

Accepted Manuscript

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PII: S0960-0760(17)30256-X
DOI: <http://dx.doi.org/10.1016/j.jsbmb.2017.09.008>
Reference: SBMB 5025

To appear in: *Journal of Steroid Biochemistry & Molecular Biology*

Received date: 8-5-2017
Revised date: 1-9-2017
Accepted date: 8-9-2017

Please cite this article as: V erena Landel, Delphine Stephan, Xiaoying Cui, Darryl Eyles, Franois Feron, Differential expression of vitamin D-associated enzymes and receptors in brain cell subtypes, *Journal of Steroid Biochemistry and Molecular Biology* <http://dx.doi.org/10.1016/j.jsbmb.2017.09.008>

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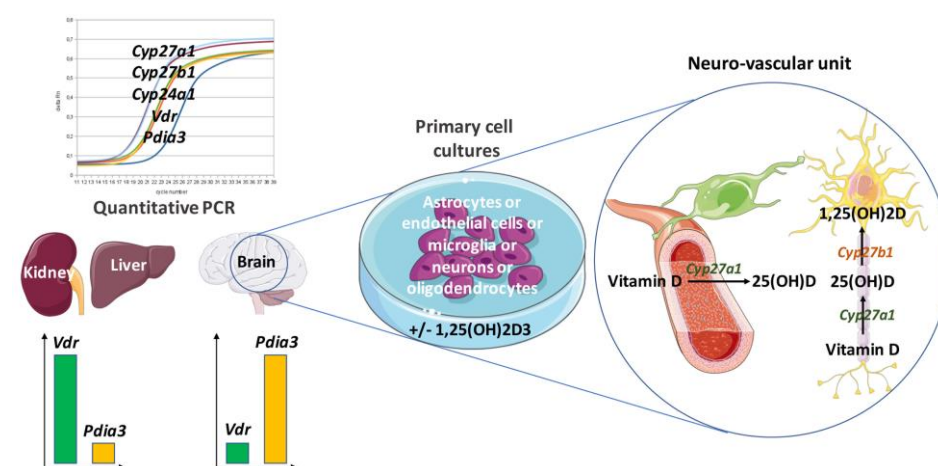
Differential expression of vitamin D-associated enzymes and receptors in brain cell subtypes

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Graphical abstract



HIGHLIGHTS

- In comparison to the kidney and liver, the brain produces low levels of the transcripts coding for the enzymes *Cyp27a1*, *Cyp27b1*, *Cyp24a1*.
- High levels of *Pdia3* mRNA are found in cortex and hippocampus compared to kidney and liver.
- The transcript coding for *Vdr*, the genomic vitamin D receptor, is mainly expressed in astrocytes.
- The strongest expression of *Pdia3* mRNA is observed in neurons, astrocytes and endothelial cells.

- An increased production of Cyp24a1 is found in microglia and endothelial cells after treatment with 1,25(OH)₂D₃.
- In the brain, Pdia3 may be considered as the main vitamin D receptor.

Abstract

Accumulating evidence indicates that the active form of vitamin D, 1,25(OH)₂D₃, can be considered as a neurosteroid. However, the cerebral expression of vitamin D-associated enzymes and receptors remains controversial. With the idea of carrying out a comparative study in mind, we compared the transcript expression of *Cyp27a1*, *Cyp27b1*, *Cyp24a1*, *Vdr* and *Pdia3* in purified cultures of astrocytes, endothelial cells, microglia, neurons and oligodendrocytes. We observed that endothelial cells and neurons can possibly transform the inactive cholecalciferol into 25(OH)D₃. It can then be metabolised into 1,25(OH)₂D₃, by neurons or microglia, before being transferred to astrocytes where it can bind to VDR and initiate gene transcription or be inactivated when in excess. Alternatively, 1,25(OH)₂D₃ can induce autocrine or paracrine rapid non-genomic actions via PDIA3 whose transcript is abundantly expressed in all cerebral cell types. Noticeably, brain endothelial cells appear as a singular subtype as they are potentially able to transform cholecalciferol into 25(OH)D₃ and exhibit a variable expression of *Pdia3*, according to 1,25(OH)₂D₃ level. Altogether, our data indicate that, within the brain, vitamin D may trigger major auto-/paracrine non genomic actions, in addition to its well documented activities as a steroid hormone.

