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Vitamin D_3 enhances ATRA-mediated neurosteroid biosynthesis in human glioma GI-1 cells

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Emerging evidence indicates that vitamin D (VD) is an important modulator of brain development and function. To investigate whether VD modulates neurosteroid biosynthesis in neural cells, we investigated the effect of VD_3 on steroidogenic gene expression in human glioma GI-1 cells. We found that VD_3 enhanced CYP11A1 and 3β-hydroxysteroid dehydrogenase gene expression. The induction of CYP11A1 gene expression by VD_3 was dose- and incubation time-dependent. Calcipotriol, a VD_3 receptor (VDR) agonist, also induced CYP11A1 gene expression in GI-1 cells, indicating that VDR is involved in this induction. The induction of progesterone (PROG) *de novo* synthesis was observed along with the induction of steroidogenic genes by VD_3. Furthermore, VD_3 enhanced all-*trans* retinoic acid (ATRA)-induced CYP11A1 gene expression and PROG production. This suggests cooperative regulation of steroidogenic gene expression by the two fat-soluble vitamins, A and D. In addition, a mixed culture of neuronal IMR-32 cells and GI-1 cells treated with ATRA and VD_3 resulted in the induction of PROG-responsive gene expression in the IMR-32 cells. This result shows a paracrine action of PROG that is induced in and released by the GI-1 cells. The relationship between neurological dysfunction associated with VD deficiency and neurosteroid induction by VD is discussed.

Keywords: ATRA/CYP11A1/glia/neurosteroid/vitamin D.

Abbreviations: ATRA, *all*-trans retinoic acid; PREG, pregnenolone; PROG, progesterone; HSD3B1, 3β-hydroxysteroid dehydrogenase; PGR, PROG receptor; VD, vitamin D.

Introduction

Vitamin D (VD) is a secosteroid hormone that is typically associated with the regulation of phosphocalcic homoeostasis and osteogenesis (*1*, *2*). However, in the past decade a number of publications have highlighted that VD is not only associated with bone disease, but is also associated with non-bone adverse health outcomes, including immunosuppression, cancer, infections and cardiovascular diseases (*1*–*3*). These associations are related to the fact that VD has multiple biological targets on which its effect is mediated by the VD receptor (VDR) that is present in numerous cells (*1*). VD-related effects upon the central nervous system (CNS) have not been as well studied as its effects on other target organs. However, it appears that VD deficiency is associated with neurological dysfunction (*1*, *2*). The impact of VD during the natural development of the CNS during the embryonic period has been studied primarily in rodents (*4*, *5*). In humans, VD deficiency is accompanied by irritability, anxiety, depression, psychosis and defects in mental development (*6*–*8*). From these properties, VD has been recognized as a neuroactive steroid in the last decade. However, the precise molecular mechanism of VD functions in the brain remains to be clarified.

Steroids that are synthesized within the central or peripheral nervous systems have been termed 'neurosteroids' (*9*–*12*). The nervous system is an important site of steroid production within which both neurons and glial cells can synthesize steroids *de novo* from cholesterol. The mitochondrial cytochrome P450scc (CYP11A1), which is the cholesterol side chain cleavage enzyme that catalyses the *de novo* synthesis of pregnenolone (PREG), is expressed throughout the rodent brain (*13*–*15*). The 3β-hydroxysteroid dehydrogenase (HSD3B1), which converts PREG to progesterone (PROG), is also largely distributed throughout the brain and spinal cord (*16*, *17*). In addition, primary cultures of mixed glial cells can metabolize cholesterol to PREG and PROG (*18*, *19*). However, the molecular basis of the regulation of neurosteroid synthesis is not fully understood.

Previously, we have reported that vitamin A can induce neurosteroid biosynthesis in human glioma GI-1 cells, by induction of steroidogenic genes (*20*). Since VD has been associated with neuronal functions in addition to vitamin A, we initiated an investigation of the functional role of VD in neurosteroid biosynthesis in the CNS, using cultured human neural cells.

Materials and Methods

Reagents

1α, 25-dihydroxy vitamin D_3 (VD_3) and calcipotriol hydrate were purchased from Cayman Chemical Company (Ann Arbor, MI, USA). All-*trans* retinoic acid (ATRA) was purchased from Wako Pure Chemicals (Tokyo, Japan). PREG and PROG were obtained from Sigma (St. Louis, MO, USA). GI-1

and IMR-32 cells were obtained from the Riken cell bank (Tsukuba, Japan).

