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Cholecalciferol (Vitamin D_3) Reduces Rat Neuropathic Pain by Modulating Opioid Signaling

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Abstract

The impact of vitamin D on sensory function, including pain processing, has been receiving increasing attention. Indeed, vitamin D deficiency is associated with various chronic pain conditions, and several lines of evidence indicate that vitamin D supplementation may trigger pain relief. However, the underlying mechanisms of action remain poorly understood. We used inflammatory and non-inflammatory rat models of chronic pain to evaluate the benefits of vitamin D_3 (cholecalciferol) on pain symptoms. We found that cholecalciferol supplementation improved mechanical nociceptive thresholds in monoarthritic animals and reduced mechanical hyperalgesia and cold allodynia in a model of mononeuropathy. Transcriptomic analysis of cerebrum, dorsal root ganglia, and spinal cord tissues indicate that cholecalciferol supplementation induces a massive gene dysregulation which, in the cerebrum, is associated with opioid signaling (23 genes), nociception (14), and allodynia (8), and, in the dorsal root ganglia, with axonal guidance (37 genes) and nociception (17). Among the identified cerebral dysregulated nociception-, allodynia-, and opioid-associated genes, 21 can be associated with vitamin D metabolism. However, it appears that their expression is modulated by intermediate regulators such as diverse protein kinases and not, as expected, by the vitamin D receptor. Overall, several genes—*Oxt*, *Pdyn*, *Penk*, *Pomc*, *Pth*, *Tac1*, and *Tgfb1*—encoding for peptides/hormones stand out as top candidates to explain the therapeutic benefit of vitamin D_3 supplementation. Further studies are now warranted to detail the precise mechanisms of action but also the most favorable doses and time windows for pain relief.

Keywords Cholecalciferol · Analgesia · Steroid · Sciatic nerve constriction · Gene regulation · Opioid

Introduction

Vitamin D is now recognized as an active and pleiotropic neurosteroid in the brain, where it acts as a hormone and a paracrine/autocrine agent [1, 2]. In mammals, vitamin D synthesis begins with the cleavage of the B ring of 7-dehydrocholesterol, in the epidermis, under UVB radiation (ultraviolet B; 290–315 nm) producing an extremely unstable pre-vitamin D [3]. After spontaneous isomerization, this creates the precursor molecule, cholecalciferol, also commonly called vitamin D_3, which is subjected to three further hydroxylations, two of which render it bioactive, the third one allowing for inactivation. The first hydroxylation step occurs under the activity of a number of microsomal cytochrome P450 enzymes. CYP27A1, CYP2J2, and CYP3A4 are potential players for the addition of a hydroxyl group on the C25 of vitamin D, but CYP2R1 appears to be the most implicated enzyme in the conversion of cholecalciferol into 25-hydroxyvitamin D_3 [25(OH)D3], or calcidiol [4, 5]. 25(OH)D is subsequently converted to 1,25-

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dihydroxyvitamin D [1,25$(OH)_2$D] or calcitriol by another cytochrome P450 enzyme, CYP27B1, also referred to as 1α-hydroxylase. Once metabolized, 1,25$(OH)_2$D promptly upregulates the expression of a third cytochrome P450, CYP24A1, which catabolizes both 25(OH)D and 1,25$(OH)_2$D into biologically inactive, water-soluble calcitroic acid [6]. Within the whole body, calcitriol binds to the vitamin D receptor (VDR) and induces the transcription of hundreds of genes.

Evidence linking vitamin D and pain is based on in vitro, animal, epidemiological, and clinical research. For instance, four observational studies report that hypovitaminosis D is associated with various forms of chronic pain [7–10]. Rats fed during 2–4 weeks with a vitamin D-deficient diet displayed mechanical deep muscle hypersensitivity [11]. Pain relief was also observed in vitamin D-treated patients with fibromyalgia syndrome [12] or musculoskeletal complaints [13].

Although the published clinical studies are heterogeneous and sometimes of poor methodological quality, three meta-analyses of randomized, placebo-controlled trials were conducted [14–16]. They all conclude that there is a modest level of evidence that vitamin D supplementation is efficient to treat chronic pain. In line, reviews of published randomized controlled trials report that vitamin D induces a significantly greater mean decrease in pain score for patients with chronic pain, compared with placebo [17–19], especially when patients display calcidiol [25(OH)D] levels below 30 nmol/L [20].

The basis for considering vitamin D as a potential pain killer stems partially from its well-established role as a modulator of inflammatory and immune responses [21, 22]. In human lung fibroblasts, cholecalciferol, calcidiol, and calcitriol inhibit the production of prostaglandin E2 (PGE2), a key factor in inflammatory pain [23], and its level of expression is reduced in patients with musculoskeletal pain treated with high doses of vitamin D_3 [24]. However, since vitamin D supplementation could also be efficient in reducing symptoms in non-inflammatory chronic pain, other mechanisms may be at play [25].

The administration of vitamin D_3 attenuated cold allodynia and heat hyperalgesia in a rat model of neuropathic pain when delivery was started on the first day after surgery [26]. We also demonstrated that vitamin D_3 supplementation improved functional recovery and myelination [27, 28]. Subsequently, we noticed a cholecalciferol-associated diminution of pain in humans with arm and/or hand nerve trauma (unpublished data). In order to assess whether cholecalciferol is a true pain modulator and identify its potential mechanisms of action, we performed the current study, based on two rat models of pain—knee monoarthritis and mononeuropathy—that represent good examples of chronic pain associated with various degrees of inflammation. Animals were supplemented with cholecalciferol for several weeks after which their nociceptive sensitivity to mechanical and cold stimuli was quantified. At the end of the experiment, the cerebrum, dorsal root ganglia (DRG), and spinal cord were collected and analyzed by means of cDNA microarrays, quantitative PCR, and bioinformatic tools, in order to identify the vitamin D-associated genes involved in nociception.

Methods

Animals and Pain Models

Male Sprague Dawley rats weighing 250–350 g (Janvier Le Genest St. Isle, France) were housed in groups of three, under standard conditions (room temperature 22 °C; 12/12 h light-dark cycle) with ad libitum access to food and water. All experiments were conducted in conformity with the recommendations of the European Committee Council Direction of September 22, 2010 (2010/63/EU). Procedures were positively evaluated by the regional ethical committee, and experiments were conducted with an official authorization for animal experimentation from the French Department of Agriculture (License 67-116 to PP).

Knee monoarthritis Transient inflammation of the rat knee was induced by unilateral injection of 50 μL CFA (complete Freund's adjuvant (Sigma St Louis, MO, USA)) under light isoflurane anesthesia (3%), as previously published [29]. Control animals received a similar volume of mineral oil, the diluent for CFA (Sigma Aldrich, St Louis, USA).

Mononeuropathy To produce a controlled constriction of the sciatic nerve, we used the cuff model which has been well characterized previously in our laboratory [30]. The surgical procedure was carried out under aseptic conditions and ketamine/xylazine anesthesia (ketamine 87 mg/mL, xylazine 13 mg/mL, i.p. (intra-peritoneal) 10 mL/kg; Centravet, Taden, France). The common branch of the right sciatic nerve was exposed, and a 2-mm long split section of polyethylene tubing (ID = 0.86 mm, ED = 1.27 mm; PE-90, Harvard Apparatus, Les Ulis, France) was placed around it (cuff group). The shaved skin layer was then closed using suture. Sham-operated rats underwent the same surgical procedure as described above but without implantation of the cuff (sham group).