Keywords: brain, vitamin D, 1,25(OH)₂D₃, limiting enzymes, receptors, cell types, rat

Introduction

For the past two decades, vitamin D has been increasingly recognized as a potent neurosteroid with multiple actions in the brain (1,2). Based on the well-described endocrine actions of vitamin D, it is metabolized to its hormonal form 1,25(OH)₂D₃ in various organs

such as the gut and kidney and, in this form, it enters the brain via the blood brain barrier to act directly on cells containing its nuclear receptor, the vitamin D receptor (VDR) (3). However, it appears now also likely that an autocrine/paracrine system within the nervous system underlies the cerebral actions of vitamin D in brain. In the latter case, the presence of the various key enzymes and receptors is a prerequisite.

A number of studies have acknowledged the presence of enzymes and receptors across different brain regions and within the various cerebral cell types (4,5,6,7,8,9). Although there is evidence for VDR presence in the mammalian brain, there is still some dispute about the cellular and subcellular localization of this receptor within the nervous system. This debate is in part based on the work by DeLuca's group claiming the absence of VDR protein in the brain, questioning the technical tools at our disposal (10). In a recent study, we provide unambiguous evidence, using mass spectrometry, that VDR is present in the rodent brain, however it is in significantly lower amounts than in reference tissues such as the gut and kidney (11).

Although several studies demonstrated the presence of VDR and/or certain enzymes in various cell-types of the nervous system, no single study compared the relative expression of VDR, CYP450 enzymes CYP27a1, CYP27b1 and CYP24a1 among all these cell types. Protein disulfide isomerase family member3 (PDIA3), also known as 1,25 D3-MARRS or ER57, was identified as a membrane receptor of vitamin D (12). Given the relatively low abundance of VDR in the brain it may be possible that a membrane receptor plays a more important role in vitamin D signaling in this organ.

No study so far has examined the cellular distribution of metabolic enzymes and receptors in the brain. This is crucial in understanding the nature of vitamin D processing and action in this tissue. Ideally, fractionation of brain tissues followed by mass spectrometry would help with this issue. However, given the extremely low amounts of VDR previously

shown to be present, this approach would require extremely high amounts of starting material. We therefore decided to analyze transcript expression in cultured individual brain cells (astrocytes, endothelial cells, microglia, neurons, oligodendrocytes) of enzymes and receptors implicated in vitamin D metabolism.

Materials and methods

Organ collection

Livers, kidneys, cortex and hippocampus were collected from three adult Wistar rats and frozen at -80°C before being used for RNA extraction and qPCR.

Primary cell cultures

Neurons

Cells were prepared from the cerebral cortex or hippocampus of E18 Wistar rats, as previously described (13). Briefly, embryos were placed on ice and their brains were quickly removed aseptically. Blood vessels and meninges were discarded. Cerebral cortices and hippocampi were collected separately in Hanks' balanced salt solution, dissociated with trypsin and plated at a density of 1.2×10^5 cells/cm² on poly L-lysine coated coverslips. Neurons were cultured in Neurobasal medium supplemented with 2% B-27, 1% penicillin-streptomycin, and 0.3% glutamine in a humidified atmosphere containing 5% CO₂ at 37°C for 2 weeks. Cells were then exposed to 20 nM 1,25(OH)₂D₃ or 20 nM EtoH as a vehicle for 12 hours prior to RNA extraction (see below).