Cell culture

GI-1 is a human glial cell line established from a tumour specimen removed from the left frontoparietal region of a 61-year-old man and IMR-32 is a cell line derived from a human neuroblastoma. Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 10 U/ml penicillin and 10 U/ml streptomycin at 37°C in a humidified atmosphere of 5% CO_2. For vitamin treatments, exponentially growing cells were split into six-well plates 3×10^5 cells/well and cultured for 4–5 days in medium supplemented with 10% FBS. This growth medium was exchanged for DMEM containing 5% charcoal-treated serum and vitamins at various concentrations in 0.1% dimethyl sulfoxide (DMSO). For mixed culture experiments, both GI-1 and IMR-32 cells were seeded into six-well plates (1.5×10^5 cells each/well) and cultivated for 3 days, and used for the treatment with vitamins.

Real-time polymerase chain reaction (PCR)

Total RNA was isolated using the guanidium thiocyanate phenol-chloroform extraction method. First strand cDNA was synthesized from 5 μg of total RNA using 100 U of reverse transcriptase (ReverTra Ace, TOYOBO, Tokyo, Japan) and random primers, according to the manufacturer's protocol. PCR was then carried out, using this synthesized cDNA as a template, with Taq polymerase (GoTaq, Promega, Madison, WI, USA). The amplification was carried out for 30 cycles of 1 min at 95°C, 1 min at 58°C and 1 min at 72°C. Quantitative real-time-PCR was performed using an ABI-Prism 7,300 thermal cycler and a SYBR green PCR reagent kit (Roche Diagnostics K. K., Tokyo, Japan). Ct (cycle at which threshold fluorescence is reached) values for each sample were then collected at a threshold level of fluorescence set within the linear phase of amplification. Calculations of the initial amounts of mRNA were performed according to the cycle threshold method (*21*). The mRNA levels were normalized using the 18 S rRNA levels, which had been quantified by real time-PCR. The PCR primers used to amplify the steroidogenic cDNAs were designed from published DNA sequences using Primer Express *ver*. 3.0 (Applied Biosystems, Foster City, CA, USA). The sequences of the primers used are as follows; 18 S rRNA, forward 5′-TGG TTG CAA AGC TGA AAC TTA AAG-3′ and reverse 5′-AGT CAA ATT AAG CCG CAG GC-3′; StAR, forward 5′-CCA CCC CTA GCA CGT GGA T-3′ and reverse 5′-ATT GTC CTG CTG ACT CTC CTT CTT-3′; CYP11A1, forward 5′-AGG AGG GGT GGA CAC GAC-3′ and reverse 5′-TTG CGT GCC ATC TCA TAC A-3′; AKR1C1, forward 5′-CCT GTG AGG GAG GAA GAA AGA A-3′ and reverse 5′-CCA ACC TGC TCC TCA TTA TTG TAT AA-3′; AKR1C3, forward 5′-TAA AGC CAG GTG AGG AAC TTT CA-3′ and reverse 5′-GGA TGA CAT TCT ACC TGG TTG CA-3′; SDR5A1; forward 5′-GCC ATG TTC CTC GTC CAC TAC-3′ and reverse 5′-TTA ACC ACA AGC CAA AAC CTA TTA GA-3′; HSD3B1, forward 5′-TCA TCC GCC TCT TGG TGA AG-3′ and reverse 5′-AGC ACT GTC AGC TTG GTC TTG TT-3′.

Radiolabelling of steroid hormones and analysis by thin layer chromatography (TLC)

To label cholesterol and steroid molecules, 1 mCi/ml of [1−^{14}C]acetic acid sodium salt (57 mCi/mmol, Moravek Biochemicals, Brea, CA, USA) was added to the culture for 24 h. The culture medium was then collected and steroids were extracted as described previously (*22*). Briefly, an equal volume of ethyl acetate:isooctane (1:1 v/v) was added to the media. This mixture was then centrifuged and the upper organic phase was collected. The extraction procedure was repeated twice and the organic phase was dried and resuspended in 20 μl ethyl acetate. The steroids (5 μl) were separated on silica-gel 60 F_{245} plates (Merck, Darmstadt, Germany) with chloroform:ethyl acetate (4:1) as the mobile phase. The radioactive lipids on the TLC plate were visualized using an FLA7000 device (Fuji Film, Tokyo, Japan). A non-radioactive standard mixture was included on the plate and visualized by UV or phosphomolybdic acid. The migration of standard steroids was used to identify the radioactive steroids on the same plate.

Statistical analysis

Data were statistically analysed using Student's *t*-tests. For the multiple comparisons, data were analysed using Dunnett's multiple comparison tests. Results were considered significant when $P < 0.05$.

