips per treatment were imaged giving a total of approximately 12 cells per treatment.

Images of striatal-mesencephalic explant co-cultures were acquired using Axio Imager standard fluorescent microscope equipped with a 10X magnification lens. Three images of each mesencephalic explant were acquired from the side proximal to the striatal explant.

The 10 longest TH+ projections in each image were measured using the Simple Neurite Tracer plugin on FIJI software, according to the protocol described by (Schmidt et al., 2012).

Sholl analysis was used for synaptic location (distance from soma) in all cultured cells. As mesencephalic cells are multipolar compared with TH+ SH-SY5Y/VDR+ cells we used Sholl analysis to also assess neuritic branching.

2.6 | DA measurement in TH+ SH-SY5Y/VDR+ cells

The DA content of the cells and the culture media were analysed using HPLC (Agilent Technologies) with electrochemical detection as previously described (Cui, Lefevre, et al., 2015; Petty et al., 2019).

2.7 | Real-time PCR (qPCR)

SH-SY5Y/VDR+ cells were collected and RNA was extracted using RNeasy Plus mini kit (Qiagen, #74106) according to the manufacturer's instructions. One micrograms of RNA per sample were used for cDNA synthesis using SensiFAST™ cDNA Synthesis Kit (Bioline, #BIO-65054) according to manufacturer's instructions, and quantitative PCR was performed in LightCycler® 480 System (Roche Diagnostics) using SensiFAST SYBR Green Master Mix (Bioline, #BIO-98020) under the following conditions: initial denaturation at 95°C for 10min followed by 40cycles of amplification (95°C for 15s, then 60°C for 30s, then 72°C for 20s). Primer pairs for all transcripts are shown in Table S1. Thermal cycling conditions were as follows: a denaturation step at 95°C for 10min and then amplification for 45cycles (95°C for 10s, 60°C for 30s and 72°C for 20s). Gene expression was assessed using the $\Delta\Delta C_t$ method. The relative expression of examined human genes was normalised to that of the endogenous control hypoxanthine-guanine phosphoribosyltransferase (HPRT).

2.8 | DA release studies in SH-SY5Y/VDR+ cells

All live imaging experiments were performed using a two-photon laser scanning microscope (LSM 710; Carl Zeiss Pty Ltd.) equipped with a Mai Tai titanium-sapphire pulsed laser and a Plan-Apochromat 63x/1.4 NA oil immersion objective lens. GFP fluorescence (TH marker) was detected at 495–591nm and FFN200 at 420–480nm. 16-bit images were captured with 0.25 μ m intervals (18-slice z-stack) and 512 \times 512-pixel resolution. The pixel dwelling time was 1.27 μ sec. A z-stack was acquired every 60s for a total of 19 min. Image acquisition was performed using Zen software (Carl Zeiss).

Cells were incubated with 10 μ M FFN200 (Tochris Bioscience, #5911) in a culture medium for 45min at 37°C under 5% CO₂. Cultures were then transferred to a prewarmed imaging chamber and perfused with aCSF (0.65 mL min⁻¹; 10x ringer's solution, 1M

MgCl₂, 1M CaCl₂) for 10min before imaging. During the 10min aCSF perfusion, one TH-GFP AAV1 virus labelled cell-containing FFN200 per dish was selected according to the following criteria: TH-GFP-positive soma with short, branched dendrites and the presence of an axonal branch extending from the cell soma in which there was an obvious accumulation of FFN200. A single z-stack was obtained to track the TH+ cell before and after the recording to account for cell drift. After acquiring an initial baseline (first four-time bins) with aCSF, to assess the acute effects of vitamin D, cells were perfused with either vehicle or 20nM 1,25OHD. To assess the chronic effects of vitamin D on cells cultured with 1,25OHD, after obtaining a baseline vehicle and 1,25OHD treated cultures were perfused with aCSF containing 45 μ M amphetamine to induce FFN200 release over 15 minutes. aCSF-perfused cells were also assessed to monitor FFN200 bleaching during the experiment.

For the image processing, the mean fluorescence intensity of FFN200 in the TH+ cell of choice was assessed over time. The cell chosen was cropped from the background using ImageJ (Image processing and Analysis in Java, National Institutes of Health). Fluorescent puncta were then identified by defining a size threshold (estimate XY diameter=0.5 μ m, estimated Z elongation/diameter=1 μ m) and shape constraints in Imaris (Bitplane, version 9.4). Live imaging data were then normalised to the background rate of FFN200 signal decay over time established from the baseline aCSF perfusion. For an outline of the experimental design, see Figure S3.