Nociceptive Tests

Mechanical nociceptive threshold Mechanical nociceptive thresholds were measured using a calibrated forceps (Bioseb, Vitrolles, France), as previously described [31]. All animals were habituated to the room and the tests, at least

1 week before the initial experiments. Habituated rats were loosely restrained, with a towel masking the eyes, in order to limit the stress induced by environmental stimulations. The tips of the forceps were placed on each side of the paw and a gradually increasing force was applied. The pressure, in grams, producing withdrawal of the paw or, in some rare cases, vocalization of the animal, was considered as the nociceptive threshold value. This manipulation was performed three times for each hind paw, and values were averaged. All tests were performed between 10:00 AM and 4:00 PM, prior to drug treatment.

Cold allodynia Thermal cold allodynia was assessed by scoring the aversive behaviors of rats using the acetone test, as previously published [32]. Rats were placed on a wire mesh delimited by a Plexiglas™ cage and allowed to accommodate for at least 15 min. A drop of acetone was then placed on the ventral side of the hind paw, producing a non-noxious decrease in temperature during evaporation. The rat behavioral response was scored during 20 s following acetone application as follows: 0, no response of the animal; 1, quick withdrawal, flick, or stamp of the paw; 2, prolonged withdrawal or repeated flicking of the paw; and 3, repeated flicking of the paw with licking of the paw. Manipulation was performed three times for each paw and values were added (maximal score 9).

Drugs and Treatments

Vitamin D_3 (cholecalciferol) was provided in the diet (monoarthritic model) or by gavage (monoarthritic and neuropathic models). Animals were fed with either a control diet (1000 IU/kg), a vitamin D_3–deficient diet (0 IU/kg), or a vitamin D_3–supplemented diet (7500 IU/kg) (INRA, France). If we consider that 300–350-g rats eat approximately 20 g of chow, the administered doses of vitamin D_3 were 60 IU/kg/day, 0 IU/kg/day, and 450 IU/kg/day, respectively. Six weeks were required to reach vitamin D–deficiency status. In parallel, a group of rats was vitamin D_3–supplemented by a weekly gavage (50,000 IU/mL, 200 μL/week) (Uvedose, Crinex). Control animals received the same volume of triglycerides, the diluent for vitamin D_3. Supplementation, through diet or gavage, was performed for 4 weeks.

ADVIA Centaur® Vitamin D Total Analysis

This assay is a one-step, automated direct competitive chemiluminescent immunoassay that detects 25-OH vitamins D_2 and D_3 in serum. It employs a proprietary releasing reagent, an anti-fluorescein monoclonal mouse antibody covalently bound to paramagnetic particles, an anti-25(OH)D monoclonal mouse antibody labeled with acridinium ester, and a vitamin D analog labeled with fluorescein. There was an inverse relationship between the resulting chemiluminescent signal detected by the system and the amount of vitamin D present in the sample. This assay is standardized against LC-MS/MS (liquid chromatography coupled to tandem mass spectrometry). The assay range is 9.3 to 375 nmol/L.

Microarray Assay

Lower lumbar DRG, lumbar spinal cord, and cerebrum were collected from all groups. Rats were anesthetized with isoflurane before sacrifice, and tissues were isolated from each animal, before being snap-frozen in liquid nitrogen and stored at − 80 °C until use. Total RNA was isolated from the snap-frozen tissues 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 was assessed on an Agilent 2100 Bioanalyzer (Agilent Technologies, Les Ulis, France).

RNA samples from three animals in each group were pooled for microarray hybridization. Unwanted genomic DNA was removed using a DNase set kit (#79254, Qiagen). Purified total RNAs were processed for hybridization on genome-wide DNA microarrays within 1 month. Cyanine-3-labeled cDNA was generated from 300 ng of RNA using the One-Color Low RNA Input Linear Amplification Kit (Agilent Technologies) according to the manufacturer's instructions, followed by purification on a RNeasy column (Qiagen). All amplified cDNAs were checked for dye incorporation, cDNA yield, and amplification profile. Only those fitting all quality criteria were fragmented for further hybridization onto microarrays. Samples were then carefully hybridized onto Agilent Whole Rat Genome (4644 K) Oligo Microarrays (G4131F). Microarrays were scanned using an Agilent DNA microarray scanner G2505B. Data are available on the ArrayExpress database (accession number E-MTAB-6801).

Microarray Data Analysis

Individual microarray quality was evaluated based on quality control report, pair-wise MA-plots (with M as the log ration and A as the mean average), and box plots. Intra-array normalization of raw signals from the eight microarrays (corresponding to the four above-mentioned conditions in duplicate) was performed using Feature Extraction software 9.1.3.1 (Agilent Technologies®). Microarray normalized data were further exported into the limma package, for inter-array normalization using the quantile method. Statistical analysis was performed using the TIGR MeV (MultiExperiment Viewer) v4.4 software (http://www.tm4.org/mev.html) and the GeneANOVA program. The multi-way ANOVA (analysis of variance) model was implemented: first, to identify differentially regulated genes when accounting for the multiple

sources of variation in the microarray experiment and, second, to evaluate the effect of the main variable, i.e., the addition of cholecalciferol. Multiple test correction was further carried out using the false discovery rate method. Cluster and Tree View software were used for unsupervised hierarchical clustering.

Biological interpretation of the data was performed using Ingenuity Pathway Analysis (IPA, Ingenuity Systems). The main criterion to validate a differentially expressed gene was a fold change over 1.5 or under − 1.5 when considering expression values in the group supplemented with vitamin D_3 relative to the control diet group, whether considering control or experimental animals. Upregulated and downregulated genes were analyzed in the same data sets to obtain the biologically relevant function categories. Right-tailed Fisher's exact test was used to calculate a p value determining the top statistically significant biological functions assigned to the data set.

Real-Time Quantitative PCR

Tissues from cerebrum and lower lumbar DRG of all animal groups were collected, reconstituted in a guanidine thiocyanate/β-mercaptoethanol preparation using ultraturax, and stored at − 80 °C. Total RNA was extracted according to a protocol derived from the original procedure of Chomczynski and Sacchi [33], consisting of two independent total RNA extractions separated by a DNAseI treatment (TURBO DNaseTM, Ambion, Life technologies, Saint Aubin, France), as previously described in detail [34]. Eight hundred nanograms RNA was used for reverse transcription with the RT iScript kit (Bio-Rad, Marnes-la-Coquette, France). Real-time quantitative PCR (RT-qPCR) was performed using SYBR Green Supermix (Bio-Rad), on the iQ5 real-time PCR System (Bio-Rad). Amplifications were carried out in 42 cycles (20 s at 95 °C, 20 s at 60 °C, and 20 s at 72 °C). Serial dilutions of samples were used to create standard curves, after which relative gene expression was calculated as the ratio between cDNA concentration of the gene of interest and that of the housekeeping gene. To specifically amplify mRNA encoding various rat proteins, we designed all our specific primer sets (sense and antisense respectively) to get an optimal annealing efficacy at 60 °C using Oligo6.0 and M-fold software for selected genes. Primer sequences and their spanning regions in NCBI sequences are given in Supplementary Table 1.

To standardize the experiments, four classic housekeeping genes were tested, i.e., beta2-microglobulin (B2m), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), β-glucuronidase, and hypoxanthine guanine phosphoribosyl transferase (HPRT). Preliminary experiments showed that HPRT transcripts were very stable between samples. Therefore, HPRT was selected as the housekeeping gene. Samples were accurately dispensed in duplicates using a robotic workstation (Freedom EVO100, Tecan, Lyon, France), and amplification efficacy given by standard curves was always close to 100% (± 2%), while amplification specificity was assessed by a melting curve study.

Statistics

All data are expressed as mean ± standard error of the mean (SEM). Two-way repeated-measures analysis of variance (RM tw ANOVA), followed by Bonferroni's post-hoc tests were used to analyze the effects on mechanical/thermal nociception. RT-qPCR results were analyzed with one-way ANOVA followed by Sidak's post-hoc test. Differences were considered statistically significant for $p < 0.05$.