Astrocytes

Astrocytes were prepared from 2-day old newborn rat cerebral cortices, as described before (14). After removal of the meninges, the brain tissue was dissociated with trypsin. Dissociated glial cells were seeded into cell culture flasks and the culture medium (Dulbecco's Modified Eagle's Medium supplemented with 10% fetal bovine serum, 100 units/mL antibiotic penicillin, and 100 µg/mL streptomycin) was replaced three times a week. After 1 week of

proliferation, the glial cells were gently shaken for 24 hours to remove the microglial cells. After 3 weeks of culture, astrocytes were shaken once again overnight before treatment with 1,25(OH)2D3. Cells were then exposed to 20 nM 1,25(OH)2D3 or 20nM EtoH as a vehicle for 12 hours prior to RNA extraction (see below).

Microglia

Mixed glial cells were obtained as described for astrocyte cultures without shaking. After 3 weeks of culture, microglia were isolated with gentle trypsin 0.25% in DMEM F12 for 20-45 minutes until the intact layer of astrocytes was fully detached. Microglial cells that had remained attached were then kept overnight before treatment with 1,25(OH)2D3 and RNA extraction.

Brain endothelial cells

Brain endothelial cells were prepared from 6 week-old male Wistar rats as previously described (15). Briefly, cells were seeded on type IV collagen and fibronectin and maintained in DMEM/Ham's F12 supplemented with 20% bovine platelet-poor plasma-derived serum named Endothelial Cell Media, composed of bFGF (2 ng/mL), heparin (100 µg/mL; Life Technologies), gentamicin (50 µg/mL; Life Technologies) and HEPES (4-[2-hydroxyethyl]-1-piperazineethanesulfonic acid; 2.5 mM; Life Technologies) for 1 week before treatment with 1,25(OH)2D3 and collection.

Oligodendrocytes

Oligodendrocytes were prepared as previously described (16). Briefly, cortical precursors were isolated from E15.5. Oligodendrocyte precursors were enriched in serum-free oligodendrocyte growth medium supplemented with bFGF (20ng/ml) and PDGF-AA. Cells were then cultured in oligodendrocyte differentiation medium without mitogens for 10 days before treatment and collection.

Vitamin D treatment

1,25(OH)2D3 was resuspended in 100% EtoH and kept away from light at -20°C. Each cell culture was treated with either 20nM 1,25(OH)2D3 or ethanol for 12h and then collected for RNA extraction.

RNA extraction

Total RNA was isolated from the purified cell types using RNeasy Mini kit (Qiagen, Courtaboeuf, France), according to the manufacturer's instructions. RNA concentration was determined using a Nanodrop 2000 spectrophotometer (Life Technologies ThermoFisher Scientific, Villebon sur Yvette, France) and RNA integrity assessed on an Agilent 2100 Bioanalyzer (Agilent Technologies, Les Ulis, France).

qPCR

Reverse transcription was performed on 1µg of RNA using the high capacity RNA-to-cDNA mix (applied biotechnologies). qPCR was performed on 25ng of cDNA using the following primers:

<i>Hprt</i>	Forward	GCTCGAGATGTCATGAAGGAGA
	Reverse	TCAGCGCTTTAATGTAATCCAGC
<i>Beta actin</i>	Forward	CACTATCGGCAATGAGCGGT
	Reverse	ATTTGCGGTGCACGATGGA
<i>Cyp27a1</i>	Forward	GGAAGGTGCCCCAGAACAA
	Reverse	GCGCAGGGTCTCCTTAATCA
<i>Cyp27b1</i>	Forward	GAGATCACAGGCGCTGTGAAC
	Reverse	TCCAACATCAACACTTCTTTGATCA
<i>Cyp24a1</i>	Forward	TGGATGAGCTGTGCGATGA
	Reverse	TGCTTTCAAAGGACCACTTGTTT
<i>Vdr</i>	Forward	TGACCCACCTACGCTGACT
	Reverse	CCTTGGAGAATAGCTCCCTGTACT
<i>Pdia3/Marrs/Erp57</i>	Forward	ATTGTCTGACTTTGGCTTAG
	Reverse	CAGCATTCACTATGTCATCA