3 | STATISTICS

The effect of 1,25OHD treatment on mean neurite length, synaptic number, synaptic localisation, cellular and supernatant DA content and SV2C or Syp-1 intensity within TH+ SH-SY5Y/VDR+ cells was analysed using an ordinary one-way analysis of variance (ANOVA). Gene expression in cell cultures was assessed using FDR-corrected multiple unpaired t tests. The distribution of synaptic proteins across the neurite of individual TH+ cells was analysed using a general linear model with a repeated measure for neurite length. For neurite length in cultured mesencephalic cells or explants, a simple unpaired t test was used. For live imaging experiments, to examine the chronic effects of 1,25OHD on FFN200 release from TH+ SH-SY5Y/VDR+ cells, three groups were compared: aCSF perfusion (photobleaching control), amphetamine-perfused 1,25OHD-treated group and amphetamine-perfused vehicle-only group. The rate of FFN200 release (mean fluorescence intensity) over time was compared using repeated measure ANOVA. SPSS software and Bonferroni's correction for multiple comparisons were used for each test. Pearson's correlations were used to test the relationship between VDR intensity in TH+ SH-SY5Y/VDR+ cells and neurite outgrowth. A statistically significant result was reported, where $p < 0.05$. We have used these cells for experiments examining the impact of vitamin D on dopamine differentiation several times before. The sample size was based on our prior publications (Cui, Lefevre, et al., 2015; Pertile



et al., 2016; Pertile et al., 2018). No sample points were excluded from any experiment. All data were normally distributed. For *t* tests, a Shapiro-Wilks test confirmed variances were normally distributed. All *t* tests were two-tailed. For all ANOVAs, Brown-Forsythe tests were used to show variances were similar between groups.

4 | RESULTS

4.1 | Vitamin D increases neurite outgrowth and branching in TH+ cells

TH+ SH-SY5Y/VDR+ cells treated with 20nM 1,25OHD formed significantly longer neurites $F_{(2,79)}=11.7$ compared with RA treatment ($p<0.0001$) or vehicle $p<0.01$ (Figure 1a). Although this same treatment in cultured E14.5 mesencephalic TH+ neurons did not significantly increase either neurite length or total branching per se, Sholl analysis indicated that there was an interaction with greater overall neuritic branching produced by 1,25OHD $F_{(198,4356)}=2.1$ ($p<0.0001$) (Figure 1b). TH+ Neurite length was also increased by vitamin D in the mesencephalic striatal explant co-cultures $t=7.1$ ($p<0.0001$) (Figure 1c).

The intensity of expression of the transfected VDR in the TH+ SH-SY5Y/VDR+ cells is variable. Therefore, cells with more intense

VDR expression could produce greater effects when cultured in the presence of the 1,25OHD ligand. Therefore, we examined the relationship between the intensity of VDR protein expression in TH+ SH-SY5Y/VDR+ cells with neurite length. However, we show VDR intensity did not correlate with neurite length in either 1,25OHD-treated ($r^2=0.016$, $p>0.05$ $n=44$) or vehicle ($r^2=0.0015$, $p>0.05$, $n=45$)-treated cells thus removing this potential source of variation (Figure S4).

4.2 | Vitamin D increases synapse number and changes distribution along the neurite in TH+ cells

The expression of SV2C and Syp-1 was examined as an index of synaptic-like development in TH+ SH-SY5Y/VDR+ cells. 1,25OHD treated cells had about double the number of puncta for both synaptic proteins $F_{(2,95)}=14.9$ (SV2C) and $F_{(2,79)}=14.9$ (Syp-1) compared to both RA- and vehicle-treated cells ($p<0.001$) (Figure 2a,c). As an increase in synapse number could simply reflect the longer neurites found in 1,25OHD cultured cells, we used sholl analysis to visualise synapse density per unit of neurite length. Repeat measure ANOVA clearly showed the density of puncta for both presynaptic markers was increased along the length of the neurite for SV2C $F_{(1144,57)}=17.7$ and Syp-1 $F_{(1430,71)}=50.1$ compared with RA treatment or vehicle ($p<0.0001$). This also revealed