Results

Vitamin D_3 Supplementation Reduces Mechanical Hyperalgesia in Monoarthritic Rats

The modulatory action of vitamin D_3 on pain symptoms was first evaluated using a model of transient inflammatory pain induced by a single intra-articular injection of CFA. This classical model of knee monoarthritis rapidly induces a local inflammation associated with mechanical hyperalgesia of the ipsilateral hind paw (Fig. 1a). Compared with control values (just before injection at $t = 0$:295.8 ± 2.4 g), mean mechanical nociceptive thresholds dropped significantly to a minimal value of 168.1 ± 5.9 g, 24 h after CFA injection ($n = 6$) in control animals receiving normal food. Mean mechanical nociceptive threshold remained unchanged for the ipsilateral hind paw of rats injected with the vehicle of CFA (Fig. 1b) and for all contralateral hind paws (data not shown). When present, mechanical hyperalgesia persisted for about 3 weeks since mechanical nociceptive thresholds returned to control values 21 days after CFA injection.

Plasma concentrations of the active metabolite of calcitriol (1,25-dihydroxyvitamin D) were 48.94 ± 3.42 ng/mL ($n = 11$) for rats under normal diet and around 120 ng/mL for rats supplemented for 4 weeks (food 119.93 ± 6.25 ng/mL, $n = 6$; gavage 135.20 ± 9.14 ng/mL, $n = 5$). Data were statistically different from normal food rat group at $p < 0.001$ with the Kruskal-Wallis test (KW = 16.10) followed by Dunn's post-hoc test (normal food vs food supplementation $p < 0.01$; normal food vs normal food + gavage $p < 0.001$).

After 4 weeks of supplementation with vitamin D_3 in their food or by weekly gavage, we found that rat mechanical hyperalgesia was significantly reduced compared with those under normal diet (RM tw ANOVA, time × treatment, $F_{(15, 75)} = 5.59$, $p < 0.001$). Twenty-four hours after CFA injection, thresholds reached a minimal value of 215.6 ± 6.9 g and of 222.4 ± 10.7 g for rats receiving vitamin D_3 food

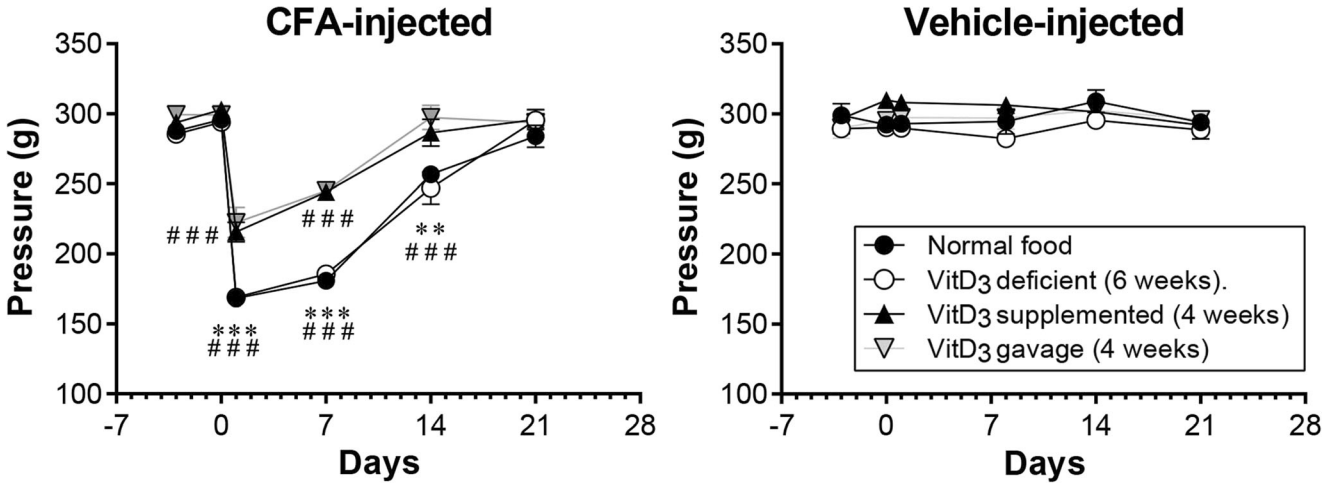

Fig. 1 Time course of mechanical nociceptive thresholds measured from the hind paw of rats injected with CFA (complete Freund's adjuvant) (**a**) or its vehicle (**b**) in the knee joint at day 0. The comparison is shown for rats fed with a normal or vitamin D_3 (vitD_3) deficient diet, or vitamin D_3–supplemented through diet or by gavage. Comparisons between supplemented and unsupplemented groups are indicated by asterisks at each time point. #Statistical significance when comparing values, for each time point, between vehicle- and CFA-injected animals receiving a similar diet. Statistical significance was assessed with Bonferroni's post-hoc test, illustrated as follows: $^{**}p < 0.01$ and $^{***/\#\#\#}p < 0.001$

supplementation ($n = 6$) and gavage ($n = 6$), respectively. Normalized to the mean threshold observed in rats under normal diet, these values corresponded to a 30–40% reduction in hyperalgesia. Accordingly, at the end of week 2, no sign of hyperalgesia was observed in supplemented animals whereas rats from the other groups still displayed significantly lower mean mechanical nociceptive thresholds compared with baseline. No difference in the time course of pain symptoms was observed between the two groups of unsupplemented and vitamin D_3–deficient rats (Fig. 1a).

Vitamin D Limits Neuropathic Pain Symptoms and Accelerates Recovery

We then investigated the consequences of vitamin D_3 supplementation, using rat gavage for 4 weeks, on a neuropathic pain model induced by a chronic constriction of the sciatic nerve. This well-characterized model of neuropathic pain is associated with long-lasting mechanical hyperalgesia and cold allodynia (Fig. 2). After the cuff surgery, mechanical hyperalgesia developed as indicated by a decrease in the mean mechanical nociceptive threshold (Fig. $2a_1$). Thresholds were significantly different between the cuff- and sham-operated groups (Fig. $2a_1$: RM tw ANOVA, time × surgery, $F_{(5,\ 25)} = 11.25$, $p < 0.001$; $n = 6$ rats per group). In respect with cold allodynia measured with the acetone test, differences between sham and cuff groups were also observed (Fig. $2a_2$: RM tw ANOVA, time × surgery, $F_{(5,\ 25)} = 29.88$, $p < 0.001$; $n = 6$ rats per group).

Similarly to the monoarthritic model, animals receiving vitamin D_3 supplementation displayed significantly reduced mechanical hyperalgesia (RM tw ANOVA, time × treatment, $F_{(5,\ 25)} = 8.57$, $p < 0.001$; $n = 6$ rats per group) and cold allodynia (RM tw ANOVA, time × treatment, $F_{(5,\ 25)} = 6.73$, $p < 0.001$; $n = 6$ rats per group) when compared with the unsupplemented group. After 1 week, hyperalgesia-associated mechanical thresholds raised significantly (control food 159.97 ± 8.44 g; control food + gavage 215.11 ± 8.13 g; $n = 6$) while cold allodynia scores diminished significantly (control food 6.00 ± 0.68 g; control food + gavage 3.50 ± 0.56 g; $n = 6$). Overall, vitamin D_3–associated modulations of pain symptoms, after 1 week of treatment, were about 40.6% and 45.5%, respectively for mechanical and cold modalities. Mechanical sensitivities and behavioral scores in response to acetone reached similar values to sham-operated control animals 3 weeks after surgery. No withdrawal (i.e., reappearance of pain symptoms) was observed during the following days (data not shown). In this model, spontaneous recovery is observed after 60 to 90 days. Altogether, these data suggest that vitamin D_3 supplementation accelerates the recovery of these animals as demonstrated by the rapid reduction in pain symptoms.