Results

Induction of the CYP11A1 gene by VD_3 in GI-1 cells

We previously reported that ATRA induces neurosteroid biosynthesis in GI-1 cells via activation of several steroidogenic genes such as *StAR, CYP11A1* and *HSD3B1,* and that this activation is mediated by retinoic acid receptor (RAR) and retinoid X receptor (RXR) (*20*). Because VD has been shown to affect CNS functions (*4–7*) and RXR, which is activated by 9-*cis* retinoic acid, is known to crosstalk with VDR (*23*), we investigated the possible involvement of VD in the regulation of steroid biosynthesis in neural cells. We measured the effect of VD_3 on the expression of genes involved in steroid biosynthesis (Fig. 1) using real-time PCR analysis. We found that CYP11A1 and HSD3B1 genes were induced by VD_3 in GI-1 cells (Fig. 2A). As the reaction catalysed by CYP11A1 is recognized as the first and rate-limiting step of steroid biosynthesis in the classical steroidogenic tissues, we focused on the effect of VD_3 on CYP11A1 expression. The level of CYP11A1 gene induction by VD_3 was comparable with that induced by ATRA (1.8-fold versus 3- to 5-fold). In contrast, the level of HSD3B1 induced by VD_3 was very low compared with that previously reported to be induced by ATRA (1.9-fold versus 50-fold) (*20*). The induction of the CYP11A1 gene was VD_3 dose- and incubation

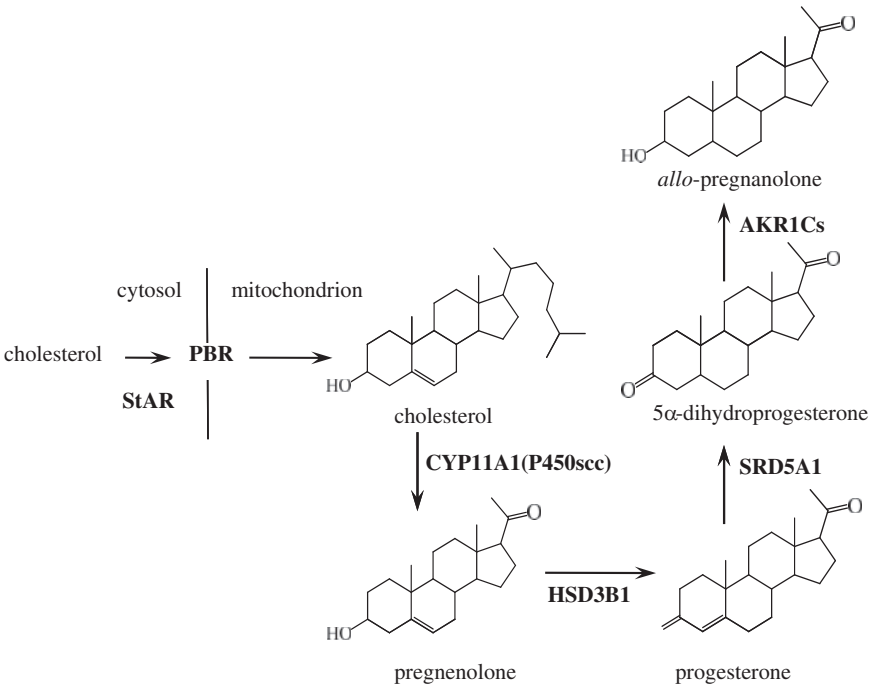

Fig. 1 Steroid biosynthetic pathway. StAR, steroidogenic acute regulatory protein; PBR, peripheral-type benzodiazepine receptor; SRD5A, steroid 5α-reductase; AKR1C, aldo-keto reductase 1 C.