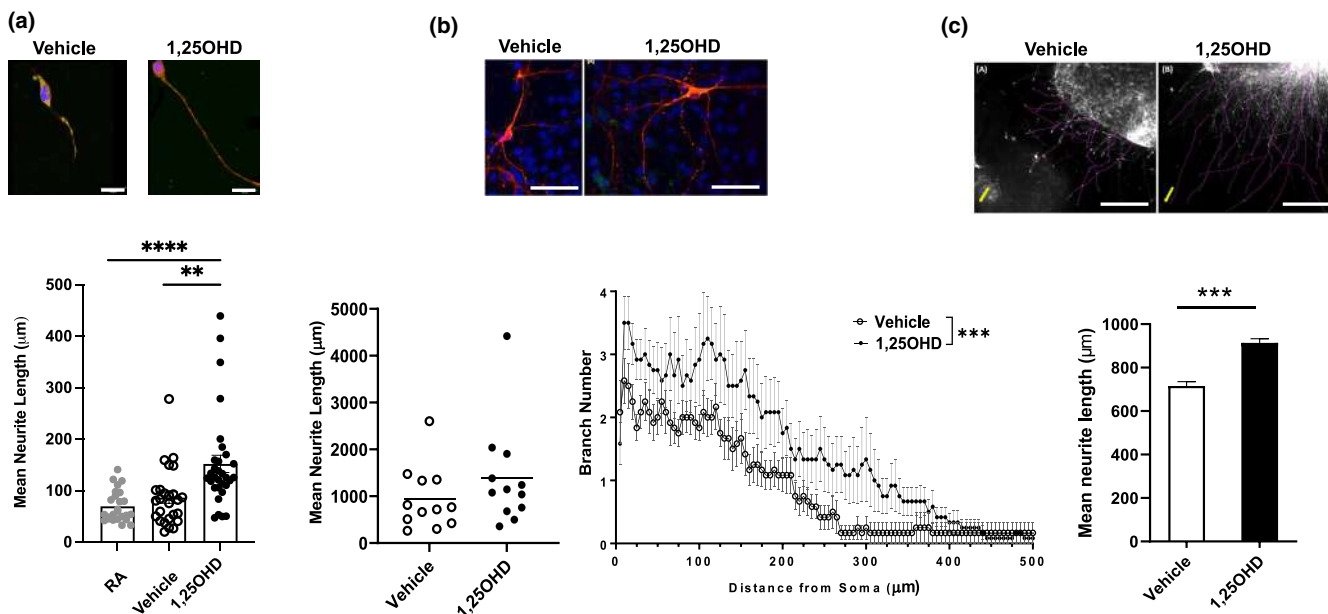


FIGURE 1 Vitamin D increases neurite outgrowth and branching in TH+ cells (a) TH+ SH-SY5Y/VDR+ cells treated with 1,25OHD formed significantly longer neurites $F_{(2,79)}=11.7$ compared with RA treatment ($p<0.0001$) or vehicle $p<0.01$. (insets represent neurites from vehicle and 1,25OHD-treated cells) (RA 25, Veh 26, 1,25OHD 31 individual cells from at least three separate cultures) (b) TH+ E14.5 mesencephalic neurons treated with 1,25OHD did not significantly increase neurite length. Sholl analysis indicated that there was an interaction with greater overall neuritic branching produced by 1,25OHD $F_{(198,4356)}=2.1$ ($p<0.0001$) (insets represent neurites from vehicle and 1,25OHD-treated cells) ($n=12$ cells from 3 separate cultures). (c) TH+ Neurites in mesencephalic striatal explant co-cultures treated with 1,25OHD were significantly longer, $t=7.1$ ($p<0.0001$) (insets represent TH+ (purple) neurites from vehicle and 1,25OHD-treated mesencephalon, yellow arrows indicate striatal target) [$n=30$], that is 10 longest neurites from three separate co-culture]). Scale bars: A=20μm, B and C=100μm.