Primers purchased from IDT were resuspended in water as indicated by the manufacturer to obtain 100µM concentrations of each primer. For qPCR, primers were used at a final concentration of 500nM (2µl of reverse and 2µl of forward primer was resuspended in 250ul H₂O). 10µl of iTaq universal SYBR green master mix was mixed with 5µl of 500nM primers

and added to 5 μ l cDNA in 96 well plates. For the comparisons between tissues and cell types, expression levels were calculated using the Δ Ct method where the expression level of the mRNA of interest is given by $2^{-\Delta\text{CT}}$ with $\Delta\text{CT} = \text{CT target mRNA} - \text{CT reference mRNA}$ (*Hprt*), in the same sample. For the cultures treated with 1,25(OH)2D3, relative expression levels were determined according to the $\Delta\Delta$ Ct method where the expression level of the mRNA of interest is given by $2^{-\Delta\Delta\text{CT}}$ where $\Delta\Delta\text{CT} = \Delta\text{Ct}_i$ for treated cultures - ΔCt_i for untreated cultures.

Statistical analysis

All baseline inter-organ and inter-cell type data were analyzed by Anova test followed by the Least significant difference (LSD) post-hoc test. For the experiments based on the addition of 1,25(OH)2D3 in the culture medium, the cell type-based pairwise comparisons were performed using the Wilcoxon test.

Results

Quantitative comparison of gene expression was performed by comparing the expression of the gene of interest to two housekeeping genes, namely *Hprt* (hypoxanthine-guanine phosphoribosyltransferase) and *beta actin*. Quantification with both reference genes provided similar results and we report here the data obtained with the gene whose level of expression was closer to those of vitamin D-associated genes, that is *Hprt*.

Hprt was expressed at very similar levels in every organ and every cell subtype, including cells cultivated with 1,25(OH)2D3.

Transcripts coding for vitamin D-associated enzymes and VDR are less abundant in brain tissue but Pdia3 is highly expressed.

As expected the expression of *Cyp450* enzymes relevant to vitamin D metabolism were less abundant in the brain compared to classic vitamin D target organs. This was also the case for the *Vdr*. Interestingly, the expression of *Pdia3* was greater in the brain, when compared to the

two other organs (Fig. 1).

Neurons are equipped to transform cholecalciferol and 25(OH)D3 into 1,25(OH)2D3

As shown on Figure 2, the transcripts coding for CYP27a1, CYP27b1 and CYP24a1 were comparatively highly expressed in neurons. Overall, *Cyp27a1* was expressed in every cell type, with a high expression in endothelial cells. However, *Cyp27b1* and *Cyp24a1* transcript expression was close to zero in astrocytes, oligodendrocytes and endothelial cells. Microglia were in intermediate position with a consistent but not elevated expression of the three enzymes.

Vdr is expressed mostly in astrocytes but Pdia3 is present in every cell type

Figure 2 indicates that the overall expression of the transcript coding for the VDR was very limited in all cell types, except in astrocytes. Conversely, *Pdia3* mRNA was present in relative abundance in every brain cell subtype and peaked in endothelial cells.

1,25(OH)2D3 treatment induces a very limited effect on Vdr and Pdia3 expression

In order to assess a putative effect of 1,25(OH)2D3 on the vitamin D machinery-associated transcripts, we measured their expression after a 12 hour-treatment. Figure 3 shows that *Vdr* and *Pdia3* expression was unaffected by a short term addition of 1,25(OH)2D3 in the culture medium, except in microglia and endothelial cells where a statistically significant modification was observed for *Vdr* and *Pdia3*, respectively. In regard to the enzymes, only the expression of *Cyp24a1* transcript was increased but only in astrocytes and microglia.

Discussion

The current study is the first to exhaustively assess the cellular expression of genes coding for vitamin D-associated metabolic enzymes and receptors, at the level of the neuro-vascular unit. It now requires to be confirmed at the protein level and in the brain. However, our data allow

us to draw a schematic view in which neurons and, possibly, microglia transform the inactive cholecalciferol into 1,25(OH)₂D₃ (Figure 4). The active form of vitamin D is then transferred to astrocytes where it can bind to VDR and initiate gene transcription or be inactivated when in excess. Alternatively, 1,25(OH)₂D₃ can induce autocrine or paracrine rapid non-genomic actions since all brain cell types express *Pdia3*, the other membrane receptor of vitamin D. Of note, brain endothelial cells stand out as a cell type able to transform cholecalciferol into 25(OH)D₃ and exhibiting a variable expression of *Pdia3*, when exposed to 1,25(OH)₂D₃.