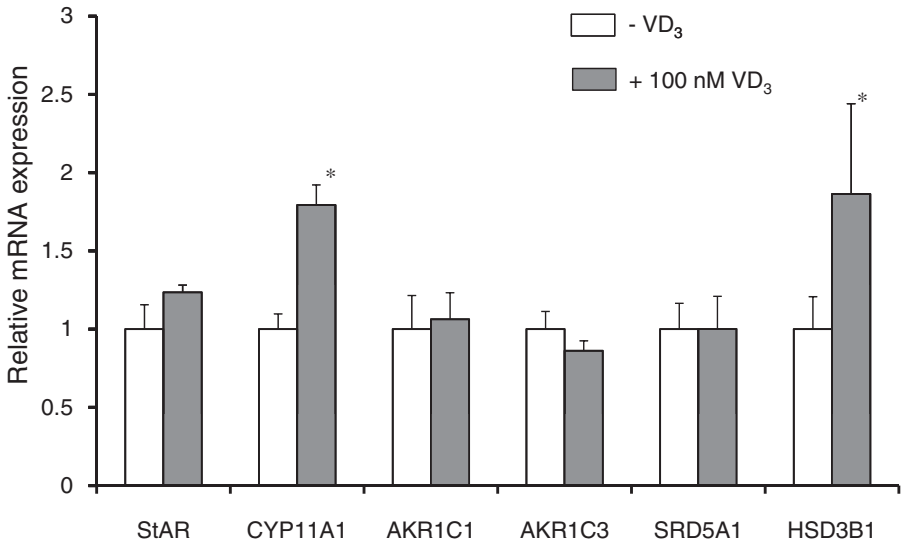

Fig. 2 Effect of VD_3 on the expression of steroidogenic genes in GI-1 cells. The cells were treated with or without 100 nM VD_3 for 48 h and the expression of steroidogenic genes was measured using real-time PCR analysis. $*P<0.05$ indicates a significant difference compared with the negative control (DMSO). ($n=3$). StAR, steroidogenic acute regulatory protein; SRD5A, steroid 5α-reductase; AKR1C, aldo-keto reductase 1 C. Data are presented as the mean ± SD.

time-dependent as shown in Fig. 3A and B, although the values varied at 10 nM. Calcipotriol, an agonist for VDR, was also able to induce the same level of CYP11A1 gene expression in GI-1 as VD_3, indicating that VDR is involved in the induction of this gene (Fig. 3C).

VD_3 enhanced the de novo biosynthesis of neurosteroids in GI-1 cells

We next measured the *de novo* biosynthesis of neurosteroids in the VD_3-treated GI-1 cells by the method as previously described (*20*). GI-1 cells were treated with VD_3 for 48 h and [1−^{14}C]acetic acid was then added to the culture medium for 24 h prior to collection of the medium. Radiolabelled steroids were extracted from the culture media samples, separated by TLC and visualized. As shown in Fig. 4A, in addition to cholesterol, low but significant levels of PREG and PROG were synthesized and secreted into the culture medium. Following the addition of VD_3, the *de novo* synthesis of PROG increased, whereas the synthesis of PREG and cholesterol did not change. The level of production