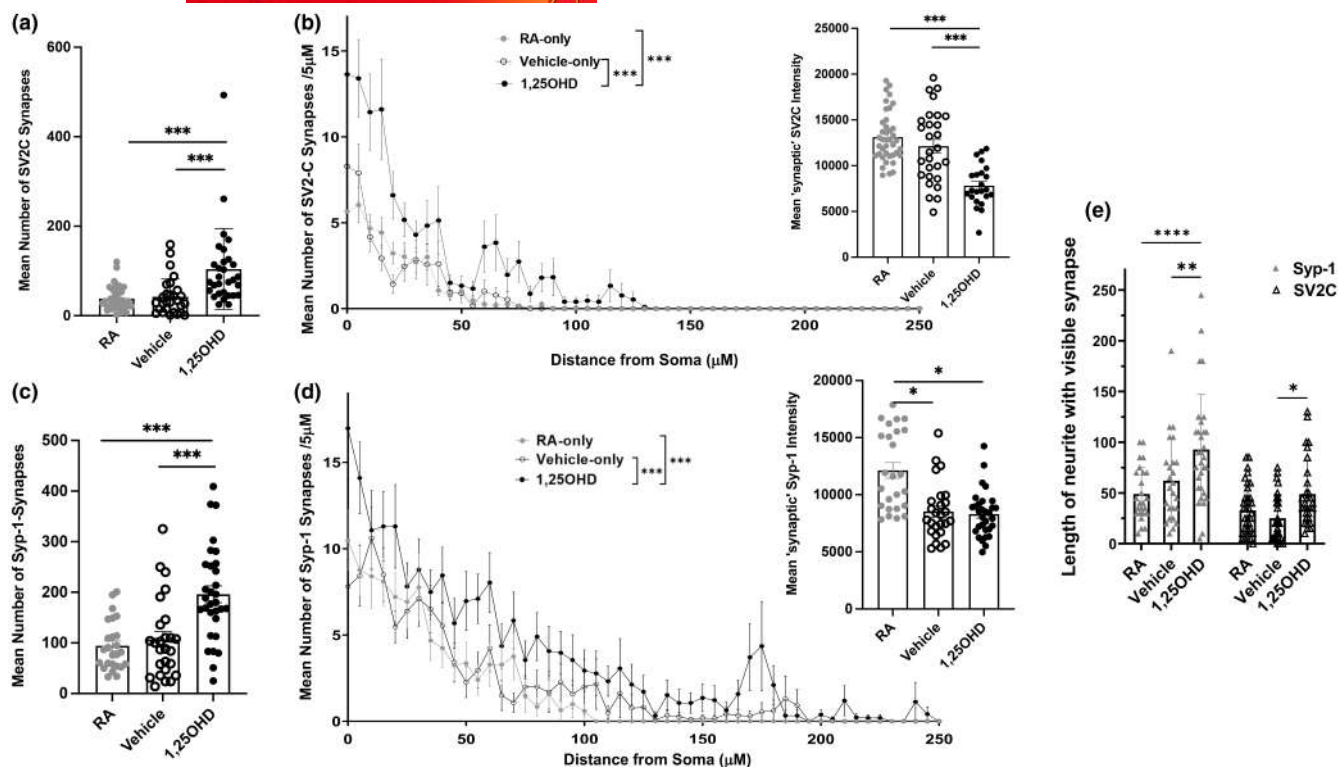


FIGURE 2 Vitamin D increases synapse number and changes distribution along the neurite in TH+ cells. (a, c) TH+ SH-SY5Y/VDR+ cells treated with 1,25OHD had a greater number of presynaptic protein puncta for both SV2C $F_{(2,95)} = 14.9$ and Syp-1 $F_{(2,79)} = 14.9$ compared to both RA and vehicle-treated cells ($p < 0.001$) (b, d) Sholl analysis clearly showed the density of puncta for both presynaptic markers was increased along the length of the neurite for SV2C $F_{(1144,57)} = 17.7$ and Syp-1 $F_{(1430,71)} = 50.1$ compared with RA treatment or vehicle ($p < 0.0001$). This also revealed synaptic densities were the greatest closer to the soma and that SV2-C synapses were restricted more closely to the soma compared to Syp-1 $F_{(2,170)} = 13.1$ ($p < 0.0001$). The intensity of the immunohistochemical response was decreased relative to both controls for SV2C $F_{(2,94)} = 25.5$ ($p < 0.001$) but unaltered compared to vehicle for Syp-1. (e) 1,25OHD treatment led to increased distribution of Syp-1 synapses further along the neurite compared with either vehicle or RA treatment ($p < 0.01$) and SV2-C synapses compared to vehicle-treated cells ($p < 0.05$) [n, SV2C data RA 40, Veh 28, 1,25OHD 30; and in SYP-1 data n RA 25, Veh 26, 1,25OHD 30 individual cells from at least three separate cultures].

synaptic densities were the greatest closer to the soma and that SV2-C synapses were restricted more closely to the soma compared to Syp-1 $F_{(2,170)} = 13.1$ ($p < 0.0001$) (Figure 2b,d). We also measured the intensity of the immunohistochemical response for synaptic protein expression. RA increased the intensity of Syp-1 puncta, perhaps consistent with its role as a neuronal differentiation agent. However, it had no effect on SV2C puncta intensity. Surprisingly despite 1,25OHD treatment leading to an increase in apparent number of synaptic puncta, the intensity of the immunohistochemical response was decreased relative to both controls for SV2C $F_{(2,94)} = 25.5$ ($p < 0.001$) but unaltered compared to the vehicle for Syp-1 (Figures 2b,d). There was also a main effect of 1,25OHD on the distribution of both presynaptic proteins $F_{(2,170)} = 30.7$ ($p < 0.0001$) with Syp-1 synapses being distributed further along the neurite compared with either vehicle or RA treatment ($p < 0.01$) and SV2-C synapses being distributed further along the neurite compared to vehicle-treated cells ($p < 0.05$) (Figure 2e). Taken together, these data indicate that 1,25OHD has altered the overall distribution and recruitment particularly of SV2C in TH+ SH-SY5Y/VDR+ cells.

4.3 | Vitamin D increases TH, DA but not noradrenalin levels in SH-SY5Y/VDR+ cultures

The increase in SV2C-labelled synapses in TH cells and the putative functional role of SV2C on DA release led us to analyse the levels of TH on DA and noradrenalin (NA) in the cell lysate and extracellular medium of SH-SY5Y/VDR+ cells exposed to the different treatments. 1,25OHD increased DA both within cells $F_{(2,21)} = 27.86$ ($p < 0.0001$) and in the media $F_{(2,21)} = 43.79$ ($p < 0.0001$). As expected, RA had the exact same effect. In contrast, RA and 1,25OHD had no effect on NA levels within cells $F_{(2,21)} = 1.147$ ($p = 0.33$). 1,25OHD also had no effect on NA levels in cell media while the media from RA-treated cells had lower NA levels $F_{(2,21)} = 7.424$ compared to both 1,25OHD and vehicle-treated cell media ($p < 0.05$) (Figure 3a,b).