Because neuropathic pain may appear after surgery in some clinical cases, we also evaluated the benefits of introducing an immediate postsurgical vitamin D_3 treatment. Figure 2b illustrates the time course of mechanical nociceptive thresholds after cuff surgery. The new kinetic profile is very similar to the previous one. Mechanical allodynia was strongly and significantly reduced 1 week after surgery (RM tw ANOVA, time × treatment, $F_{(5,\ 25)} = 11.40$, $p < 0.001$). Moreover, mechanical hypersensitivity fully disappeared 2–3 weeks after surgery in vitamin D_3–treated rats whereas control rats still exhibited pain symptoms. It can thus be concluded that vitamin D_3 limits pain symptoms in these two models and accelerates recovery from persistent pain states.

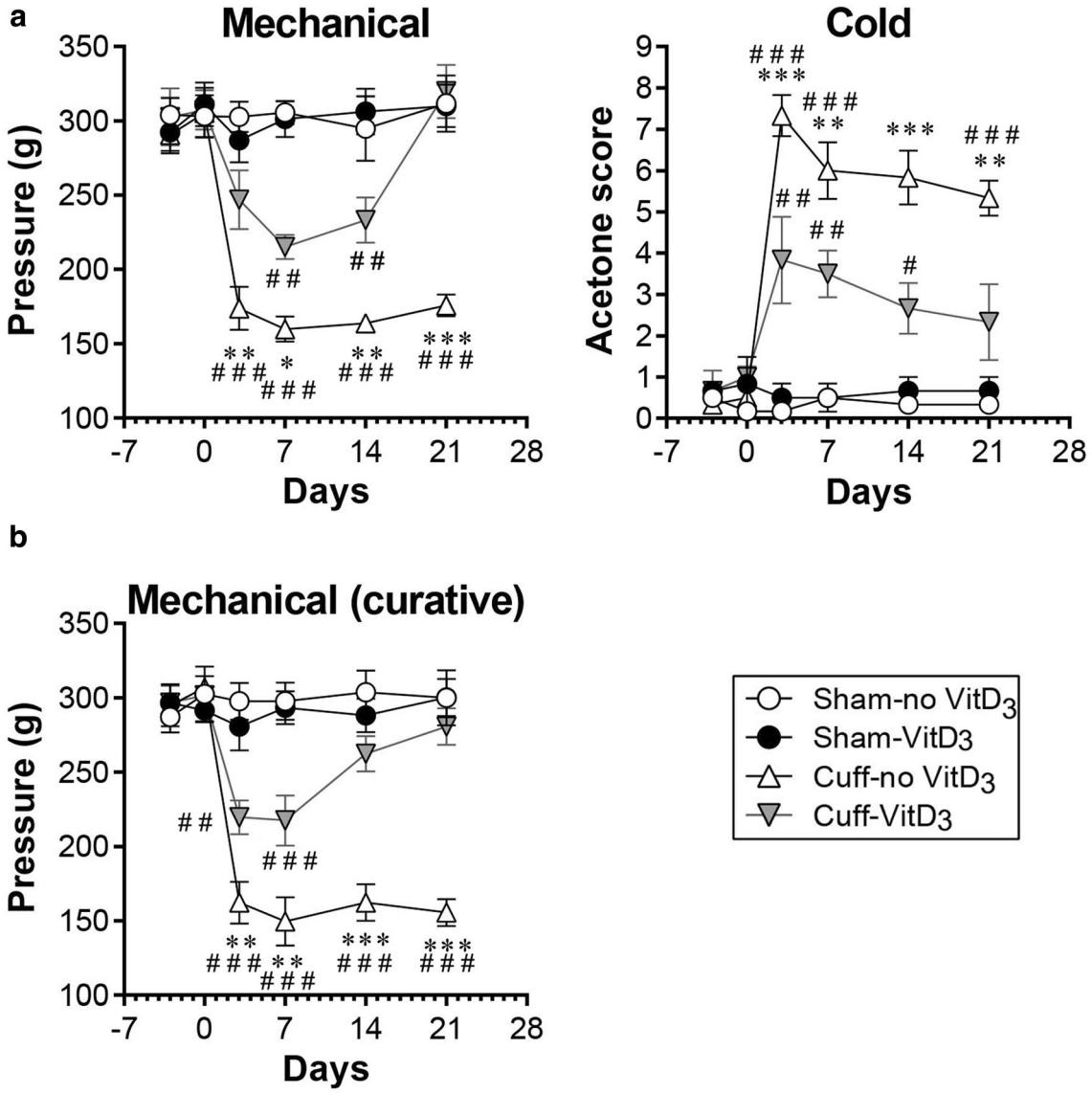

Fig. 2 **a** Time course of mechanical nociceptive thresholds (a_1) and cold allodynia scores (a_2) measured in sham-operated and cuffed rats, supplemented or not with vitamin D_3 (vitD3). **b** Time course of mechanical nociceptive thresholds of rats supplemented or not with vitamin D_3, from day 0 (time of surgery). Statistical significance was assessed with Bonferroni's post-hoc test, illustrated as follows: $^{*}p < 0.05$, $^{**}p < 0.01$, or $^{***}p < 0.001$ for comparisons between control and vitamin D_3–supplemented groups and $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, or $^{\#\#\#}p < 0.001$ for comparisons between sham and cuffed animals receiving a similar diet, at each time point

Vitamin D3 Induces a Massive Tissue-Dependent Gene Dysregulation

Since calcitriol exerts most of its biological effects by modulating gene transcription, we then performed transcriptomic experiments in order to investigate possible genomic pathways explaining its modulatory effect on pain symptoms. We analyzed three nervous tissues of interest: DRG, spinal cord, and cerebrum. Supplementary Table 2 lists the 1285 genes that were dysregulated, in at least one of the three tissues, after 4 weeks of cholecalciferol supplementation in neuropathic animals. In the cerebrum, the number of under- (187) and over-expressed (145) genes was nearly even. In contrast, in the spinal cord (69 vs 200) and DRG (278 vs 482), dysregulated genes were predominantly over-expressed.

Figure 3a reveals that vitamin D_3 supplementation induced dysregulation of more than twice as many genes in the DRG as compared with the cerebrum or the spinal cord. Interestingly, only three genes—*Mrpl43* (mitochondrial ribosomal protein L43), *Myh7* (myosin heavy chain 7), and *Syn1* (Synapsin 1)—were commonly dysregulated in the three nervous tissues, after vitamin D_3 treatment. *Mrpl43* was under-expressed in all three tissues, while *Myh7* and *Syn1* were under-expressed in the cerebrum but over-expressed in the spinal cord and DRG.