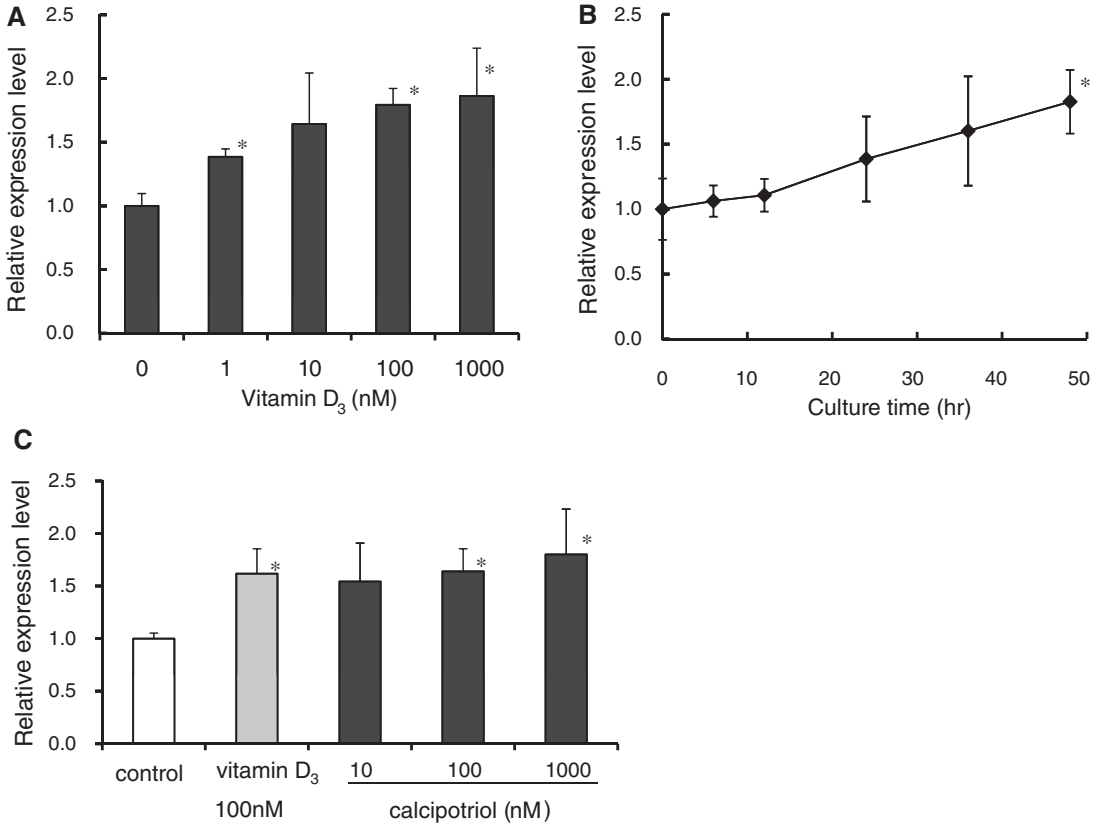

Fig. 3 Effect of VD_3 on the expression of the CYP11A1 gene in GI-1 cells. (A) GI-1 cells were treated with various concentrations of VD_3 (0, 1, 10, 100, 1000 nM) for 48 h and the expression of CYP11A1 was monitored using real-time PCR. $^*P<0.05$ indicates a significant difference compared with the negative control (DMSO). ($n=3$). (B) The cells were treated with 100 nM VD_3 for various times up to 48 h. The expression of CYP11A1 was monitored using real-time PCR. $^*P<0.05$ indicates a significant difference compared with time =0; ($n=3$) (C) Effect of calcipotriol on the expression of the CYP11A1 gene in GI-1 cells. The cells were treated with VD_3 or calcipotriol for 48 h and the expression of CYP11A1 was monitored using real-time PCR. $^*P<0.05$ indicates a significant difference compared with the negative control (DMSO), ($n=3$). Data are presented as the mean ± SD.

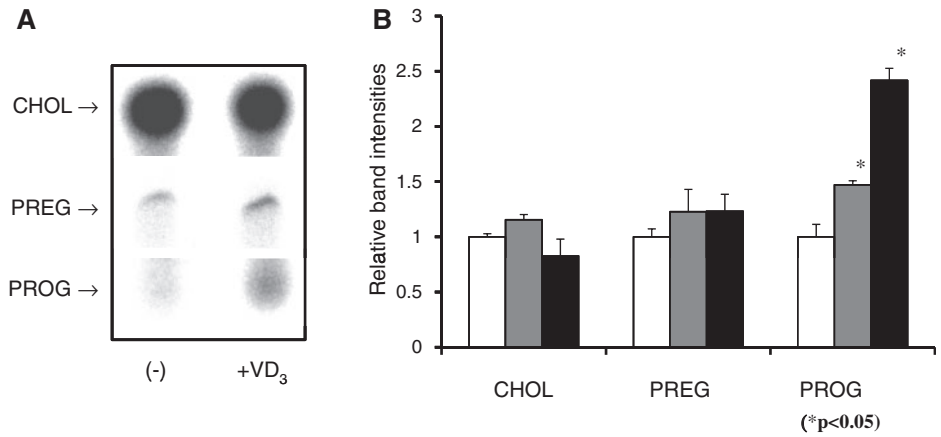

Fig. 4 Induction of neurosteroid *de novo* synthesis in GI-1 cells by exposure to VD_3. (A) GI-1 cells were treated with VD_3 (0, 100 nM) for 48 h. The ^{14}C-labelled steroids in the culture medium were extracted with ethyl acetate:isooctane (1:1 v/v) and separated by TLC. The radioactivity on the TLC plate was visualized using a FLA7000. Arrows indicate the positions of the steroid standards. (B) The intensities of the spots corresponding to CHOL, PREG and PROG were quantified using an image analyser. Open bars: $VD_3=0$ nM; grey bars: $VD_3=10$ nM; black bars: $VD_3=100$ nM. The results are presented using arbitrary units, with the control values set at 1. The values are the mean ± SD of three independent labelling experiments. $^*P<0.05$ indicates a significant difference compared with the negative control (DMSO), ($n=3$). CHOL, cholesterol.

of these steroids was quantified based on the band intensities, and the index of induction relative to the non-treated control was calculated (Fig. 4B). The combined results indicated that VD_3 enhanced the *de novo* synthesis of PROG via the upregulation of steroidogenic genes in GI-1 cells. The increase in the *de novo* synthesis of PROG almost paralleled to the expression levels of the steroidogenic genes that

are induced by VD_3. However, the levels of PREG production did not change as much as those of PROG by VD_3.

VD_3 enhanced ATRA-induced neurosteroid synthesis in GI-1 cells

To determine if VD_3 affects ATRA-induced neurosteroid biosynthesis in GI-1 cells, we measured the effect of VD_3 on ATRA-induced CYP11A1 gene expression. As shown in Fig. 5A, the fold increased in CYP11A1 gene expression that was mediated by 10 μM ATRA (1.9-fold versus control) was increased to 3.6-fold by the addition of 100 nM VD_3. The *de novo* production of PREG and PROG was also enhanced by the combination of ATRA and VD_3, compared with the effect of either agent alone, as shown in Fig. 5B.