RA is the most well-known differentiation factor for DA neurons and increases DA production by increasing the expression of the rate-limiting enzyme in its synthesis of TH (Jeong et al., 2006). We have previously shown 1,25OHD also increases TH production

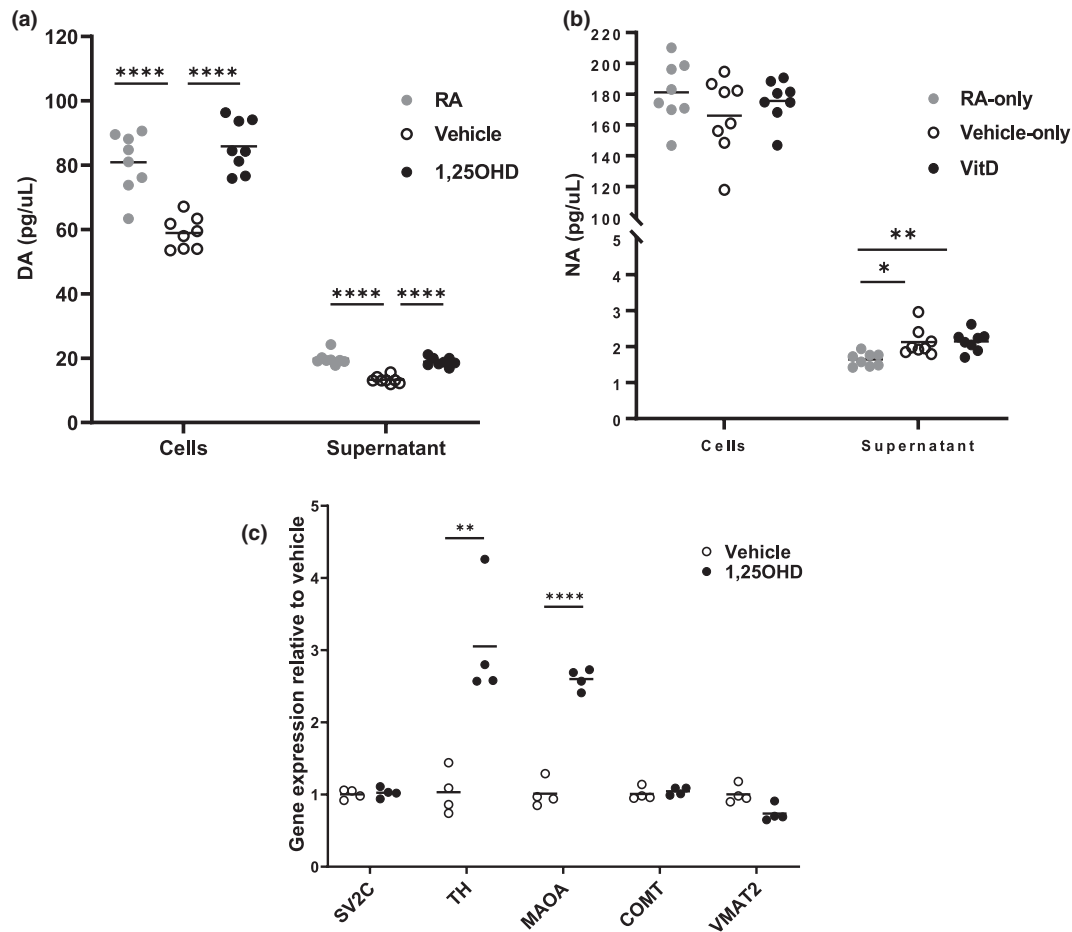


FIGURE 3 Vitamin D increases tyrosine hydroxylase and DA but not noradrenalin levels in SH-SY5Y/VDR+ cultures. (a) 1,25OHD and RA increased DA concentrations both within cells $F_{(2,21)} = 27.86$ ($p < 0.0001$) and in the media compared with vehicle $F_{(2,21)} = 43.79$ ($p < 0.0001$) ($n =$ media or harvested cells from 8 separate cultures). (b) 1,25OHD and RA had no effect on NA levels within cells $F_{(2,21)} = 1.147$ ($p = 0.33$). 1,25OHD also had no effect on NA levels in cell media while the media from RA-treated cells had lower NA levels $F_{(2,21)} = 7.424$ compared to both 1,25OHD and vehicle-treated cell media ($p < 0.05$) ($n =$ media or harvested cells from 8 separate cultures). (c) 1,25OHD increased expression of tyrosine hydroxylase (TH) ($t = 4.65$) $p < 0.01$ MAO-A ($t = 13.2$), $p < 0.00001$) and monoamine oxidase (MAO)A mRNA in these cells ($n =$ RNA from harvested cells from 4 separate cultures).

in these cells (Cui, Pertile, et al., 2015) and again confirm this here ($t = 4.65$) $p < 0.01$ along with an increase in major metabolic enzyme MAO-A ($t = 13.2$), $p < 0.00001$ (Figure 3c).

4.4 | Chronic but not acute vitamin D increases FFN200 release in TH+ SH-SY5Y/VDR+ cultures

To investigate whether acute treatment with 1,25OHD itself could induce DA release at the single-cell level, individual TH+ SH-SY5Y/VDR+ cells were located as described above and then perfused with aCSF solution containing either vehicle or 1,25OHD. Acute addition of 1,25OHD did not affect the rate of FFN200 release (-0.104% min $^{-1}$ for vehicle vs -0.754% min $^{-1}$ for 1,25OHD) ($F_{(1,20)} = 2.1$, $p = 0.15$), (n CSF = 10, n 1,25OHD = 12); Figure 4a. These results suggest 1,25OHD does not act non-genomically to induce DA release.