It is established that both 25(OH)D₃ and 1,25(OH)₂D₃ cross the blood-brain barrier (BBB)

(3). If confirmed, our study suggests that calciferols can also be metabolised by the brain since endothelial cells and neurons are equipped to transform them into 25(OH)D₃. It can therefore be envisioned that calciferols have the capacity to go through the BBB and/or to be captured by the endothelium before being released as 25(OH)D₃ within the brain parenchyma. In the former case, it would be of great interest to assess the effect of cholecalciferol on brain cell types, especially neurons; in the latter case, measuring circulating calciferols is probably as important as quantifying seric 25(OH)D₃, when assessing the role of vitamin D in brain function.

All cerebral cell types express *Cyp27a1*, although at very low level for astrocytes, microglia and oligodendrocytes, and potentially can turn pre-vitamin D into 25(OH)D₃. Conversely, neurons and maybe microglia, are possibly the only cells capable of metabolizing 25(OH)D₃ into 1,25(OH)₂D₃. These data are partially in line with two previous studies reporting that i) the protein CYP27b1 is found in human neurons (7) and ii) cultured murine microglia can metabolize 25(OH)D₃ to 1,25(OH)₂D₃ (9). Noticeably, the current study reports a very weak expression of *Cyp27b1* in astrocytes and oligodendrocytes, suggesting that these cells are less likely to produce 1,25(OH)₂D₃, unlike what was observed in one of our previous studies, based on protein detection using immuno-histochemistry (7). Nevertheless, it should be borne

in mind that our work is exclusively based on mRNA expression and it cannot be excluded that the CYP27b1 protein, produced by neurons or microglia, is transferred to glia.

We report here that the astrocytes are the predominant cell type expressing the transcript coding for the vitamin D receptor and therefore appear to be the most likely cell type to respond to 1,25(OH)₂D₃ at the genomic level. The presence and functionality of VDR in astrocytes is also backed up by another major finding: these cells undergo important increases in *Cyp24a1* after 1,25(OH)₂D₃ treatment. It is established that vitamin D and its receptor induce the expression of CYP24a1 (17), in a dose-dependent manner (4). Interestingly, CYP24a1 has been observed previously in rat glia cells (4) and in astrocytes of the human brain (18). However, the latter study also reported the presence of CYP24a1 in human neurons, a finding that is not confirmed by our work at the transcriptomic level. Such a discrepancy could potentially be explained by differential immunohistochemical response to certain antibodies.

We also show here that the expression of *Vdr* transcript remains steady when 1,25(OH)₂D₃ is added to the culture medium. This observation is coherent with previous *in vivo* studies indicating that VDR transcription in adult animals is not affected by a prenatal vitamin D depletion (19, 20) or supplementation during adulthood (21). However, a slightly different picture emerges when looking at mouse newborns that grew in the womb of vitamin D-deficient mothers. We found that *Vdr* mRNA is overexpressed in their spinal cord at birth (22). Similarly, inflammation may trigger the transcription of VDR and *Cyp27b1*, as demonstrated in an *in vitro* study (23)

Although well studied in various cell systems (24,25), very little is known about PDIA3 expression in the nervous system. It has been reported that this vitamin D receptor mediates the action of diosgenin, a plant-derived steroidal sapogenin, which induces cognitive enhancement in normal mice (26) and a reduced number of amyloid plaques and

neurofibrillary tangles in a mouse model of Alzheimer's disease (27). In line with this, the current study is the first to report that *Pdia3* is highly expressed in the brain, when compared to kidneys and livers. All cerebral cell types are concerned, indicating that, within the brain, vitamin D may preferentially act via rapid non-genomic pathways. In astrocytes, VDR and PDIA3 possibly interact, as demonstrated in osteoblasts (28). In all other brain cell types, PDIA3 is probably the only regulator of vitamin D actions. Similar studies in brain are now required to confirm that PDIA3 is a major regulator of vitamin D signaling in brain.