Paracrine action of the PROG induced in GI-1 cells towards neuronal IMR-32 cells

To elucidate a possible physiological function of the PROG that was induced by ATRA and/or VD_3 in GI-1 cells, we set up a mixed culture of human neuronal IMR-32 cells and GI-1 cells. The IMR-32 cell line is derived from a human neuroblastoma and it does not express major steroidogenic genes such as CYP11A1 and HSD3B1, even when treated with 100 nM VD_3 and/or 10 μM ATRA (data not shown). To evaluate the effect of PROG produced in GI-1 cells on neuronal IMR-32 cells, we analysed PROG induction of the PROG receptor (PGR) gene, which encodes a receptor responsible for PROG hormonal actions. As shown in Fig. 6A, in IMR-32 cells, PGR expression is controlled by PROG binding to the PGR, which is inhibited by RU-486, an antagonist of the PGR (*24*). Because GI-1 cells do not express the PGR gene (Fig. 6B), it is possible to monitor the effect of PROG on PGR gene expression in IMR-32 cells even when co-cultured with GI-1 cells. Expression of the PGR gene was induced by addition of ATRA and/or VD_3 to the mixed cell culture and it was inhibited by RU-486 (Fig. 6C). Comparison of the level of induction of the PRG gene in Fig. 6C with those induced by different amounts of PROG (Fig. 6A), indicates that the concentration of PROG in the media of cells treated with 10 μM ATRA and 100 nM VD_3 was almost equivalent to 10 nM. This concentration of PROG is equivalent to that of the plasma concentration of PROG during ovulation days in human females (*25*).

Discussion

In this report, we demonstrated that VD_3 is able to induce neurosteroid biosynthesis in human glioma GI-1 cells via induction of the two steroidogenic genes, CYP11A1 and HSD3B1, and the induction is mediated by VDR. Immunochemical studies revealed that VDR are widespread in the brain and the spinal