To examine whether chronic exposure to 1,25OHD during cell culture had any effect on DA release, TH+ SH-SY5Y/VDR+ cells were

located in cultures that had been either chronically treated with 20nM 1,25OHD or vehicle. On the day of the experiment, cells were then perfused with amphetamine to induce FFN200 release. A separate photobleaching control was used with either vehicle or vitamin D-treated TH+ SH-SY5Y/VDR+ cells which were perfused with aCSF. One-way ANOVA revealed a statistically significant increase in the rate of amphetamine-mediated FFN200 release in chronically 1,25OHD-treated cells compared with those cultured with vehicle or the untreated bleaching control ($F_{(2,17)} = 10.63$, $p = 0.004$), (-2.248% min $^{-1}$, for 1,25OHD $n = 8$; -0.380% min $^{-1}$ for photobleach control, $n = 5$; -0.6625% min $^{-1}$ for vehicle, $n = 7$), (Figure 4b). Because increased release may be because of increased uptake we also examined FFN200 puncta number and intensity in TH+ SH-SY5Y/VDR+ cells. Consistent with an absence of any effect on VMAT2 expression, the number ($t = 0.12$, $p > 0.05$, n control = 18, n 1,25OHD = 12) and intensity ($t = 0.44$, $p > 0.05$, n control = 18, n 1,25OHD = 12) of FFN200 puncta taken up by TH+ SH-SY5Y/VDR+ cells was not affected by long term exposure to 1,25OHD (Figure S5).

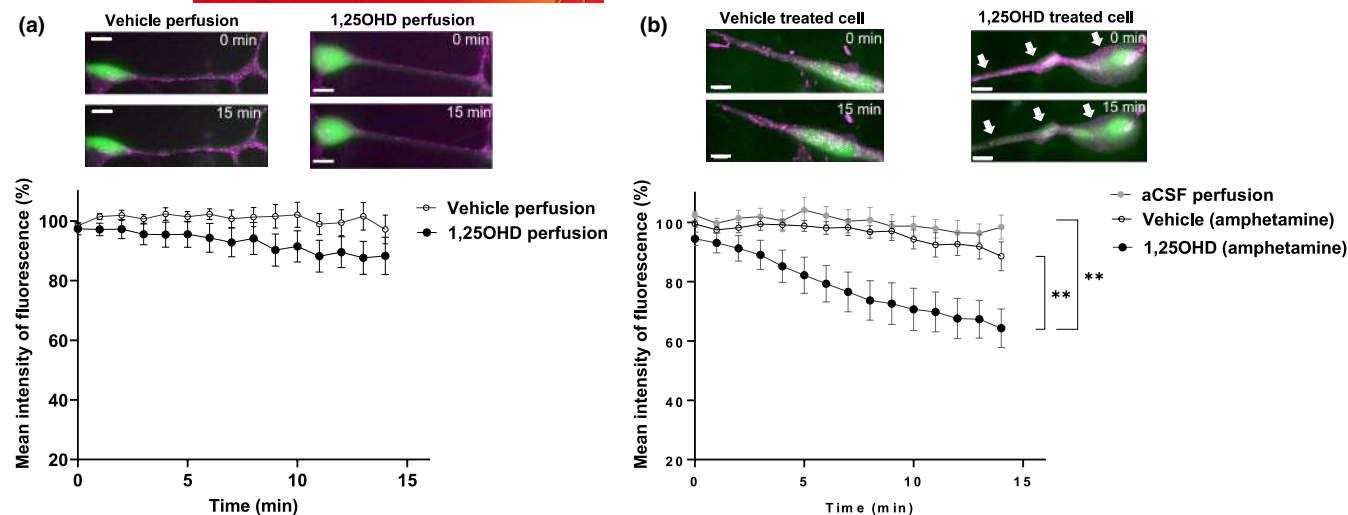


FIGURE 4 Vitamin D increases FFN200 release in TH+ SH-SY5Y/VDR+ cultures. (a) Acute addition of 1,25OHD did not affect the rate of FFN200 release ($-0.104\%/min$ for vehicle vs $-0.754\%/min$ for 1,25OHD) ($t = 1.365$, $p = 0.189$, n vehicle = 10, n 1,25OHD = 12). (b) Culturing SH-SY5Y/VDR+ cells with 1,25OHD increased amphetamine-mediated FFN200 release from TH+ cells compared with those cultured with vehicle or compared with the untreated photobleaching control ($F_{(2,17)} = 10.63$, $p = 0.004$), ($-2.248\%/min$, for 1,25OHD $n = 8$; $-0.380\%/min$ for photobleach control, $n = 5$; $-0.6625\%/min$ for vehicle, $n = 7$). White arrows on TH+ FFN200 containing cells indicate the loss of FFN200 puncta over 15 min. n values represent separate individual neurons from at least four separate cultures. Scale bars: A and B = $15\ \mu m$.