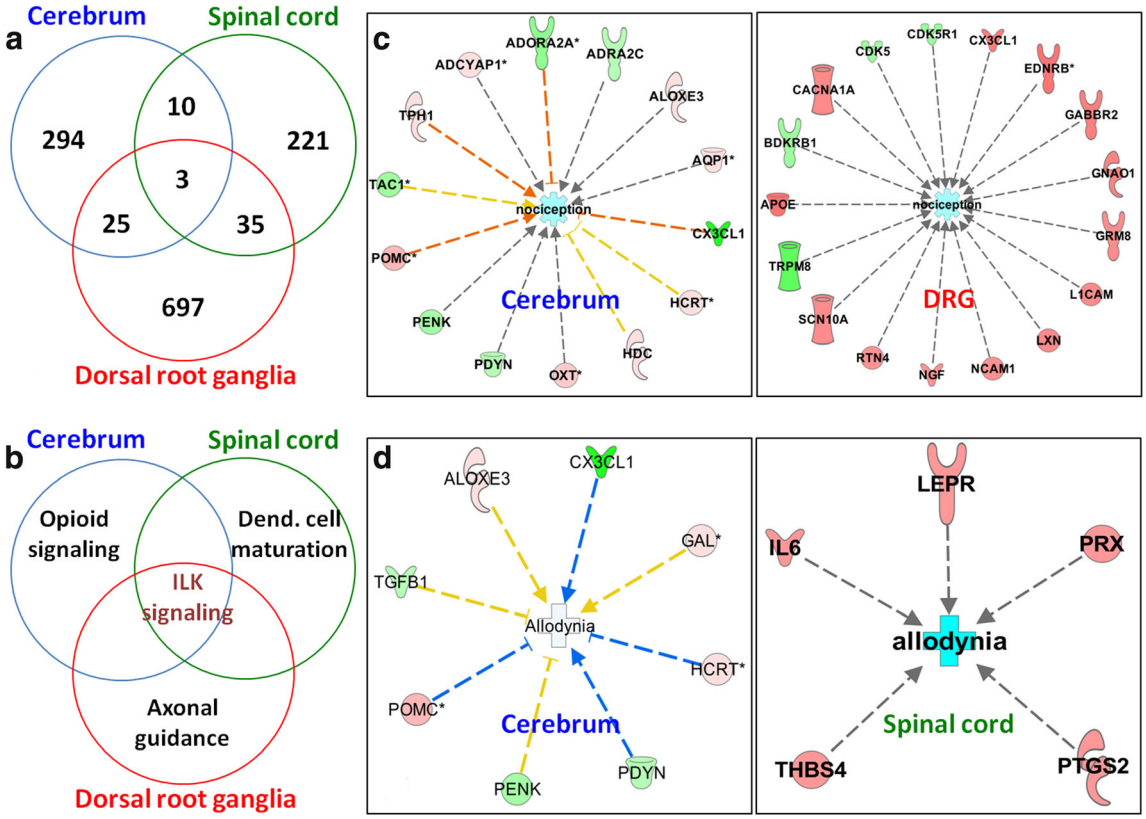

Fig. 3 Extensive transcript dysregulation in the cerebrum, spinal cord, and dorsal root ganglia (DRG) of rats, 2 days after being fed during 4 weeks with a vitamin D_3–supplemented diet. **a** Venn diagram showing the number of dysregulated genes in each tissue. The number of genes specifically dysregulated in DRG is more than twice as large as in other tissues. **b** Venn diagram indicating the top canonical pathway in each tissue (dend. cell maturation, dendritic cell maturation; ILK, integrin-linked kinase). **c** Schematic overview of the genes associated with nociception in the cerebrum and DRG. **d** Schematic overview of the genes associated with allodynia in the cerebrum and spinal cord. Fold change cut off used for the above analyses was − 1.5 > FC > 1.5

Using the IPA software, the clustering of the area-specific dysregulated genes indicated that among the top canonical pathways, the ones displaying the highest significance were the opioid signaling pathway (23 genes), dendritic cell maturation (12), and axonal guidance signaling (37), for the cerebrum, spinal cord and DRG, respectively (Fig. 3b). Furthermore, integrin-linked kinase (ILK) signaling, via its cytosolic kinase activity and/or modulatory role on nuclear gene expression, stands out as the prime common canonical pathway.

Vitamin D3 Modulates Nociception and Allodynia in a Tissue-Specific Manner

To unveil the detailed role of vitamin D_3 in nociception and allodynia, we narrowed our analysis to the genes that are directly associated with these two functions. As summarized in Fig. 3c, when comparing vitamin D3–supplemented with unsupplemented neuropathic rats, we observed 14 and 17 nociception-associated genes whose expression was dysregulated in the cerebrum and DRG, respectively. Within these two pools, only one gene—*Cx3cl1* (C-X3-C motif chemokine ligand 1)—was commonly dysregulated, although in an inverted way. In regard to allodynia, eight and five genes were dysregulated in the cerebrum and spinal cord, respectively (Fig. 3d), none of them being misexpressed in both tissues. Within the cerebrum, three genes that are involved in the modulation of nociception and allodynia—*Pdyn, Penk*, and *Pomc*—encode major ligands of the opioid signaling pathway (prodynorphin, proenkephalin, and proopiomelanocortin, respectively; Fig. 4). No allodynia-associated dysregulated gene was observed in the DRG, and no nociception-associated dysregulated gene was noticed in the spinal cord. The 23 genes associated with opioid signaling (Fig. 4) are *Adcy5*, *Ap2b1*, *Ap2s1*, *Ctnnb1*, *Fos*, *Gnal*, *Gnas*, *Gng7*, *Gsk3b*, *Itpr1*, *Myc*, *Pde1b*, *Pdyn*, *Penk*, *Plcb1*, *Pomc*, *Ppp3ca*, *Prkcb*, *Prkcd*, *Rgs3*, *Rgs9*, *Sos1*, and *Th*.

At this point, we decided to validate the observed expression of several dysregulated genes of our data set, using RT-qPCR performed on samples extracted from animals having received sham or sciatic cuff surgery with or without vitamin D_3 supplementation. We chose to sort out the data from both DRG and cerebrum tissues, using three major classes of genes. First, we focused on the opioid system (Fig. 5) in cerebrum

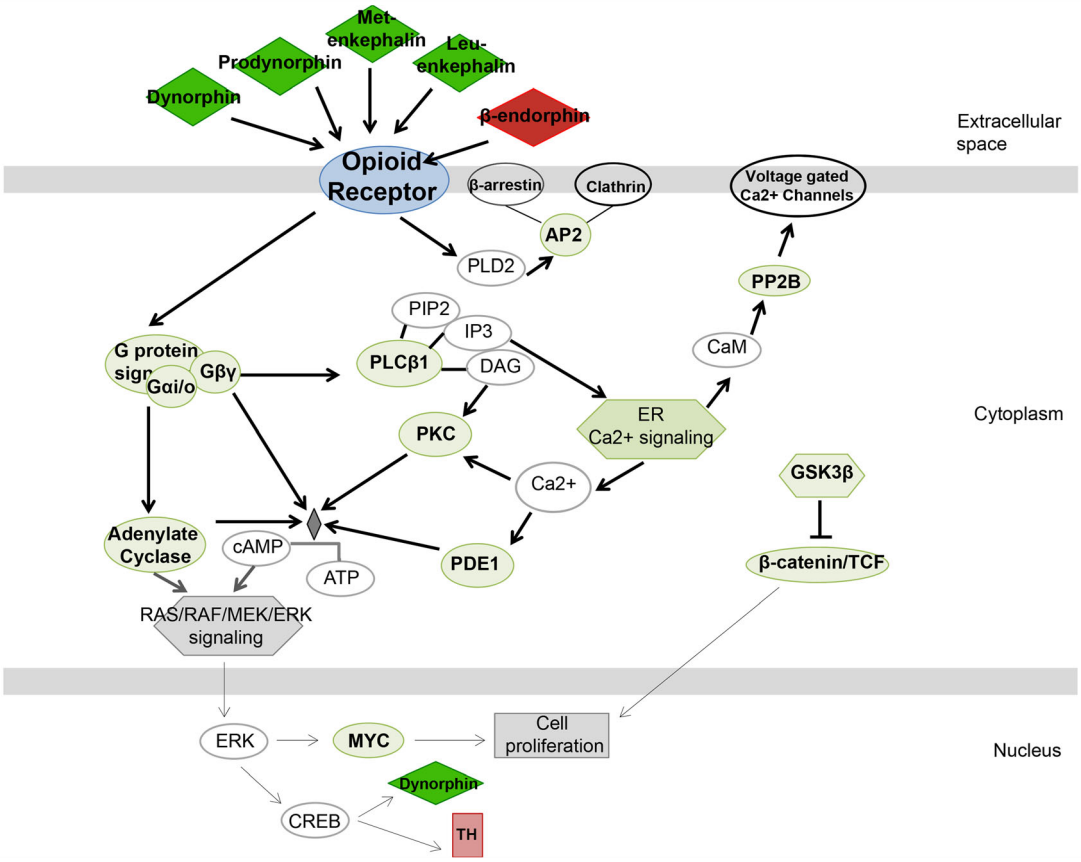

Fig. 4 Schematic opioid signaling pathway. Cerebral dysregulated genes, associated with opioid signaling, are highlighted (up: red; down: green) in a simplified view of the canonical pathway developed by Ingenuity©

samples. We observed that prodynorphin and proenkephalin transcripts were upregulated in injury conditions but returned to basal levels after vitamin D_3 treatment. Conversely, the *Pomc* gene, known to give rise to many endogenous opioid peptides including met-enkephalin and β-endorphin, was over-expressed in cuff animals supplemented with vitamin D_3. In addition, opioid receptors remained unaffected in our paradigm, promoting the hypothesis that the analgesic endogenous opioid pathways may actively participate in vitamin D actions. This activation appears quite selective since DRG samples extracted from the very same animals did not show a similar pattern.