Acknowledgments

We thank Jean Louis Franc for his input in analyzing the transcriptomic data. This work was supported by public [*Aix-Marseille Université* (AMU), *Centre National de la Recherche Scientifique* (CNRS)] and private grants [*Fondation de l'Avenir, Demain Debout, Combattre la Paralyse*]. The project leading to this publication has received funding from Excellence Initiative of Aix-Marseille University - A*MIDEX, a French “Investissements d’Avenir” programme”.

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Legends

Figure 1. Comparative expression of vitamin D-associated genes in kidney, liver and brain. When compared to kidneys and livers, the expression of the three enzymes and the *Vdr* is reduced in the cortex and the hippocampus. Conversely, *Pdia3* transcript is over-expressed in the brain. N = 3 for each organ. * = $p < 0.05$; ** = $p < 0.01$.

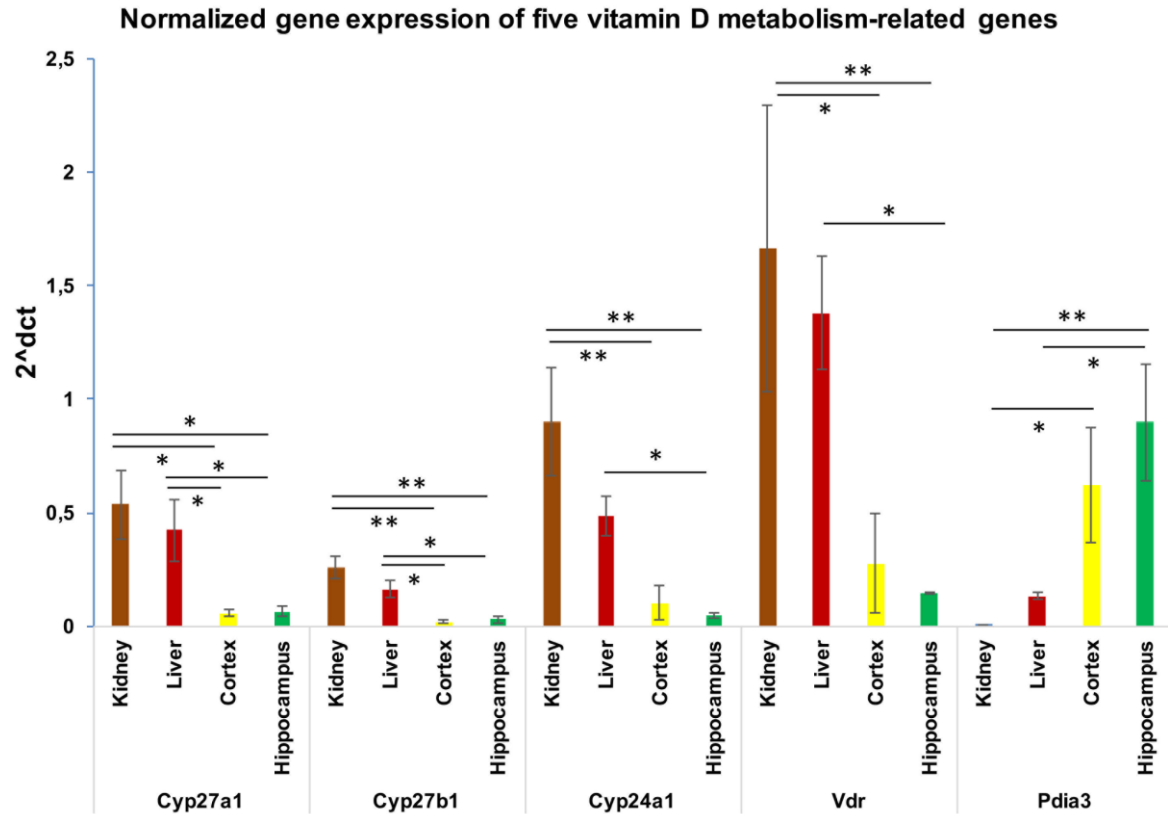


Figure 2. Comparative expression of vitamin D-associated genes in five brain cell types. *Cyp27a1* is mostly expressed in neurons and endothelial cells. *Cyp27b1* and *Cyp24a1* are nearly absent from astrocytes, oligodendrocytes and endothelial cells. *Pdia3* expression is high in almost every cell type but with a peak in endothelial cells and astrocytes while *Vdr* expression is elevated in astrocytes. N = 3 for each cell type. * = $p < 0.05$; ** = $p < 0.01$

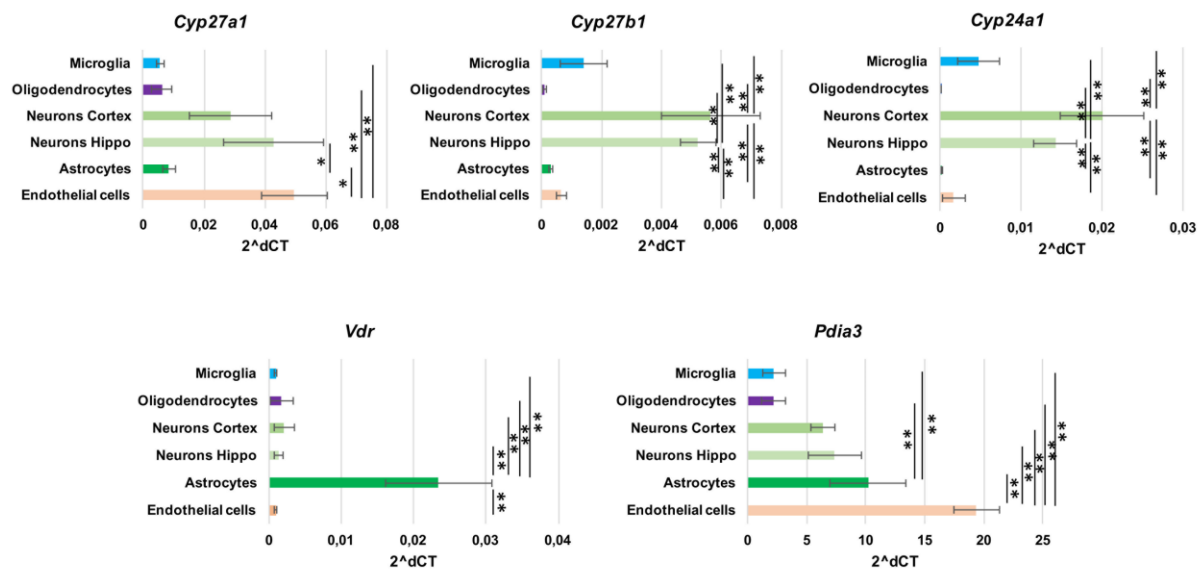


Figure 3. Effect of *in vitro* 1,25(OH)2D3 supplementation on gene expression in brain cell subtypes. An increased expression of *Cyp24a1* is observed in astrocytes and microglia while a slight reduced expression of *Pdia3* is noticed in endothelial cells. N = 3 for each cell type; * = p<0.05; ** = p<0.01.