5 | DISCUSSION

Consistent with previous data showing vitamin D is a potent differentiation agent for dopamine neurons here we provide convincing data in multiple experimental systems that 1,25OHD increases TH+ neurite length. We also show 1,25OHD increases TH neuron branching in mesencephalic cultures. In addition, we show 1,25OHD alters synapse distribution and DA production in SH-SY5Y/VDR+; neuroblastoma cells. Although calcitriol has been shown to increase drug-mediated DA release in vivo (Cass et al., 2014; Trinko et al., 2016) here we directly visualise this process for the 1st time in TH+ cells.

6 | VITAMIN D ENHANCES NEURITE OUTGROWTH

Older data suggested that treatment of embryonic rat hippocampal explants with 1,25OHD resulted in increased neurite outgrowth (Brown et al., 2003). Here we extend this to TH+ neurons within mesencephalic explants cocultured with striatal explants and to two other culture systems, cultured rat embryonic ventral mesencephalon neurons and TH+ cells from SH-SY5Y/VDR+; neuroblastoma cells. Our neuroblastomas are largely unipolar, however, primary mesencephalic TH+ cells are multipolar giving us the opportunity to also examine neurite branching which we also show to be increased by culturing cells with 1,25OHD. The molecular mechanisms for these differentiation outcomes could be numerous but one that has been repeatedly suggested is vitamin D's actions in promoting glial-derived growth factor (GDNF) production (Eyles et al., 2003; Orme et al., 2013; Shirazi et al., 2015). GDNF is an

essential factor in DA neuron differentiation (Granholt et al., 2000). Indeed we have shown previously that 1,25OHD increased GDNF mRNA production in these same SH-SY5Y/VDR+ cells along with the GDNF binding partner, the protooncogene tyrosine-protein kinase receptor Ret (C-Ret) at both the mRNA and protein levels (Orme et al., 2013; Pertile et al., 2018). Importantly this same study also showed c-Ret was directly targeted by the 1,25OHD liganded VDR using chromatin immunoprecipitation (ChIP). Consistent with this data, the absence of vitamin D in brain development leads to a reduction in embryonic forebrain c-Ret expression (Pertile et al., 2018). But the evidence for Vitamin D promoting NGF and BDNF also exists (Cui et al., 2021; Eyles, 2021b) which could also contribute to neurite growth.

Alternate epigenetic mechanisms may also be at play. We have just shown a number of microRNAs are over-expressed in embryonic DA neurons individually sorted from developmentally vitamin D-deficient embryonic brains. Many of these microRNAs have a role in suppressing neurite elongation. In particular, we show that when one such microRNA - 181c-5p is over-expressed in TH+ SH-SY5Y/VDR+ cells this leads to truncated neurite extension (Aparecida Nedel Pertile et al., 2022). Whether the addition of 1,25OHD to cultured neurons reverses this process to block the production of such an inhibitory microRNA is unknown.

7 | VITAMIN D ALTERS PRESYNAPTIC PROTEIN DISTRIBUTION

Additionally, it would appear that the addition of 1,25OHD appears to change the distribution and expression of presynaptic proteins involved in synaptic DA release. Using antibodies directed against both

Syp-1 and SV2C as presynaptic markers we show SV2C synapses were clustered closer to the soma than Syp-1. Synapse distribution across the neurite decreased with distance from soma perhaps reflecting the decrease in neurite size. 1,25OHD treatment led to an increase in synaptic puncta number for both synaptic markers. Perhaps this is to be expected given the increased neurite length in 1,25OHD treated TH+ cells. However, with increased neurite length came an increased distal distribution of these synaptic proteins. Sholl analysis showed increased synaptic number/ μm neurite in 1,25OHD treated cells. This was more prominent for SV2C. SV2C is not exclusively expressed in dopaminergic neurons. It is also found in GABAergic cells and in some cholinergic neurons (Dardou et al., 2011). However SV2C is highly expressed in dopaminergic neurons and approximately 75% of TH+ neurons from the mice substantia nigra and VTA strongly co-express SV2C (Dardou et al., 2011). SV2C is expressed in the vesicles of dopaminergic neurons and there is a significant reduction of synaptic release of DA following the genetic deletion of SV2C (Dunn et al., 2017). Nevertheless, our findings regarding the distribution of these presynaptic proteins were not specific to SV2C. We also chose to examine Syp-1 as a non-specific general presynaptic marker protein and show a similar pattern of re-distribution in response to 1,25OHD suggesting a more global differentiation effect. RA increased the immunofluorescence intensity of Syp-1 but had no effect on SV2C. Curiously, while the immunofluorescent intensity of Syp-1 puncta was unaffected by 1,25OHD, this led to a reduction in SV2C puncta intensity. Taken together our data suggest that 1,25OHD alters the overall distribution and perhaps recruitment of presynaptic proteins involved in neurotransmitter release to neurite processes.