A second class of genes (Supplementary Fig. 1) which raised our attention includes numerous neurotransmitters/neuropeptides and neurotrophic factors. Here, we show that differential patterns of secreted molecules expressed in the DRG and cerebrum are modulated by nerve injury and vitamin D_3 supplementation. Indeed, cerebral *Avp* (vasopressin), *Oxt* (oxytocin), *Adcyap1* (PACAP), *Gal* (galanin), and *Hcrt* (orexin) genes were significantly upregulated by vitamin D_3 in cuffed condition while other such as *Tac1* (tachikinin) and *Npy* (neuropeptide Y), which expression is exacerbated by the lesion, returned to basal levels upon vitamin D administration. In the DRG, similar situations have been observed with *Gdnf* (glial cell–derived neurotrophic factor), *Ngf* (nerve growth factor), *Gdf10* (growth differentiation factor 10), *Nrtn* (neurturin), and *Ngr1* (neuregulin 1) transcripts going up after vitamin D_3 supplementation of injured animals and *Nppb* (encoding the precursor of the brain natriuretic peptide) gene expression enhanced in cuffed condition but restored by vitamin D_3.

Finally, a last set of genes (Supplementary Fig. 2), dealing with inflammation and extracellular matrix remodeling, was also scrutinized in our analysis. RT-qPCR confirmed that, in the cerebrum, *Cx3cl1* and *Adam11* (metalloprotease) gene upregulation observed in cuffed condition was abrogated by vitamin D_3 treatment while *Timp4* (a member of the metalloprotease inhibitor family) was solely upregulated in vitamin D_3–treated cuffed samples. In line with these findings, we also observed changes in *Cxcl14* (C-X-C motif chemokine ligand 14), *Cx3cl1*, and *Ccl21b* (C-C motif chemokine ligand 21) chemokine transcript levels in DRG with *Cxcl14* and *Ccl21b* upregulated in cuffed condition and *Cx3cl1* mRNA by vitamin D_3 instead. A similar situation was observed for extracellular matrix remodeling with *Mmp9* and *Mmp16* (matrix metallopeptidases 9 and 16) expression enhanced by the lesion and *Adamts12* (metalloprotease with thrombospondin) solely upregulated after vitamin D_3 treatment.

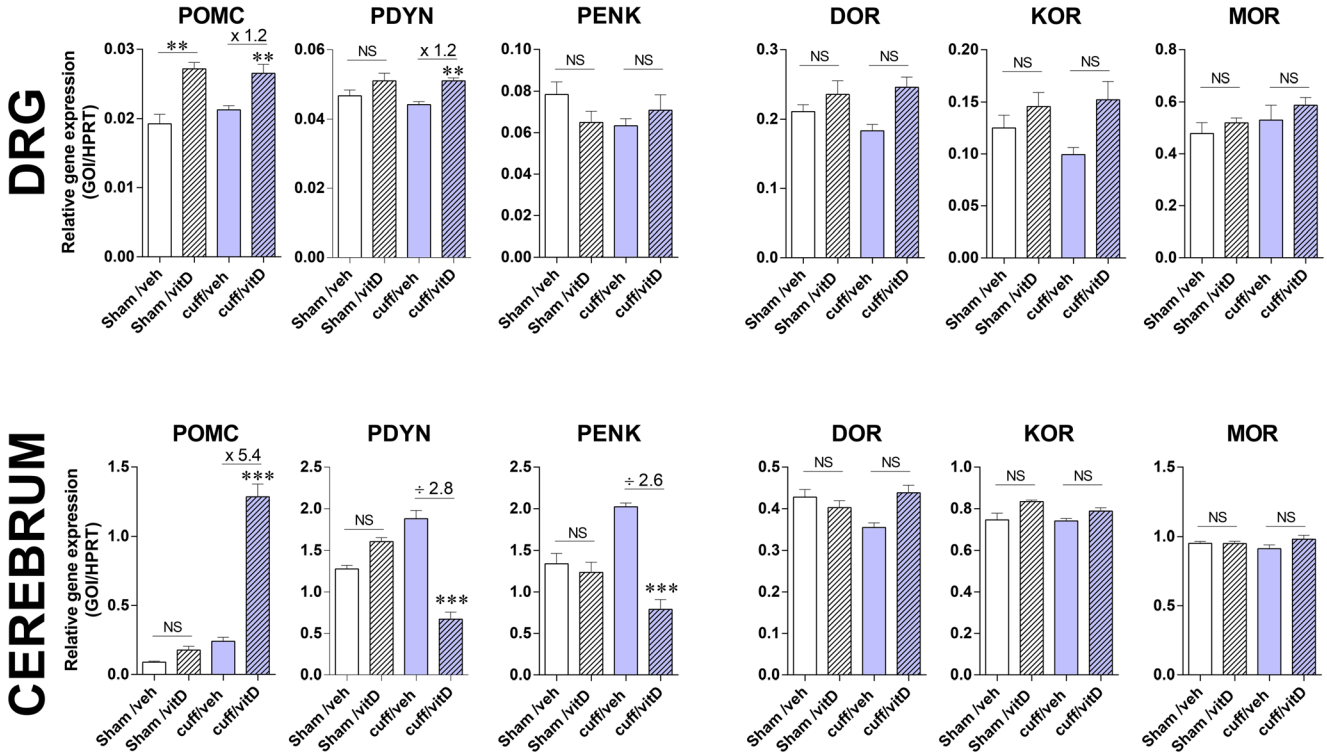

Fig. 5 RT-qPCR validation of several dysregulated transcripts in the dorsal root ganglia (DRG) (top graphs) and cerebrum (bottom graphs) of animals that received sham or cuff surgery with or without vitamin D_3 supplementation. The different graphs illustrate the relative gene transcript expression for pro- and pre-pro-opioidergic peptides (POMC, pro-opio-melanocortin; PDYN, prodynorphin; PENK, proenkephalin) as well as for the three main opioid receptors (DOR, delta opioid receptor; KOR, kappa opioid receptor; MOR, mu opioid receptor). Statistical code for Sidak's multiple comparisons test: $^{**}p < 0.01$, $^{***}p < 0.001$

Nociception and Allodynia May Be Modulated by Inactive and Active Forms of Vitamin D

To further decipher the modes of action of vitamin D and its metabolites, we used the IPA software to identify the intermediate regulators linking vitamin D_3 to the misexpressed genes that, within the cerebrum, are associated with allodynia, nociception, and opioid signaling. Our research was focused on calcitriol, the active form, but also on its two precursors: cholecalciferol and calcidiol. Figure 6 indicates that cholecalciferol, calcidiol, and calcitriol are associated with 9, 8, and 24 dysregulated genes, respectively. The three metabolites regulate a common pool of five genes: *Ctnnb1*, *Fos*, *Myc*, *Pomc*, and *Th*.