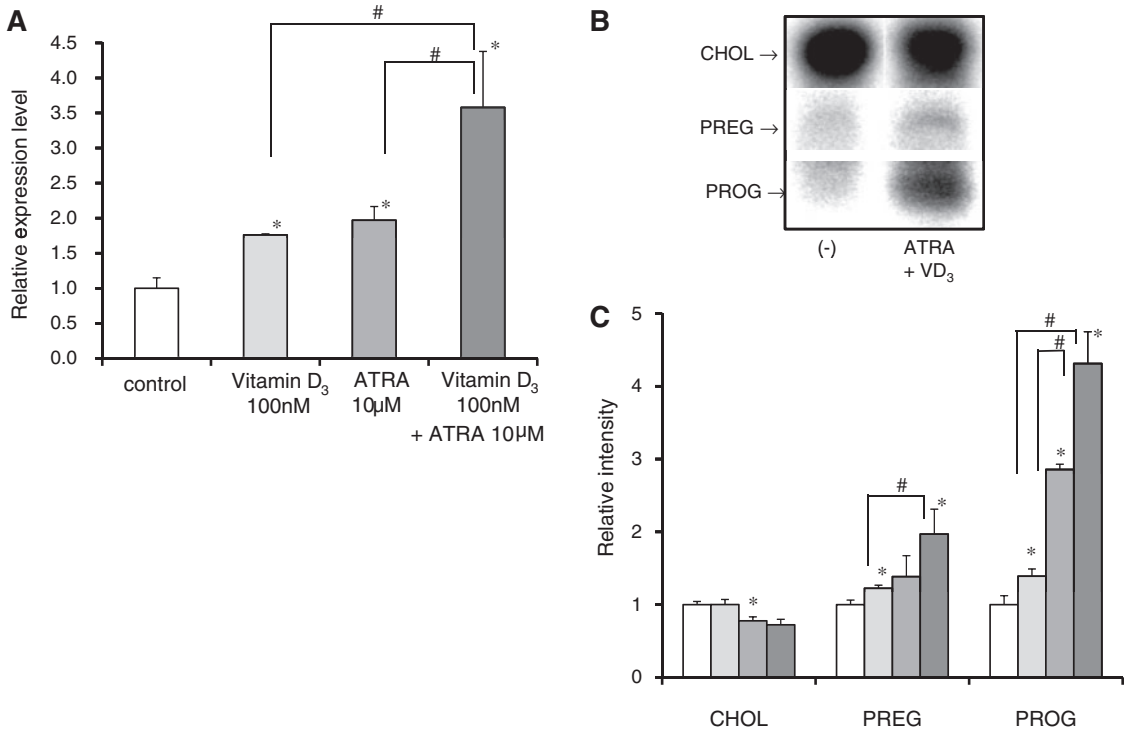

Fig. 5 Effect of VD_3 on ATRA-induced neurosteroidogenesis in GI-1 cells. (A) The cells were treated with 100 nM VD_3 and /or 10 μM ATRA for 48 h and the expression of the CYP11A1 gene was measured using real-time PCR. $^*P<0.05$ indicates a significant difference compared with the negative control (DMSO); ($n=3$). (B) After treatment of the cells together with incubation with ^{14}C-acetic acid for 48 h, the ^{14}C-labelled steroids in the culture medium were extracted and separated by TLC. The radioactivity on the TLC plate was visualized using a FLA7000. (C) The intensities of the spots corresponding to CHOL, PREG and PROG were quantified using an image analyser. Open bars: control (DMSO); light grey bars: 100 nM VD_3; mid-grey bars: 10 μM ATRA; dark grey bars: VD_3 and ATRA. $^*P<0.05$ indicates a significant difference compared with the negative control (DMSO). $^{\#}P<0.05$ indicates a significant difference between the indicated bars; ($n=3$) Data are presented as the mean ± SD.

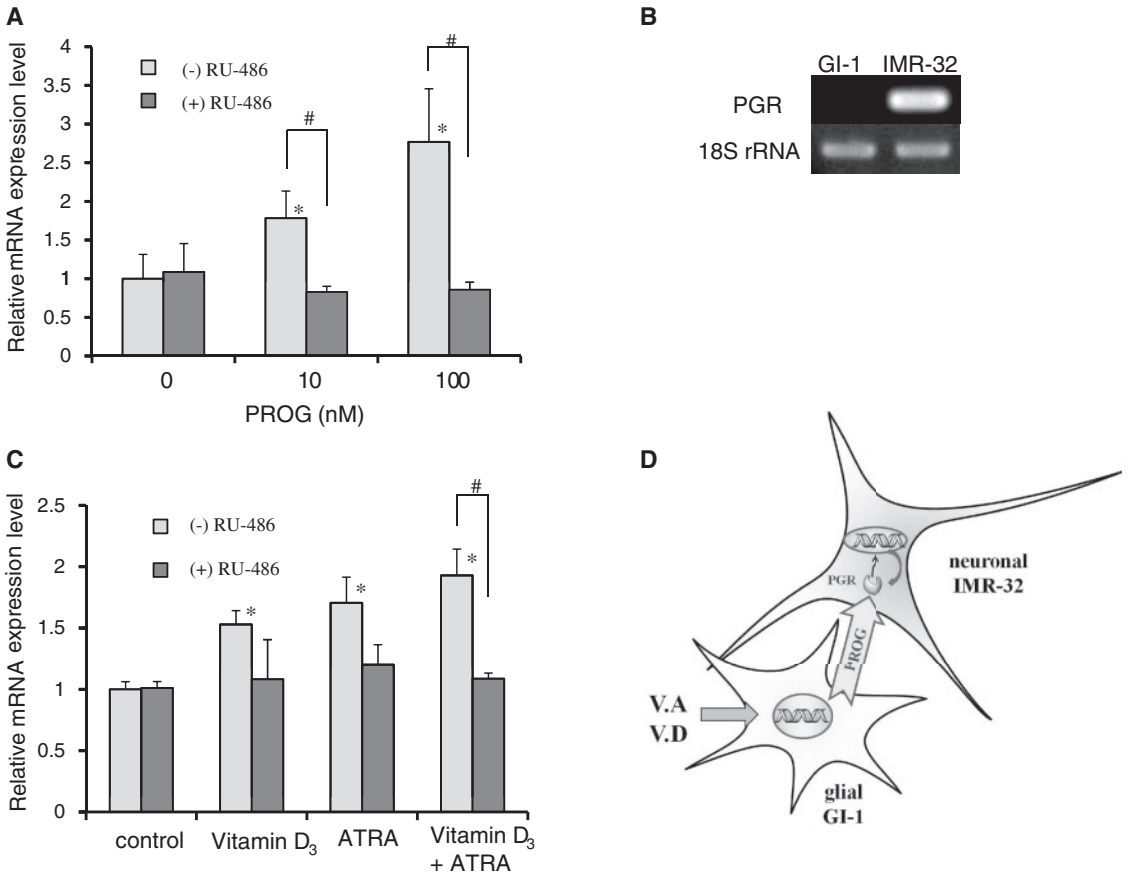

Fig. 6 Paracrine action of the induced PROG on the expression of the PGR gene in IMR-32 cells. (A) IMR-32 cells were treated with PROG with or without 1 μM RU-468, an antagonist of the PGR, for 48 h and the expression of the PGR gene was monitored using real-time PCR. (B) PCR analysis of PGR gene expression in GI-1 and IMR-32 cells. No induction of the PGR gene expression was observed in both cells, following VD_3 and/or ATRA treatment (data not shown). 18 S rRNA was used as a loading control. (C) A mixed culture of GI-1 and IMR-32 cells was incubated for 3 days. The cells were then treated with 100 nM VD_3 and/or 10 μM ATRA with or without 1 μM RU-486 for 48 h and expression levels of PGR gene were measured. $^{*}P < 0.05$ indicates a significant difference compared with the negative control (DMSO). $^{\#}P < 0.05$ indicates a significant difference between the indicated bars; ($n = 3$) Data are presented as the mean ± SD. (D) Schematic representation of the mixed culture of GI-1 and IMR-32 cells.