8 | VITAMIN D INCREASES DOPAMINE SYNTHESIS AND RELEASE

Here we confirm previous studies (Cui, Pertile, et al., 2015) which have shown that 1,25OHD increases intracellular DA levels and the rate-limiting enzyme in its synthesis TH. Presumably, the increase in the synthetic enzyme is responsible for the increase in intracellular DA. The absence of any 1,25OHD alteration to intracellular NA levels perhaps suggests minimal effects on dopamine beta-hydroxylase which is also expressed in SH-SY5Y cells. An increase in intracellular DA may also necessitate increased DA turnover to prevent reactive oxygen-mediated cell damage. Therefore, the increase in the major metabolic enzyme for DA, MAO-A is consistent with this proposal.

In this study, we also show extracellular DA is increased. This suggests that 1,25OHD may promote DA release. Our findings of increased presynaptic molecules, some of which such as SV2C have been directly implicated in DA release add further weight to this suggestion. However, increased extracellular DA could also simply reflect the excess intracellular DA leaking out of living or dying cells. Although the absence of any increase in extracellular NA levels argues against this later explanation. To investigate this, we took advantage of the recently developed fluorescent false neurotransmitters to visualise whether culturing cells with 1,25OHD

primed TH+ cells to actively release DA in response to a pharmacological challenge or indeed whether 1,25OHD itself could act non-genomically to release DA.

False fluorescent neurotransmitters were developed to directly visualise neurotransmitter uptake (Pereira et al., 2016). Molecules such as FFN200 are fluorescent DA derivatives that are taken up into cells and sequestered by any cell containing the vesicular monoamine transporter VMAT2. To date, FFN200 has been used to visualise DA release in striatal slices, cultured mesencephalic DA neurons, human kidneys and PC12 cells (Black et al., 2021; Hu et al., 2019; Pereira et al., 2016). To the best of our knowledge, our group is the 1st to report the use of this agent in differentiated SH-SY5Y neuroblastoma cells. We chose these cells as we have amassed a substantial amount of data regarding the DA-promoting differentiation effects of 1,25OHD in SH-SY5Y/VDR+ cells (Aparecida Nedel Pertile et al., 2022; Cui, Pertile, et al., 2015; Pertile et al., 2016). However, we note that although FFN200 is readily taken up into these cells, we were not able to pharmacologically release them from SH-SY5Y/VDR+ TH+ cells not exposed to 1,25OHD using amphetamine. It may be possible that the differentiation with 1,25OHD has preferentially affected presynaptic cellular components required for DA release. The increase in Syp-1 and particularly SV2C may be particularly relevant here.

The increased release of FFN200 from TH+ SH-SY5Y/VDR+ cells cultured with 1,25OHD release is independent of uptake. Given that FFN200 is a selective substrate for VMAT2-containing cells our finding that 1,25OHD did not affect VMAT2 expression across all cultured SH-SY5Y/VDR+ cells is again consistent with no alteration in FFN200 uptake. It has been suggested that 1,25OHD itself may act non-genomically via Cav1.2L-Type calcium channels to affect neurotransmitter release (Gooch et al., 2019). SH-SY5Y cells possess such channels, however, we were not able to demonstrate any effect on FFN200 release with 1,25OHD. In contrast, we show a robust increase in FFN200 efflux in TH+ SH-SY5Y/VDR+ cells cultured in the presence of 1,25OHD when DA release was pharmacologically induced. Previously, it has been suggested that only 30% of TH+ varicosities within the striatum contain active zone-like sites required for axonal DA release (Banerjee et al., 2022; Liu et al., 2018). We assessed the rate of pharmacologically induced FFN200 release across the entire TH+ SH-SY5Y/VDR+ cell so could not directly assess whether there were 'silent' synapses as found in vivo. However, we also assessed the FFN200 puncta number (data not shown) and found this declined at a similar rate to FFN200 intensity suggesting that in our model at least, FFN200 release occurred across the cell.

9 | CONCLUSION

The data presented here add to numerous prior investigations outlining the role vitamin D plays in DA neuron differentiation. We demonstrate that 1,25OHD treatment increases neurite outgrowth both in vitro and in two ex vivo models of dopaminergic development as well as increasing DA production along with the synthetic enzymes (TH), and presynaptic release proteins (SV2C)

that may be involved in DA release. Perhaps, most importantly, we also present the first evidence to suggest that differentiating cells with 1,25OHD also promotes the pharmacological release of DA from TH+ cells. DVD deficiency is an epidemiologically proven risk factor for schizophrenia (Eyles et al., 2018; McGrath et al., 2010). The work presented here further suggests developmental variations in vitamin D may subtly alter the trajectory of DA neuron development, supporting the emerging hypothesis that schizophrenia is a developmental dopaminergic disorder (Eyles, 2021a; Eyles et al., 2012). Our results also suggest possible new avenues of research aimed at investigating the connection between vitamin D and SV2C function with respect to the molecular mechanisms underlying the disrupted DA-signalling found in schizophrenia.

AUTHOR CONTRIBUTIONS

RP and RB performed the neurite outgrowth and gene expression experiments; VR and XC performed the dopamine release studies; ZD performed the catecholamine analysis; DE obtained the funding and was responsible for drafting the manuscript. All authors contributed to the writing of the manuscript.

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PEER REVIEW

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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