Each molecule acts in its own way. Cholecalciferol and calcidiol share a similar outcome but none of the intermediate regulators. Many genes associated with cholecalciferol are part of the pool of transcripts regulated by calcitriol (Fig. 6a, c). Conversely, calcidiol stands out as the only molecule that is linked to modified expression of *Itpr1* (inositol 1,4,5-triphosphate receptor type 1; Fig. 6b). Calcitriol is associated with the highest number of transcripts of interest and operates through two levels of regulators (Fig. 6c).

We next explored the tissue-specific mechanisms and compared the consequences of vitamin D_3 supplementation on gene expression. In the interest of simplification, we show here the metabolic pathways associated with cholecalciferol. Supplementary Fig. 3 reveals a similar profile with five common signaling regulators—*Erk1/2* (i.e., *Mapk3/1*), *Jnk* (i.e., *Mapk8*), *P38Mapk* (i.e., *Mapk14)*, *Prkca* and *Smad*—for the three tissues. Nevertheless, the final result differs dramatically from one tissue to another.

Discussion

Notwithstanding accumulating evidence indicating that hypovitaminosis D is a risk factor for various forms of pain, the current behavioral and transcriptomic study is, to our knowledge, the first one to assess the effects of a short-term cholecalciferol supplementation in the central (cerebrum and spinal cord) and peripheral (DRG) nervous systems of rats with chronic pain. We observed a dramatic improvement in nociceptive thresholds and allodynia scores as well as a massive and heterogeneous gene dysregulation in the three scrutinized tissues of mononeuropathic animals supplemented with vitamin D_3. A large number of dysregulated genes are associated with (i) opioid signaling, nociception, and allodynia, in the cerebrum, and (ii) axonal guidance and nociception, in the DRG. Reassuringly, 21 identified cerebral dysregulated genes are associated with vitamin D metabolites

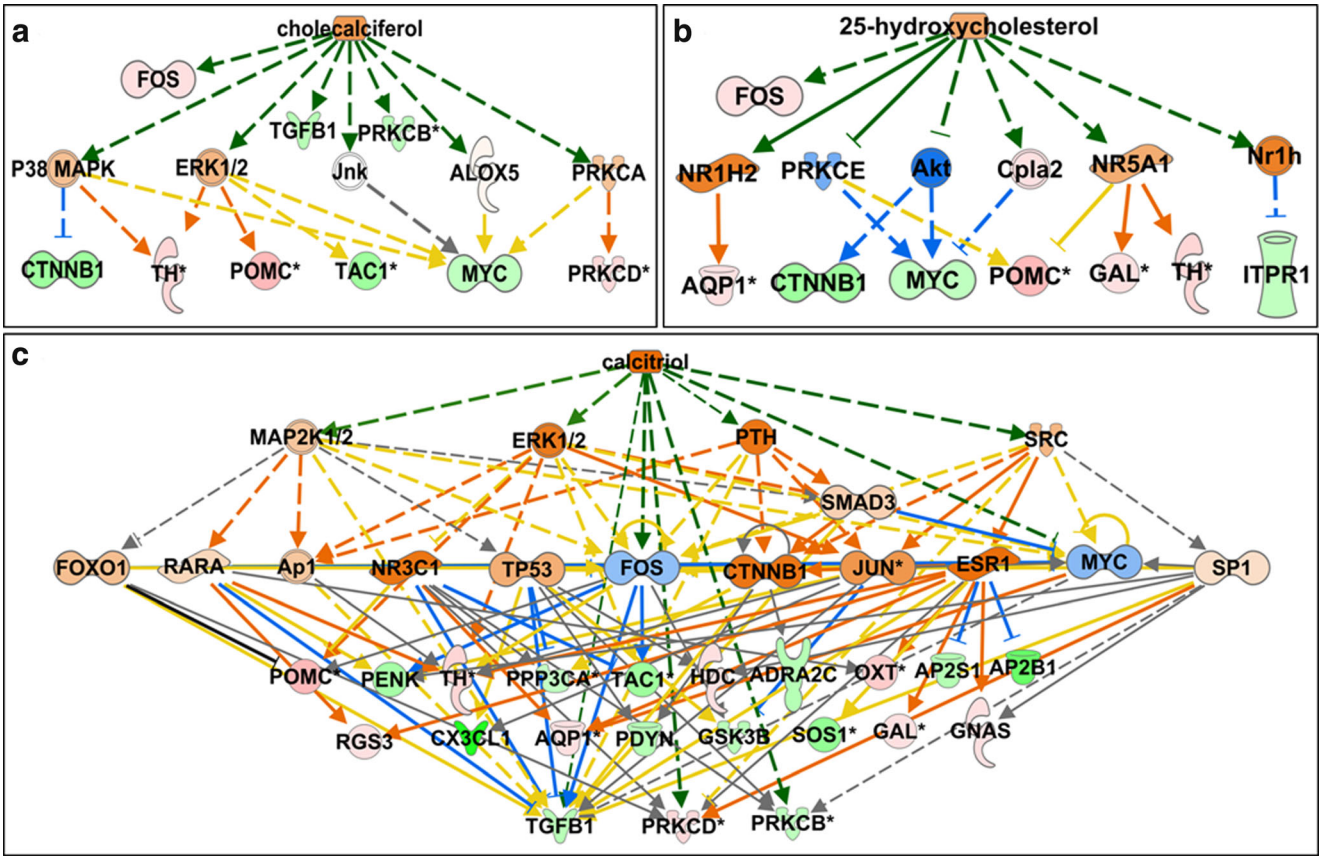

Fig. 6 Schematic view of the putative mechanisms of vitamin D_3 action in the cerebrum. Cholecalciferol, 25-hydroxycholesterol (calcidiol), and calcitriol are associated with 9, 8, and 24 nociception-, allodynia-, and opiod-associated dysregulated genes, respectively. Five genes—*Ctnnb1*, *Fos*, *Myc*, *Pomc*, and *Th*—are commonly regulated by the three metabolites. Each molecule regulates gene transcription in a specific manner. Regulators are either activated (brown) or inhibited (blue); genes are either up- (red) or downregulated (green)

and nociception and/or allodynia and/or opioid signaling. We show here that the anti-nociceptive action of vitamin D_3 in the brain involves signaling through *Erk1/2* (i.e., *Mapk3/1*), *Map2k1/2*, *Pth*, and *Src* and not, as expected, *Vdr*.

A Confirmed Immuno-Modulatory Role

To assess the pain-alleviating effect of cholecalciferol, we first opted for a knee monoarthritis model, in which a transient inflammation is induced by unilateral injection of CFA. Vitamin D being a potent immune modulator [35, 21, 22], an impact on inflammatory and immune processes can be expected. Indeed, it has been shown that calcitriol-treated T cells produced more anti-inflammatory cytokines and less proinflammatory cytokines [36–40] (for a recent review see [40]). However, unveiling the precise immune-modulatory modes of action of vitamin D was beyond the scope of this study. We chose instead to detail the molecular mechanisms underlying vitamin D_3 anti-nociceptive effects in a supposedly non-inflammatory model, namely mononeuropathy.

Claiming that this model is devoid of inflammation would be misleading, as it is associated with a moderate inflammation, highlighted by an altered expression of several inflammatory-related genes, which can be improved by vitamin D_3 supplementation. For example, *C1qa*, *C1qc*, *Il13ra1*, and *Irf2* (coding for complement C1q A and C chains, interleukin 13 receptor subunit alpha 1, and interferon regulatory factor 2, respectively), over-expressed in unsupplemented animals, are downregulated after cholecalciferol treatment. To a degree, this immuno-modulatory action may explain reduced pain symptoms.