cord (*26*, *27*). Increased grooming behaviour and anxiety in VDR-null mice are reported (*28*, *29*). VD deficiency has been reported to be accompanied by irritability, anxiety, depression, psychoses and defects in mental development (*7*, *30*). It has also been reported that control of neurosteroid concentrations is deregulated in depressed patients (*31*) and that VD deficiency is prevalent in patients with Parkinson's and Alzheimer's diseases (*32*). All of these previous studies suggest a close relationship between a reduction in neurosteroids and VD deficiency. Together with these evidence, our data suggest that VD_3 modulates the neurosteroid production in neural cells, thereby, affects the CNS functions. It should be noted that in this study we used a cell line isolated from human glioma as a glial cell model. It is not clear whether the results are applicable to the glia in CNS. To evaluate our results, further studies using other neural cells and animal studies are necessary. Measurements of neurosteroids in the brain of VDR-null mice will clarify the relationship between the VD/VDR system and the neurosteroid production and function in the CNS.

We previously demonstrated that ATRA induces neurosteroid synthesis in GI-1 cells via induction of several steroidogenic genes mediated by RXR (*20*). In the current study we found that VD_3 enhances ATRA-mediated induction of CYP11A1 gene in GI-1 cells (Fig. 5A). This is quite possible because activated VDR can associate with RXR to form heterodimers, in addition to forming VDR homodimers (*33*). Our data suggest the existence of binding sites for these receptors in the promoter regions of the induced genes, although the precise locations are not clear. In classical endocrine tissues, the nuclear protein steroidogenic factor 1 (SF-1) plays a central role in regulating steroidogenic genes, however, no expression of SF-1 gene was observed in GI-1 cells (*20*). The lack of SF-1 expression in rat glial cells has also been reported (*34*). Furthermore, Chiang *et al.* (35) reported that the SF-1 binding site is not essential for CYP11A1 promoter function in the developing mouse brain, and that upstream SF-1 binding site (−3.8 to −2.7 kb) is essential for the promoter function. These data thus suggests that ATRA and VD_3 coordinately regulate the expression of CYP11A1 gene in neural cells, in a manner that differs from that in classical steroidogenic tissues. Identification of responsible binding sites for the VDR/RXR in the upstream of the CYP11A1 promoter

is needed to understand the regulation of CYP11A1 gene expression by these vitamins.

The induction of the two steroidogenic genes, CYP11A1 and HSD3B1, resulted in the elevation of PROG production in the VD_3 and/or ATRA treated cells (Figs. 4 and 5). However, the level of PREG did not change as much as that of PROG. This suggests that HSD3B1 catalyses more rapidly than CYP11A1 in the GI-1 cells. This suggests that the regulation of HSD3B1 expression is a key step for the overall production of following neurosteroids, although CYP11A1 activity is recognized as the first and rate-limiting step of steroid biosynthesis in the classical steroidogenic tissues such as adrenals and gonads. The fact that the HSD3B1 inhibitor trilostane shows antidepressant properties in mice might be related to this property of HSD3B1 activity (*36*). Precise analysis of the regulation of HSD3B1 expression by vitamins A and D in neural cells must be performed in future.

The mixed culture study (Fig. 6) demonstrated that, in a human glioma, ATRA and VD_3 co-operatively act to induce PROG production, which can exert a paracrine action on neighbouring neuronal cells. The concentration of PROG produced in GI-1 cells was within the range of physiological concentrations. There is increasing evidence indicating that PROG, besides its effects on gonadal functions, has profound psychotropic effects (*37*, *38*). At the cellular level, the effect of PROG on neurotransmitter release has been examined in a number of studies (*37*), and a large number of studies indicate that PROG exhibits neuroprotective properties (*38–41*). Impaired motor performance in mice lacking VDR has been reported (*42*) and this might be related to the VD_3 function to produce PROG in glial cells. This action of VD_3 on neural cells might be closely correlated to pleiotropic neuronal actions of VD.

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Conflict of interest

None declared.

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