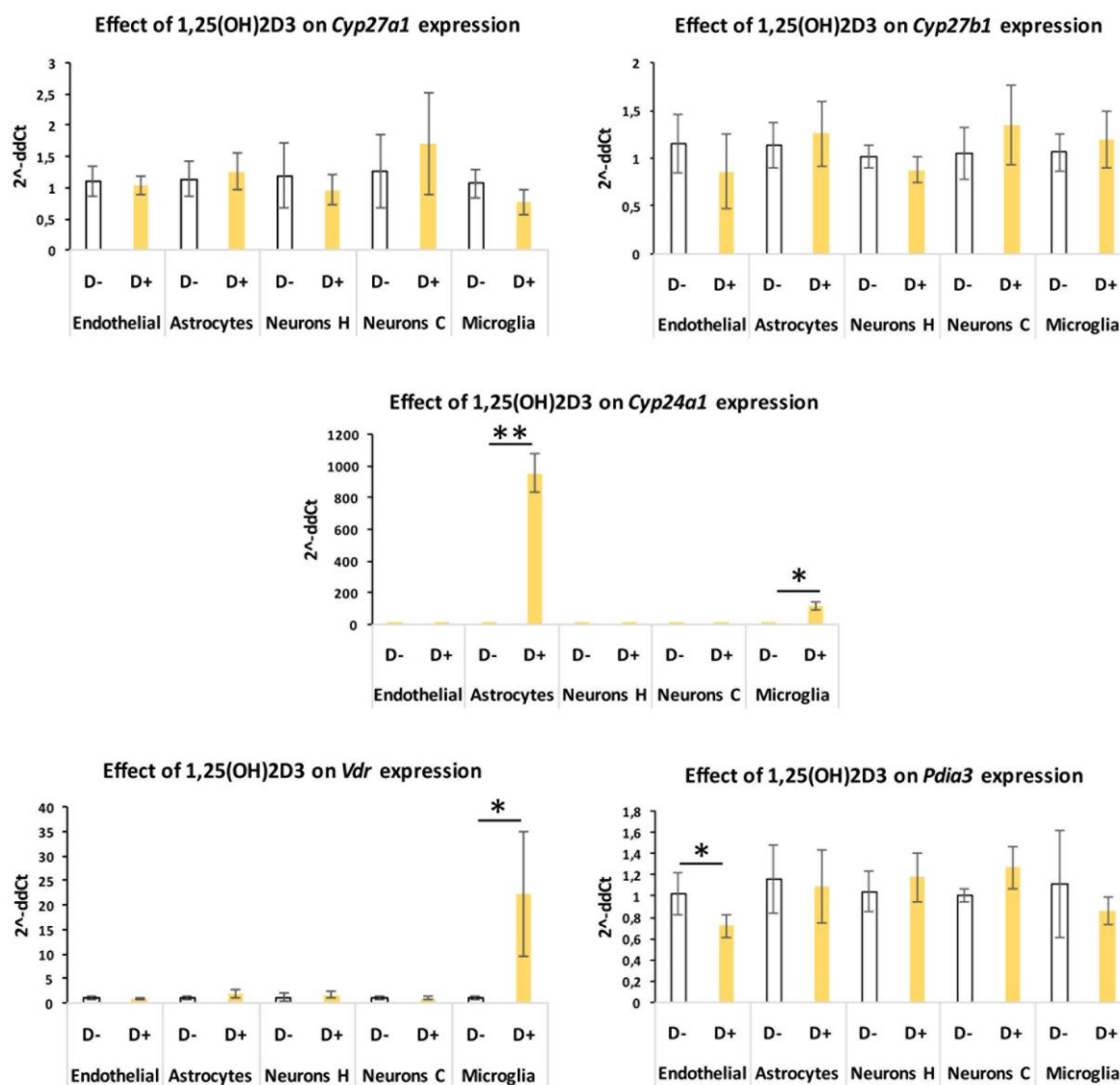


Figure 4. Schematic summary. Within the neuro-vascular unit, endothelial cells and neurons are the cells that express the most abundant number of transcripts coding for *Cyp27a1*. They can turn vitamin D into 25(OH)D which becomes active after hydroxylation into 1,25(OH)₂D in neurons and, possibly, microglia. 1,25(OH)₂D, the active form of vitamin D, triggers either *Vdr*-associated genomic actions or *Pdia3*-related non genomic actions. However, in basal conditions, *Vdr* is poorly expressed and *Pdia3* appears as the main cerebral vitamin D receptor.