A Pomc-Related Pain Relief

The modulation of opioid signaling by vitamin D_3 is one of the major findings of our study. Among the 23 cerebral dysregulated genes that are part of this pathway, three—*Pdyn*, *Penk*, and *Pomc*—are noticeable in the cerebrum since they code for endogenous opioids—prodynorphin, dynorphin, leu-enkephalin, met-enkephalin, and β-endorphin—that are involved in the sensory perception of pain [41]. An intriguing finding is the imbalanced expression of the P trio: *Pomc* (+5.4) is upregulated while *Pdyn* (−2.8) and *Penk* (−2.6) are downregulated. Although a few studies indicate a differential modulation of these three transcripts [42, 43, 3], such a

disparity is hard to comprehend. However, *Pdyn*, *Penk*, and *Pomc* belong to the opioid/orphanin gene family but they are not (i) located on the same chromosome, (ii) expressed by identical brain areas or cell types, or (iii) regulated by the same molecules. For instance, *Pdyn* and *Penk* expressions are potentially modulated by *Adora2a* (adenosine A2a receptor) and *Drd2* (dopamine receptor D2) [44] while *Pomc* is regulated by *Avp* [45]. Interestingly, the latter is over-expressed in the cerebrum and the formers are under-expressed. Likewise, *Fos*, which modulates the expression of *Pomc* [46], is over-expressed whereas *Ctnnb1*, a potential regulator of *Pdyn*, is under-expressed. This differential regulation could explain the results obtained in this study.

In addition to the altered expression of three opioid agonists, not associated with a modified transcription of opioid receptors, our study reveals a substantial dysregulation of G proteins subunits and second messengers. When delivered acutely, opioid agonists inhibit adenylyl cyclase (AC) activity [47], as observed here for *Adcy5*. Conversely, a chronic opioid treatment increases AC levels, inducing a superactivation of cAMP which leads to opioid tolerance and dependence [48]. After binding of the agonists, opioid receptors go through a phosphorylation process triggered by G protein–coupled receptor kinases (GRK) and second messenger-regulated kinases (PKC, PKA, CaMK—protein kinases C and A and Ca^{2+}/calmodulin-dependent protein kinases). Here, we observed a downregulation of *Prkcb* (protein kinase C beta) and an upregulation of *Prkcd* (protein kinase C delta). These findings are in accordance with the observed cerebral underexpression of IGF2 (insulin-like growth factor 2) and overexpression of tumor necrosis factor (TNF), potential specific regulators of each protein kinase, respectively [49, 50].

Finally, in order to further understand the potential role of POMC-derived cleavage peptides in pain alleviation, it is of prime importance to quote a study that assessed the role of β-endorphin in pain control [51]. When exposed to UV radiation, mice produce the POMC-derived peptide, β-endorphin, which in turn increases pain-related thresholds. It is therefore conceivable that the reported pain relief is a consequence of increased production of vitamin D, the collateral advantage of UV exposure.

A Tissue-Specific Reaction to Painful Stimuli

If we exclude the three genes coding for opioids, 11 and 5 cerebral transcripts are associated with nociception and allodynia, respectively. In most cases, their up- or downregulation is in accordance with their reported effect. The inhibition of *Cx3cl1* [52] and the knockout of *Adora2a* [53, 44] and *Tac1* genes [54] increase the threshold of mechanical and/or thermal nociception, while the central administration of oxytocin enhances analgesia [55] and the activation of orexin A (product of *Hcrt*) reduces hot plate nociceptive responses [56]. Contrariwise, the knockout of *Tph1* (tryptophane hydroxylase 1) decreases the threshold of thermal nociception [57] and *Aloxe3* (arachidonate lipoxygenase 3) expression is increased in peripheral inflammation [58]. Aquaporin 1, alpha-2 adrenoreceptors (Adra2c), and histidine decarboxylase (Hdc) are also associated with nociception but their overexpression should not induce hypo-nociception [59–61]. In contrast, up- and downregulation of *Aloxe3*, *Gal*, and *Tgfb1* do not fit with previous reports [62, 58, 63].

In recent years, vitamin D has been described as a cell-, tissue-, time-, individual-, dose-, pathology-, and gender-dependent neurosteroid [3, 1]. Here, discrepancies observed with the transcriptomic study are exclusively correlated with the variety of examined tissues. The differences are far from trivial since not a single allodynia-associated dysregulated gene in the cerebrum is detected in the spinal cord and vice versa. Once again, some observations are in accordance with published data. Being that the knockout of *Ptgs2* and *Prx* increases allodynia [64, 65], it can be expected that overexpression of these genes would lead to reduced allodynia. Conversely, a putative anti-allodynic effect of over-expressed *Il6*, *Lepr*, and *Thbs4* is more difficult to comprehend since the interference of active interleukin 6 and thrombospondin 4 (TSP-4, a product of *Thbs4*) and the knockout of *Lepr* and *Thbs4* decrease allodynia [66–68].

The nociception-associated transcriptomic profile in the DRG is also radically different from that observed in the cerebrum. Only one gene (*Cx3cl1*) out of 17 is commonly dysregulated in DRG and the cerebrum, although in an inverted way. Here again, alleviated pain could result from the over- or under-expression of most of the dysregulated genes. For example, it has been observed that the knockouts of *Cdk5r1*, the increase of *Trpm8* [69, 70], and the knockout of *Gnao1* decrease the threshold of thermal nociception [71], three findings in accordance with their respective under- and over-expression in the DRG. Neural cell adhesion molecule (NCAM) signaling mediates the analgesic effect of GDNF which we found over-expressed in the cerebrum [72], NOGO-66 (product of *Rtn4*) reduces neuropathic pain after sciatic nerve transection [73], and NGF is involved in the sensory perception of pain [74].

New Clues on Vitamin D_3–Related Molecular Mechanisms in the Nervous System

Studies have shown that the VDR is very poorly expressed in the central nervous system [75, 76, 2] and only a restricted number of cerebral genes contain an upstream VDRE (Vitamin D response element) [77]. In conformity with these previous findings, the current study indicates that, at most, the expression of three nociception- and cholecalciferol-related genes is regulated by the VDR. And, out of three, only one (*Ngf*) displays a VDRE [77].

The current transcriptomic study provides new clues on potential and/or established intermediate regulators. Instead of up-

or downregulating gene transcription via a VDR/VDRE mechanism, calcitriol may trigger activation or inhibition of some key proteins. This first row of regulators (MAP2K 1/2, ERK 1/2 (i.e., MAPK3/1), SRC, PTH, SMAD3) activates or inhibits a second row of proteins which, in turn, modulates the expression of the final effectors. This is in line with results from other studies done in human hepatocytes [78], chondrocytes, [79–81], keratinocytes, and colonocytes [82]. Our study also suggests that calcidiol may not be as "inactive" as it is supposed to be and does not need to be further hydroxylated to be efficient. This is in accordance with a study in which 25-hydroxycholesterol was shown to amplify inflammatory signaling by mediating the recruitment of the activator protein 1 (AP-1) components of *Fos* and *Jun* to the promoters of a subset of Toll-like receptor-responsive genes [83]. It should be noted that the metabolic pathway suggesting that cholecalciferol may be active as well should be analyzed with caution as the proposed scheme comes from publications in which [1,25-hydroxycholesterol] (calcitriol) is mislabeled as cholecalciferol.

Recommendations for a Clinical Translation

Since recent reviews and meta-analyses deliver a mixed message about the potential therapeutic benefit of vitamin D_2 or D_3 supplementation against acute and chronic pain, reliable randomized, placebo-controlled clinical trials with undisputed conclusions remain to be performed. Among the major pre-requisites, vitamin D and its time/mode of administration, as well as the patient's geno-/phenotype, should be taken into account.

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Compliance with Ethical Standards

Conflict of Interest Statement The authors declare that they have no conflict of interest.

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